MICROBIAL ATTACHMENT TO FOOD AND FOOD CONTACT SURFACES

JOSEPH F. FRANK

Center for Food Safety and Quality Enhancement
Department of Food Science and Technology
University of Georgia
Athens, GA 30602
USA

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I. INTRODUCTION

The ability of microorganisms to adhere to surfaces has significant implications for food science. Microorganisms attached to plant and animal tissues can affect food safety and spoilage. Microorganisms can adhere firmly and are therefore difficult to remove or inactivate without damaging the underlying tissue. This is not of concern for most processed foods, but it is for foods that are to be sold as raw or minimally processed. Disease outbreaks associated with *Salmonella* on chicken and fresh produce and *Escherichia coli* 0157:H7 in apple juice, alfalfa seed sprouts, and lettuce may be related to the inability of sanitizer and washing treatments to remove or inactivate attached pathogens. Microbial attachment to food contact surfaces is also of significance for food safety and spoilage. Microorganisms attached to processing equipment may escape cleaning and sanitizing procedures and proceed to contaminate processed product. Pathogens originating with raw products can attach to food preparation surfaces, which, if not adequately cleaned before reuse, can serve to recontaminate cooked foods.

The ability to attach to and subsequently detach from surfaces is a characteristic of all microorganisms. Attachment is advantageous and perhaps necessary for survival in the natural environment, as it allows microorganisms to exert some control over their nutritional environment, and offers protection from environmental stresses. Attachment is also the initial event in microbial infections, for if a cell fails to adhere it will be carried away from its potential host. Therefore, microbial attachment is a process that has been extensively studied. Detachment is just as basic to microbial survival as attachment, as an organism that cannot detach from an environmental surface cannot find new growth niches when the environment becomes unfavorable. In the food industry, the fact that attached microorganisms can detach is the very reason that food contact surfaces must be cleaned and sanitized. Detachment of microorganisms from food tissues is also a requirement for many microbial analyses.

Attachment of microorganisms is influenced by the cell surface, the attachment surface, and the surrounding medium. Considering the complexity of biological surfaces (both of the food tissue and microbial cell) it is not surprising that microbial attachment is a complex phenomenon. Its true complexity cannot be appreciated without knowledge of the bacterial cell envelope, that portion of the cell that is in direct contact with the environment. Consequently, this review will start with a discussion of the cell envelope, proceed to a description of the mechanisms of microbial attachment, and then discuss microbial attachment to and detachment from foods and food contact surfaces. The goal of this review is to increase understanding of the food safety implications of microbial attachment.

II. PROPERTIES OF THE BACTERIAL CELL THAT AFFECT ATTACHMENT

A. THE BACTERIAL CELL ENVELOPE

The cell envelope consists of the outermost structures of the cell, and thus has great influence on adherence. Bacteria isolated from natural systems produce polymers that extend from or coat the cell wall; there is no selective pressure to produce these materials when growing in laboratory media, so the cell wall of laboratory-grown cultures is often unnaturally exposed directly to the environment. Such cultures are also less adhesive (Costerton *et al.*, 1985). The cell wall, though considered part of the cell envelope, does not normally, contact the adherent surface in a natural system. Rather, various components of the envelope (surface-active polymers) are anchored to the cell in such a way that they provide a bridge to the surface. The three-dimensional organization of the cell envelope allows penetration by large molecules in varying degrees. For example, the presence of structural polymers beyond the cell wall does not necessarily prevent the reaction of antibodies with cell wall antigens (Rogers, 1979).

The structure of the cell envelope has been elucidated by using conventional electron microscopy techniques including negative staining, thin sectioning, shadow-casting, freeze-etching, and computerized image enhancement (Beveridge and Graham, 1991). These techniques require chemical fixation that can result in damage to highly hydrated, delicate envelope polymers. New electron microscopy techniques utilizing cryofixation, immunogold labeling, and nondenaturing plastics provide a more accurate view of the cell envelope (Beveridge and Graham, 1991). Cell envelope structures described below are those most frequently encountered in food-borne bacteria and most likely to be involved in attachment. They should not be viewed as components of a static system, as the cell envelope is constantly engaging the environment, reacting to it and with it. This concept will be discussed in a later section.

1. Capsules

A capsule is a gel-like substance that completely surrounds the cell wall. Capsules are composed of water and sugar-containing polymers, which are anchored to the cell surface. Their attachment to the cell distinguishes capsular polysaccharides from extracellular slime, which freely dissociates from the cell. Capsule polymers radiate from the cell and are rarely cross-linked to one another, though acidic types may be linked by divalent metal ions (Beveridge and Graham, 1991). Since capsules consist mostly of

water, they readily collapse when fixed for electron microscopic examination (Costerton *et al.*, 1985). This often makes them appear in electron micrographs as fibers extending from the cell or as blebs on the cell surface. Capsule polymers often contain acidic residues such as uronic, hyaluronic, acetic, pyruvic, glucoronic and glutamic acids (Sutherland, 1985; Joklik *et al.*, 1988). These residues impart a net negative charge to the cell surface. Capsules bind to metal ions and positively charged amino acids, possibly functioning to bring nutrients close to the cell (Costerton *et al.*, 1985). Capsules can be either adhesive or antiadhesive; a hydrophilic capsule can mask hydrophobic components of the cell envelope, preventing adhesion of the cell to hydrophobic surfaces (Ofek and Doyle, 1994).

2. *Fimbriae*

Fimbriae (pili) are threadlike projections from the cell anchored to the outer membrane. Fimbriae can be thick (7–11 nm diameter) or thin (1–4 nm), rigid or flexible, and most are 0.5 to 10 μm in length (Ofek and Doyle, 1994). They are composed of repeating protein subunits, with a lectin-containing protein at the tip that recognizes eukaryotic carbohydrate receptors, the most common being composed of oligomannose (Finlay and Falkow, 1989; Sharon and Lis, 1989). The amino acids of some fimbriae proteins contain numerous nonpolar side chains imparting hydrophobicity to the structure (Corpe, 1980). The role of fimbriae in bacterial adhesion through hydrophobic interactions has been reviewed by Irvin (1990). Fimbriae of enteric bacteria are involved in adhesion to fungi, plant, and animal (epithelial and red blood) cells (Corpe, 1980). Fimbriae can also serve as host specific adhesins (Gaastra and De Graaf, 1982; Brook and Myhal, 1991) and are involved in cell clumping.

3. *Outer membrane polymers*

Surface active compounds associated with the outer membrane include lipopolysaccharides, lipoproteins, lipoteichoic acid, and lipomannan. The orientation of these molecules (whether the hydrophilic or hydrophobic region is exposed to the environment) influences the surface hydrophobicity of the cell (Neu, 1996). Most gram negative bacteria (wild types) have long polysaccharide structural regions of their lipopolysaccharide extending outward from the cell (Ofek and Doyle, 1994) producing a hydrophilic effect, whereas some gram positive organisms, such as group A streptococci, have the lipid portion of lipoteichoic acid extending away from the cell, resulting in a hydrophobic surface (Wicken, 1980;

Neu, 1996). Various proteins anchored to the outer membrane can also take part in surface interactions. For example, for porin proteins to confer selective permeability to the cell they must interact with the cell's environment (Nikaido and Vaara, 1985). Treatment of cells with proteases or inhibition of protein synthesis often decreases cell surface hydrophobicity (Bar-Or, 1990b). Carbohydrate-binding proteins are involved in adhesion of enteric lactobacilli to the gut (Adlerbeith *et al.*, 1996; Henriksson *et al.*, 1991).

4. S layers

S layers are high molecular weight proteins and glycoproteins that form regular arrays on the external surface of some bacteria including members of the *Pseudomonas, Aeromonas. Bacillus,* and *Clostridium* genera (Austin *et al.*, 1990; Beveridge and Graham, 1991). S layers have a neutral surface charge and are held to the cell surface by electrostatic and hydrophobic interactions (Beveridge and Graham, 1991). Their potential role in attachment is unknown.

B. EXCRETED SUBSTANCES

Bacteria can excrete surface-active substances into the environment which can then coat surfaces, conditioning them to allow attachment and subsequent colonization, or promote detachment (Neu, 1996). The most common of these compounds are polysaccharide slimes. Slimes that have a role in surface attachment are referred to as "slimectins" (Ofek and Doyle, 1994). Their composition and synthesis has been described by Sutherland (1985). Some bacteria produce slimes only after attachment, where they, along with capsular material, become a constituent of the biofilm glycocalyx.

Phytopathogenic coryneforms and pseudomonads excrete compounds that aid in tissue colonization. Viscosin, produced by *Psuedomonas fluorescens*, is sufficiently surface-active to allow wetting of hydrophobic broccoli leaves and initiate the decay/disease process (Laycock *et al.*, 1991). In contrast, a lipopeptide excreted by *Serratia marcescens* converts hydrophilic surfaces to hydrophobic (Matsuyama *et al.*, 1992), so that hydrophobic cells can attach. Polymers excreted by bacteria can also condition a surface to inhibit adhesion. *Acetobacter calcoaceticus* produces emulsan, a polyphilic polymer that changes hydrophobic surfaces to hydrophilic. Hydrophilic bacteria but not hydrophobic ones will attach to such a surface (Rosenberg *et al.*, 1983).

C. CHANGES IN RESPONSE TO THE ENVIRONMENT

Since the envelope provides the means by which bacteria interact with their environment, it is not surprising that it adapts to changing environments, thus allowing the cell to maintain viability under stress. Brown and Williams (1985) reviewed the adaptability of the bacterial envelope as it affects pathogenicity. They concluded that cells respond to adverse conditions by modifications to the cell envelope that not only enhance survival, but also change the adhesive properties of the cell. Just as pathogenic bacteria growing in standard laboratory media (near ideal growth conditions) eventually lose virulence, so too bacteria adept at surface adherence can lose this ability upon repeated culture. Neu (1996) reviewed numerous studies that demonstrate the cell's ability to adapt through the production of a variety of surface-active compounds that affect adhesion capability. Therefore, any characterization of bacterial adhesion or definition of a cell's surface properties is only meaningful in the context of a specific growth environment (Brown and Williams, 1985).

1. Effect of growth conditions

Staphylococcus aureus can exhibit either a hydrophobic or hydrophilic cell surface depending on the growth media. Growth in blood agar induces a more hydrophilic cell surface in coagulase-negative staphylococci regardless of whether a capsule can be visualized (Malmo *et al.*, 1987). However, most strains involved in bovine mastitis produced a hydrophobic cell surface in stationary phase regardless of growth conditions. There are two groups of *Staphylococcus epidermidis,* those that exhibit lower surface hydrophobicity when grown in sugar supplemented media, and those that do not respond to sugar supplementation (Wadstrom, 1990). McEldowney and Fletcher (1986a) found that changes in nutrient conditions affect the surface properties and attachment ability of individual species differently. This conclusion was based on the study of freshwater isolates of *Pseudomonas fluorescens, Enterobacter cloacae,* and *Flexibacter* sp. Growth in dilute media tends to encourage adhesion, a result of increased cell hydrophobicity or capsule production (Singh and Vincent, 1987). *Coryneform glutamicum* grown in high phosphate media is more hydrophobic than are phosphate-depleted cells. This is a result of increased synthesis of lipoteichoic acid under high-phosphate conditions (Buchs *et al.*, 1988). Sucrose induces lipoteichoic-associated adhesiveness in oral streptococci (Rolla *et al.*, 1980). Kim and Frank (1994) observed increased attachment to stainless steel of *Listeria monocytogenes* grown in tryptic soy

broth as opposed to cells grown in a chemically defined medium. This increased attachment ability was attributed to the presence of peptone in the growth medium.

2. *Stationary phase – adaptation to starvation stress*

Microorganisms in food and the food processing environment are often in stationary phase due to lack of appropriate nutrients or growth environment. Most research on the attachment of stationary phase cells employs starvation conditions. Bacteria in aquatic habitats often respond to starvation by increasing surface hydrophobicity which results in greater attachment ability and aids the cells in scavenging nutrients adsorbed to the surface (Kjelleberg *et al.*, 1983; Dawson *et al.*, 1981; Kjelleberg and Hermansson, 1984). However, Fletcher (1977) found that log phase cells of a marine pseudomonad attached to polystyrene in greater numbers than those in stationary phase, indicating a reduction in surface hydrophobicity. In addition, starvation stress generally decreased attachment of *Salmonella typhimurium, L. monocytogenes*, and *Escherichia coli* to beef tissue (Dickson and Frank, 1993).

III. MECHANISMS OF MICROBIAL ATTACHMENT

Various authors, including Ofek and Doyle (1994), Marshall (1985), Marshall and Bitton (1980), Bryers (1987) and Fletcher (1996) have summarized research on the mechanisms of microbial attachment. The review of Boulange-Petermann (1996) is specifically directed at attachment to stainless steel. Marshall *et al.* (1971), described microbial attachment as a two-stage process, an instantaneous reversible stage followed by a time-dependent irreversible stage. Bacteria have the size, low density, and surface charge of colloidal particles, so the initial reversible attachment phase can be described in terms of colloidal chemistry (Norde and Lyklema, 1989; Marshall, 1992). The DLVO (named after the authors' initials) theory of colloidal stability (Derjaguin and Landau, 1941; Verwey and Overbeck, 1948) can be used to assess potential for microbial attachment by estimating electrostatic and long range London-Van der Waals forces. Although microorganisms have many characteristics of colloidal particles, application of the model requires treating microorganisms as discreet, geometrically uniform, stable particles, which, of course, they are not. Therefore DLVO theory has limitations for quantitative predictions of attachment potential (Boulange-Petermann, 1996).

The initiation of attachment can also be predicted on the basis of the

surface-free energies of the bacterial cell and attachment surface, and the surface tension of the surrounding medium (Mozes *et al.*, 1987; Bellon-Fontaine *et al.*, 1990; Marshall 1992). If the surface tension of the bacteria is greater than that of the surrounding medium, the cells will more likely adhere to hydrophilic (high surface tension) surfaces (Absolom *et al.*, 1983). More commonly, the surface tension of the bacteria is less than that of the surrounding medium, and attachment to hydrophobic surfaces is observed. Surface-free energy relates more directly to the binding force than to the number of cells bound per unit area (Norde and Lyklema, 1989), although Dexter *et al.* (1975), observed differences in attachment numbers of 1.5 to 3 orders or magnitude due to surface-free energy (as determined by substrate wettability).

Forces involved in attachment are limited to electrostatic, hydrogen bonding, hydrophobic attraction and, for some biological surfaces, attraction of coordination complexes involving multivalent metal ions (Ofek and Doyle, 1994). Most biological and inert surfaces have a net negative charge and therefore should repel bacteria. This repulsion is greatest at low electrolyte concentrations, but as electrolyte concentration increases it can be overcome by longer range Van der Waals attractive forces (Marshall *et al.*, 1971). In fact, the attached cell surface never actually contacts the substratum but maintains a distance of 10 to 100 nm (Fletcher, 1988). This distance is a function of electrolyte concentration of the surrounding solution.

Reversible attachment becomes irreversible when, over time, the cell produces extracellular binding polymers (glycocalyx) or in the case of some biological substrata, when specific binding reactions develop (i.e. lectin-carbohydrate binding). Apparent instantaneous irreversible attachment of bacteria to food tissues also results from physical entrapment of cells by capillary action. These cells are not attached to the tissue, in the strict sense of adhering to an interface, but neither are they readily removed from the tissue. This phenomenon will be addressed later.

A. CONDITIONING FILMS

All microbial attachment in natural systems occurs on surfaces with an adsorbed layer of biological macromolecules, often protein-containing polymers. Both hydrophobic and attractive electrostatic associations are responsible for protein binding (Al-Makhlafi *et al.*, 1995b). The conditioning film forms regardless of the surface chemistry of the underlying material. Some proteins, for example bovine serum albumin and gelatin, will bind to stainless steel even though they have similar surface charges (Fukuzaki, 1995). This is because these proteins undergo structural

alterations that overcome electrostatic repulsion (Norde and Lyklema, 1989). Hydrophilic proteins will also bind to hydrophobic surfaces (van Oss *et al.*, 1986). Adsorbed proteins can either stimulate or inhibit bacterial adhesion (Fletcher, 1976; Al-Makhlafi *et al.*, 1994). For example, the absence of a protein conditioning film created by using minimal media (no complex nutrients) completely inhibited attachment of *L. monocytogenes* to a polyester floor sealant material, whereas in hydrolyzed protein media, attachment was profuse (Blackman and Frank, 1996). Surface characteristics are not completely masked by the conditioning film, as different surfaces with similar protein layers can differ greatly in their ability to bind specific bacteria (Baier, 1980). The strength which cells are bound, but not necessarily the number per unit area, is highly dependent on the underlying substratum surface energy (Baier, 1980; Van Pelt *et al.*, 1985).

As previously discussed, some bacteria excrete polymers that coat surfaces, providing their own conditioning film. A variation on this phenomenon is the effect of microbial "footprints" (reviewed by Neu, 1992). After bacteria detach, they leave behind adhering substances that change the subsequent ability of other bacteria to adhere. Microbial footprints are either structural components of the cell surface left behind through enzymatic cleavage (adhesive polymers), or substances released by the cell to facilitate detachment (polysaccharides or other biosurfactants). Incomplete removal of microbial footprints by cleaning solutions has implications for maintenance of hygienic food contact surfaces, as bacteria may adhere more readily to these polymer-coated areas. Schwach and Zottola (1984) noted that sodium hypochlorite treatment of stainless steel does not dissolve microbial attachment fibrils.

The presence of adhering bacteria can influence attachment of cells subsequently exposed to the surface. Biological tissues may have specific attachment sites, which, if filled by existing cells, will interfere with the ability of another species to colonize (Bibel *et al.*, 1983). Preattachment of cells to solid inert surfaces can either inhibit, stimulate, or have no effect on subsequent attachment of other species (McEldowney and Fetcher, 1987). Bacteria such as *Pseudomonas*, that readily colonize surfaces and produce extensive glycocalyx, can create conditions that enhance attachment of less adhesive species such as *L. monocytogenes* (Sasahara and Zottola, 1993).

B. HYDROPHOBIC AND ELECTROSTATIC EFFECTS

Adherence of bacteria to a surface is best explained by a combination of hydrophobic bonding and electrostatic attraction/repulsion. Hydrophobic interactions are usually dominant in facilitating bacterial adhesion.

Rosenberg and Kjelleberg (1986) and Doyle and Rosenberg (1990) reviewed this area. Hydrophobic cells are generally more adherent than hydrophilic ones (van Loosdrecht *et al.*, 1987b) and most bacteria preferentially adhere to hydrophobic surfaces (Pringle and Fetcher, 1983). For example, encapsulated *S. epidermidis* are less hydrophobic and are found to adhere less to hydrophobic surfaces than nonencapsulated strains (Hogt *et al.*, 1983). McEldowney and Fletcher (1986b), van Loosdrecht *et al.* (1987a, 1989) and Fletcher and Loeb (1979) concluded that both hydrophobic and electrostatic interactions were important in explaining bacterial adhesion to inert surfaces.

Surface charges associated with substratum and cells are not good predictors of adherence ability. Electrostatic interactions between surfaces of like charge can occur due to the formation of an electrostatic double layer on each surface. The formation of this double layer depends on the ionic strength and pH of the surrounding medium (Oliveira, 1992). Cells will, of course, also adhere to surfaces of opposite charge. For example, *Staphylococcus aureus* attaches more to positively charged polymeric surfaces than those with a negative charge, consistent with the observation that these cells are negatively charged (Hogt *et al.*, 1986). Gilbert *et al.* (1991) observed a direct relationship between adhesiveness and surface electronegativity and hydrophobicity for *Staphylococcus epidermidis,* but an inverse relationship with *E. coli.* Cell structures such as pili participate in hydrophobic interactions and maintain the cells at a sufficient distance from the surface to overcome electrostatic repulsion (Heckels *et al.*, 1976). Cell surface hydrophobicity and electronegativity will also not always provide useful predictions of adherence. Although *L. monocytogenes,* when grown in trypticase soy broth, has a hydrophilic surface, it still attaches in greater numbers to some hydrophobic as compared to hydrophilic surfaces (Mafu *et al.*, 1991). The behavior of most microorganisms is consistent with multiple attachment mechanisms that allow them to attach to various types of seemingly incompatible surfaces (for example, hydrophobic and hydrophilic or positively and negatively charged) (Costerton, 1984; Neu, 1996; Paul and Jeffrey, 1985). Differences observed in attachment (cells per cm^2) to different surfaces are usually on the order of only one to two degrees of magnitude.

C. IRREVERSIBLE ATTACHMENT

Irreversible nonspecific attachment (as opposed to irreversible specific interactions such as lectin:carbohydrate binding to biological tissues) is a result of the microbial production of exocellular polymers that bridge the gap between the cell and the substratum. Attachment ability has often been

associated with production of exocellular polymers (Rutter and Leech, 1980; Dawson *et al.*, 1981). These polymers may be present on the cell surface before attachment, assisting in this process, or may be produced after attachment (Fletcher and Floodgate, 1973). Production of such polymers may be controlled by genes induced upon the cell's arrival at a surface. For example, alginate biosynthesis genes of *Pseudomonas aeruginosa* are induced upon attachment (Boyd and Chakrabarty, 1995). The adsorption properties of microbial exocellular polymers can be used to predict attachment to different surfaces (Pringle and Fletcher, 1986). If surface growth is initiated, a microcolony coated with adhesive material results (Allison and Sutherland, 1987). It is unclear if this biofilm polymer has the same composition as the initial adhesive polymer (Marshall *et al.*, 1989), but both are often acidic polysaccharides. For additional information on the composition of microbial adhesive polymers see the reviews of Cooksey (1992) and Christensen (1989).

IV. ATTACHMENT TO FOOD CONTACT SURFACES

It follows from the previous discussion that most microorganisms should be able to attach to all types of food contact surfaces with varying degrees of success. In fact, this appears to be the case. Generally, if a microorganism contacts a surface with sufficient time for attachment mechanisms to engage, irreversible attachment of a portion of the population will occur. The public health significance of such an attachment event depends on the degree of microbial control associated with the food and the food processing system. The origin of pathogenic bacteria in many food-borne disease outbreaks remains unknown, but adherent cells are sometimes implicated, though usually with little or no direct evidence. The low levels of *Salmonella* associated with cheese implicated in a 1989 outbreak (Hedberg *et al.*, 1992) and ice cream implicated in a 1994 outbreak (Hennessy *et al.*, 1996) are examples of such incidents. The effectiveness of food contact surface sanitation was questionable in both cases, but *Salmonella* were not isolated from the questionable surfaces. Pathogens in micro-scale growth niches would be expected to produce very low levels of contamination. In addition, testing is done days after the implicated foods are processed, when evidence may no longer be relevant.

A. ARRIVAL AT THE SURFACE

Before they can attach, microorganisms must reach a food contact surface, either under their own power or by being transported in food or water.

Motility can aid some microorganisms in reaching surfaces (Meadows, 1971). This is probably a chemotactic response to a nutrient gradient associated with the surface (Marshall, 1985). In food processing such gradients might exist on newly cleaned equipment, but would disappear with food contact. Herald and Zottola (1989) found that motile and nonmotile *Pseudomonas* attached to stainless steel in similar amounts when in buffer solution. Stanley (1983) reported that motile *Pseudomonas* attached more than nonmotile, but only in the absence of agitation. Dickson and Daniels (1991) observed motile *L. monocytogenes* to attach to glass more readily than nonmotile, but the motile cells also had a greater surface charge than the nonmotile ones. The effect of motility on attachment, when observed, is not more than 10-fold, so it probably has little practical significance. Even inactivated cells will attach to stainless steel and other surfaces, though at a reduced rate from their living counterparts (Meadows, 1971; Stanley, 1983).

Often in food processing, the food material flows through the system creating a shear force. Duddridge *et al.* (1982) reported that an increase in surface shear decreased attachment of *Pseudomonas fluorescens* to stainless steel. In contrast, in a low nutrient system, the opposite effect was observed (Mittelman *et al.*, 1990), possibly due to stimulation of exopolysaccharide production. Surface roughness can increase attachment by protecting cells from removal by shear forces while they are still reversibly attached (van Loosdrecht *et al.*, 1989). However, Stone and Zottola (1985) found that shaking the cell suspension had no effect on attachment rate to stainless steel, possibly because the shear force applied by this method was too low.

B. ATTACHMENT TO STAINLESS STEEL

The surface of stainless steel is marked with numerous channels and crevices, a result of the mechanical polishing process, which serve to entrap microorganisms and food residues (Zoltai *et al.*, 1981; Speers *et al.*, 1984). Stainless steel is moderately hydrophilic with a negative surface charge. Irreversible attachment of microorganisms to stainless steel occurs rapidly (less than one minute) and increases with time. Microorganisms appear to attach via exocellular polysaccharides or produce polysaccharides after attachment (Lewis *et al.*, 1987; Beech and Gaylarde, 1989; Mafu *et al.*, 1990; Vanhaecke *et al.*, 1990). Figure 1 shows *Pseudomonas fluorescens* attached to a stainless steel surface. Attachment appears to be random relative to the position of the polishing grooves. This indicates that this microorganism has sufficient preformed polysaccharide to adhere firmly to all portions of the surface. *Pseudomonas* spp. are especially prolific at exopolysaccharide production after initial attachment (Zoltai *et al.*, 1981).

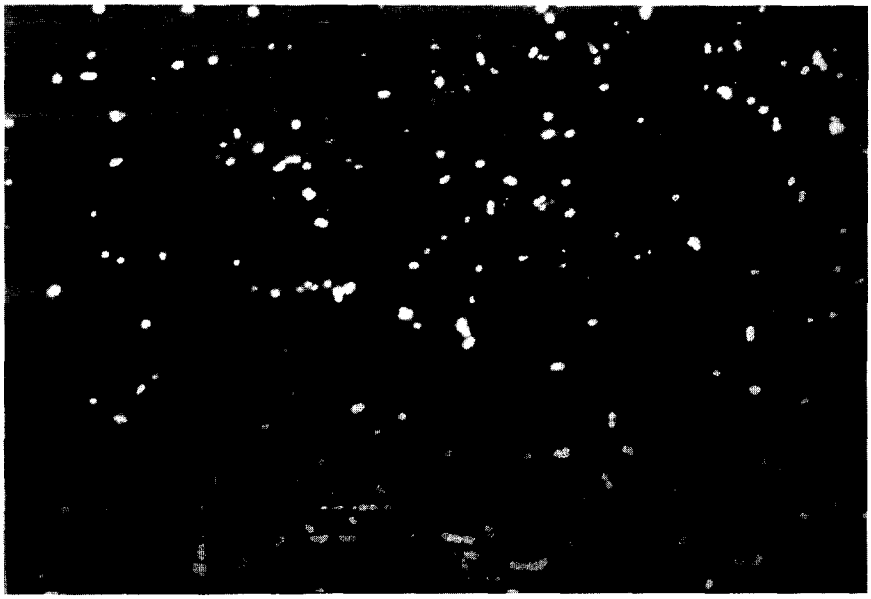

FIG. 1. CSLM micrograph of psychrotrophic *Pseudomonas* spp. attached to stainless steel. Grooves in metal resulting from mechanical polishing can also be seen. Bar = 100 μm. (Unpublished data of A. N. Hassan and J. F. Frank.)

Attachment fibrils can be visualized using scanning electron microscopy; these fibrils are probably the result of condensation of the exopolysaccharide that occurs during sample preparation (Lewis *et al.*, 1987; Mafu *et al.*, 1990). Lectins, glucosidases, and proteases can, in some cases, reduce or reverse attachment to stainless steel (Herald and Zottola, 1986; Beech and Gaylard, 1989), confirming the significance polysaccharide-mediated adherence and implicating a role for surface proteins. *Pseudomonas* attaches to stainless steel primarily through hydrophobic bonding with minimal role for electrostatic interactions (Vanhaeck *et al.*, 1990). *L. monocytogenes* apparently has multiple attachment mechanisms. Though attachment to stainless steel occurs at 10, 21, and 35°C, attachment polysaccharide is observed only at 21°C (Herald and Zottola, 1986). The microorganism has a low surface hydrophobicity which promotes adherence to stainless steel; however, the cells can also adhere to hydrophobic surfaces (Mafu *et al.*, 1991). *L. monocytogenes* and *S. typhimurium* attach in greater numbers to stainless steel than buna-N rubber (Helke *et al.*, 1993). *L. monocytogenes* adheres to and survives on cast iron used for floor drains (Spurlock and Zottola, 1991).

C. ATTACHMENT TO CUTTING BOARDS

Cutting boards are used in food processing for manual cutting operations and are also widely used in institutional and home food preparation. Although plastic (primarily polyethylene and nylon) cutting boards have long been recommended over wood because of perceived ease of cleaning, recently some investigators claimed that wood may be more sanitary. This topic has recently been reviewed by Carpentier (1997). Bacteria present on foods are readily transferred from food to the cutting board surface and then to other foods being prepared on the same surface. Attachment and detachment rates are similar for wood and polyethylene (Miller *et al.*, 1996). Ak *et al.* (1994a) reported that bacteria applied to wood are not recovered as well as bacteria applied to plastic. This is because wood is porous and entraps cells within its pores. Since bacteria penetrate less into wet wood than dry, the cells are probably transported by capillary action. Entrapped bacteria, although not always recovered by surface swabs, can retain viability (Abrishimi *et al.*, 1994). Plastic cutting boards, though they are nonporous, can entrap bacteria and food residues in superficial knife cuts. These food residues and bacteria are not readily removed by hand washing, making plastic cutting boards potential sources of pathogens, and encouraging Ak *et al.* (1994a,b) to conclude that plastic cutting boards are less sanitary than wood. This conclusion, however, is based on methodology that did not detect the viable entrapped microorganisms present in wood. ATP bioluminescence tests indicate that plastic cutting boards are easier to clean than wood (Welker *et al.*, 1997). Under actual use, however, there appears to be little difference in the cleanliness achieved (Carpentier, 1997).

D. SELECTIVE ATTACHMENT

Microorganisms will differ in their ability to attach to any given surface. In addition characteristics of the attachment substratum affect the strength of attachment so that different microorganisms will detach at different rates (Eginton *et al.*, 1995). Therefore, a surface exposed for a period of time to food could select from this food a specific microbial type. Glass surfaces in milk will select for *Pseudomonas* spp. (Dunsmore and Bates, 1982). In a milk processing system, *Acinetobacter* spp. predominated in the attached microflora, even though the raw milk flowing through the system had a predominately gram positive microflora (Lewis and Gilmour, 1987).

E. EFFECT OF FOOD MATRIX

Food carrying microorganisms can have an effect on attachment by coating either the attachment surface or the microbial cell. Serum proteins will coat hydrophobic polymeric surfaces such as nylon, polyethylene and polyvinyl chloride making them more hydrophilic and thus reducing attachment of hydrophobic bacteria such as *S. aureus* (Carballo *et al.*, 1991). Surfaces exposed to milk will develop a surface film of milk proteins that affects microbial attachment (Kirtley and McGuire, 1989). Skim, 2% fat, chocolate milk, and diluted whole milk all inhibited attachment of *L. monocytogenes* and *Salmonella typhimurium* to stainless steel and buna-N gasket material, a result attributed to the adsorption of casein and whey proteins (Helke *et al.*, 1993). Suarez *et al.* (1992) found that whole milk inhibited attachment of bacteria to various surfaces, whereas Speers and Gilmour (1985) found no effect of whole milk on attachment, and reported that lactose and non-casein protein enhanced attachment. Diluted proteins also reduced attachment to glass surfaces (Meadows, 1971). However, Czechowski (1990a) observed that bacteria in dilute solutions attach more readily than those suspended in normal strength milk. Further investigations are needed to determine why milk inhibits microbial attachment whereas many milk components or diluted milk promote attachment. This phenomenon does appear to be related to cellular changes in surface chemistry (Suarez *et al.*, 1992).

F. SIGNIFICANCE IN FOOD PROCESSING SYSTEMS

Since all raw foods contain microorganisms, and since most, if not all, microorganisms have some ability to attach to food contact surfaces, we can expect that food processing equipment will, over time, develop a population of attached microorganisms. This population may develop at the equipment surface or within food residues deposited on the surface. Austin and Bergeron (1995) observed microbial attachment to rubber and Teflon gaskets in a dairy processing line, in spite of the proper application of cleaning and sanitizing procedures. Older gaskets allow for greater attachment. Most microorganisms attach to gaskets at the inner edge, where the gasket is in contact with the product (Czechowski, 1990b). *Pseudomonas* can attach to stainless steel equipment soon after milk is introduced and remains attached during processing (Stone and Zottola, 1985). Optimum cleaning and sanitizing conditions are required to inactivate and remove these organisms. An acid rinse assists in removal of the attachment polymer, perhaps by chelating divalent cations required to link the polymer to the surface.

When assessing the significance of attached microorganisms in a food processing system, the potential for growth of the attached microflora should be assessed. For example, in some dairy processing operations, hot product flows over a surface without interruption for periods often greater than 16 hours. Under such conditions, *Bacillus* spp. or other thermoduric microorganisms can not only attach, but also grow to significant numbers (Hull *et al.*, 1992; Lehman, 1992). A variety of bacteria including a coliform, a streptococcus and an aerobic sporeformer attached to the interior wall of a heat exchanger tube that was heating milk to 80°C within 1.5 minutes (Langeveld *et al.*, 1995). Attachment was a function of wall temperature. When growth can occur on a surface, the extent to which microorganisms attach becomes less relevant. Attachment capabilities of microorganisms generally vary by less than 100-fold, a difference that becomes less significant after a few generations of growth. Since no systems exist to completely prevent microbial attachment, control must be achieved by lowering the growth rate of attached cells, or effective cleaning and sanitizing of the system before significant surface growth occurs. In many systems, build-up of food residues that provide a microbial growth niche is a greater problem than microbial attachment. A system that is designed so that food residues accumulate on its surfaces must be effectively cleaned at intervals short enough to insure that high microbial loads do not contaminate the final product. Attachment and growth of pathogenic microorganisms on the exterior surfaces of equipment can also be a hazard. The risk of these microorganisms contaminating the product depends on the degree of physical protection the processing system provides the product as well as the extent to which the pathogen will grow on the contaminated surface.

V. ATTACHMENT TO FOOD TISSUES

A. MEAT TISSUE

When bacterial suspensions contact meat tissue, some attachment occurs immediately and most occurs in the first minute (Butler *et al.*, 1979; Bouttier *et al.*, 1997). This indicates that initial attachment is not a physiological response of the bacteria to surface contact. Piette and Idziak (1992) confirmed this by demonstrating that inactivating cells have no effect on their attachment to tendon. Different bacterial species have different abilities to attach to meat, for example, *Pseudomonas* spp. attach in high numbers (Firstenberg-Eden, 1981; Farber and Idziak, 1984; Kim and Slavik, 1994) whereas lactobacilli attach poorly (Piette and Idziak, 1989).

In addition, different types of meat tissue allow attachment at different levels. Firstenberg-Eden *et al.* (1978) found that chicken breast fascia allows the greatest attachment with cut chicken muscle, cut beef muscle, cow teats, and beef fascia allowing attachment in descending order. The number of bacteria that attach to meat is proportional to the concentration of cells in the surrounding suspension (Chung et al, 1989; Dickson, 1991). Attachment sites are sufficiently plentiful that competition between bacterial types for these sites is not significant (Chung *et al.*, 1989). Following initial attachment, most bacteria are capable of increasing the strength of the attachment bond by production of extracellular polymers (Firstenberg-Eden, 1981).

1. Relative attachment to lean and adipose tissue

Most studies indicate that bacteria attach equally well (generally within 0.5 log units) to adipose and muscle tissue (Chung *et al.*, 1989; Piette and Idziak, 1989; Benedict *et al.*, 1991; Dickson and MacNeil, 1991; Dickson and Frank, 1993; Cabedo *et al.*, 1997). These studies included *E. coli* 0157:H7, *Salmonella* spp., *Pseudomonas* spp., *Enterococcus faecalis L. monocytogenes S. aureus* and others. Although, Bouttier *et al.* (1997) and Dickson (1991) observed significantly greater attachment of bacteria to muscle as compared to fat, the difference was less than 0.5 log units. The similar abilities of adipose and muscle tissue to attract bacteria can be attributed to both tissue types having hydrophobic and hydrophilic attachment sites, and the bacteria utilizing multiple attachment mechanisms. Dickson (1991) observed that bacteria grown at 25°C attached to fat in higher numbers than bacteria grown at 37°C or at 37°C followed by refrigeration at 5°C, whereas attachment to lean tissue was unaffected by growth temperature. Although the effects observed in this study were relatively small, they indicate that different mechanisms predominate in the attachment to different tissue types.

2. Attachment and physical entrapment

Bacteria on meat tissues not removed by thorough rinsing are often considered attached. In fact, only a portion of these cells are truly attached; others are entrapped between muscle fibers and collagen bundles (Benedict *et al.*, 1991). Generally, meat exposed to bacterial cells is simultaneously exposed to aqueous solutions causing the tissue to take up water and swell. Bacterial cells are drawn into the tissues with the water (Thomas and McMeekin, 1984) and entrapped between swollen fibers (Thomas and McMeekin, 1981a). The relative importance of physical entrapment as

opposed to true attachment is unknown, but evidence indicates that entrapped cells are difficult to remove (McMeekin *et al.*, 1984). Scanning electron micrographs (SEM) appear to show entrapped cells in meat tissue, but some entrapped cells may appear to be attached due to the dehydration effects of sample preparation. (The potential for confocal laser scanning microscopy to overcome this problem will be discussed in a later section.)

3. Importance of bacterial motility to attachment

The presence of flagella may assist in bacterial attachment, but observed effects are often small or insignificant. Piette and Idziak (1991) found that adherence to tendon was greater for flagellated bacteria. Bouttier *et al.* (1997) observed that attachment to beef tissue was reduced after removal of flagella, but found no evidence for specific flagellar attachment sites. Lillard (1986b) and Fratemico *et al.* (1996) found that flagella and fimbriae (pili) had no effect on attachment. Farber and Idziak (1984) observed that nonmotile bacteria attached to meat tissue in fewer numbers, but with greater strength than motile bacteria.

4. Attachment sites

Studies with *Salmonella*, *E. coli* 0157:H7 and *Pseudomonas fluorescens* indicate that bacteria preferentially attach to connective tissue with collagen fibers being the preferred bacterial attachment sites (Benedict *et al.*, 1991; Piette and Idziak, 1992, Walls *et al.*, 1993; Fratemico *et al.*, 1996). Given a sufficiently high cell concentration, bacteria will almost completely cover a tendon surface, indicating that any specific binding is to either collagen or proteoglycan molecules (Piette and Idziak, 1992). *E. coli* and enteropathogenic *Yersinia* have outer membrane proteins that mediate specific binding to collagen and fibronectin (Parry and Craig, 1984; Ljungh *et al.*, 1991; Schulze-Koops *et al.*, 1992, 1993). Lipoteichoic acid of streptococci mediates adherence to fibronectin, a mechanism that could apply to many gram positive bacteria (Courtney *et al.*, 1983). *S. aureus* also has specific binding sites for collagen and fibronectin (Speziale *et al.*, 1986). Evidence for different binding mechanisms for gram positive and gram negative bacteria has been supplied by Vercellotti *et al.* (1985), who found that gram positive but not gram negative bacteria will aggregate with extracellular matrix proteins (fibronectin, laminin, and type IV collagen). Although lectins may be involved in specific binding mechanisms, mannose (a lectin binding agent) only slightly inhibited attachment of *Salmonella* to poultry meat (Benedict *et al.*, 1991).

5. *Nonspecific forces involved in the adherence of bacteria to muscle*

Specific binding to collagen or other connective proteins as described in the previous section is probably the predominant interaction responsible for bacterial attachment to meat. However, nonspecific electrostatic or hydrophobic interactions may also be involved. The significance of hydrophobic interactions is unclear, as cells with either hydrophilic or hydrophobic surfaces will adhere to meat tissue. Data of Dickson and Crouse (1989) showing that an electrical current increased attachment of *Salmonella* to meat is an indication that electrostatic forces are involved. Electrostatic interactions were not significant in attachment of S. *choleraesuis* to beef muscle (Bouttier *et al.*, 1997); however, Dickson and Koohmaraie (1989) found that the net negative charge on the cell surface correlated with attachment of S. *typhimurium*. Electrostatic and hydrophobic forces may play a greater role increasing the strength of the adhesive bond rather than increasing the numbers of attached cells (van Pelt *et al.*, 1985). Electrostatic interactions are dependent on the ionic strength of the surrounding medium. Adhesion of P. *fluorescens* increases slightly as the ionic strength of the solution increases (to <100 mM) regardless of the cations present (Piette and Idziak, 1992). Piette and Idziak (1992) interpreted the reports of Stanley (1983), Jones *et al.* (1981), Ørstavik (1977) and Marshall *et al.* (1971) that adhesion increases with increased concentration of divalent or monovalent cations as confirming this conclusion. Ions in solution reduce the thickness of the electrical diffuse double layer on each surface which allows negatively charged cells to more closely approach the negatively charged meat tissue. Consequently other attractive forces (i.e. van der Waals forces) or surface appendages can overcome the force of electrostatic repulsion.

6. *Bacterial penetration of muscle after attachment*

As rigor develops, muscle fibers undergo radial shrinkage and pull away from surrounding endomysia. The resulting gaps provide a route for bacterial penetration (Gill *et al.*, 1984). Cell motility enhances penetration especially in the presence of excess water, which may enlarge these gaps (Thomas *et al.*, 1987b). Proteolysis aids penetration (Gill and Penney 1977, 1982) but is not a requirement (Sikes and Macxy, 1980; Thomas *et al.*, 1987b). Thomas *et al.* (1987b) conclude that the fluid in gaps between muscle fibers in chicken meat presents little barrier to the passage of motile bacteria. They also found that the presence of proteolytic bacteria can speed up the penetration of nonproteolytic types. The ability of bacteria to penetrate meat tissues has significant implications for the effectiveness of

decontamination processes, as these cells will be difficult to remove and will be protected from the effects of sanitizing chemicals.

7. Microbial response and consequences of attachment

After attachment, bacteria often secrete exocellular polymers that are associated with an increased strength of attachment (Butler *et al.*, 1980). Under SEM (in the dehydrated form), these take the form of fibrils (Butler *et al.*, 1980, Schwach and Zottola, 1982; Yada and Skura, 1982). The presence of an extracellular polymer may have significance through its ability to protect cells from sanitizing chemicals and by making the attached cells more difficult to remove. The polymer-producing and tissue-penetrating ability of bacteria indicate the importance of rapid intervention if surface microflora on animal carcasses is to be removed or inactivated, as these protective mechanisms become more significant with time.

Bacteria attached to meat tissues and chicken skin are more resistant to heat (Notermans and Kampelmacher, 1975a), chlorine (Read *et al.*, 1975; Butler *et al.*, 1980) and acid washes (Tamblyn and Conner, 1997). This increased resistance might be a result of protective effects of the tissue environment or exocellular polymers.

8. Effect of cultural conditions

Bacteria grown in liquid media under near-optimal conditions tend to lose their ability to attach to inanimate, nonporous surfaces (Costerton et al., 1985). This is a result of loss of attachment mechanisms nonessential to growth in laboratory media. The significance of this phenomenon in bacterial attachment to meat tissues has not been extensively studied. Notermans *et al.* (1980) found that bacteria from feces of freshly infected chicks attached to chicken breast meat in higher numbers and more firmly than bacteria cultured in laboratory media. However, Cabedo *et al.* (1997) observed no difference in the attachment to beef adipose and muscle tissue of *E. coli* 0157:H7 grown in sterile manure extract as compared to tryptic soy broth. Bacteria in the environment are often exposed to nutrient limitations resulting in starvation stress. Such stress can stimulate attachment to environmental surfaces, allowing the cell to take advantage of nutrients adsorbed to these surfaces. However, Dickson and Frank (1993) found that starvation decreased attachment of bacteria to beef tissue.

9. *Transfer of adherent bacteria from meat tissues*

An inherent risk in handling raw meat is that pathogens associated with the product will contaminate cutting boards, utensils or other contact surfaces, thus spreading the pathogen to other foods and throughout the food processing/preparation environment. Schwach and Zottola (1982) demonstrated the ability of *Pseudomonas fragi* and native microflora to be transferred from beef to stainless steel. Transfer was demonstrated to occur in four hours at 4°C and was greater after 20 hours. Carson *et al.* (1987) observed transfer of *S. typhimurium* from chicken skin to stainless steel; 3–10% of firmly attached cells could detach and adhere to the metal. Dickson (1990) observed transfer of *L. monocytogenes* and *S. typhimurium* between beef surfaces. There was greater transfer from fat than lean for contact times of less than one minute and greater transfer from lean as opposed to fat tissue with greater contact times. The greater transfer potential of lean tissue at longer incubation times was ascribed to cells being released into the surface water film. Bacteria in longer contact times with meat tissue are expected to be more firmly attached, and therefore should be less likely to be released upon contact with another surface. In practice, this effect is not observed, perhaps because during incubation daughter cells are readily released from microcolonies produced on the tissue surface.

B. POULTRY SKIN

Poultry skin is composed of two tissues, the epidermis (outermost epithelial layer) and the dermis (inner layer) (Fig. 2). The outer portion of the epidermis is a layer of flattened non-living horny cells (*stratum corneum*), followed by a transitional layer (*stratum intermedium*) and a finally a germinal basal layer (*stratum basale* or *stratum germinativum*) (Matolsky, 1969). The dermis is mainly composed of fibrous connective tissue containing collagen, fibrous protein and elastin. Acidic glycoproteins are associated with the collagen (Spearman, 1971). The outer layer of the dermis is primarily collagen (Clark, 1993). Poultry skin is not smooth, but contains numerous crevices and feather follicles. The poultry skin surface can change markedly during carcass processing as a result of scalding, abrasion during defeathering, and water uptake.

1. *Attachment to poultry skin*

When poultry skin is immersed in a bacterial suspension, some cells become physically entrapped, others will adhere directly to the skin tissue,

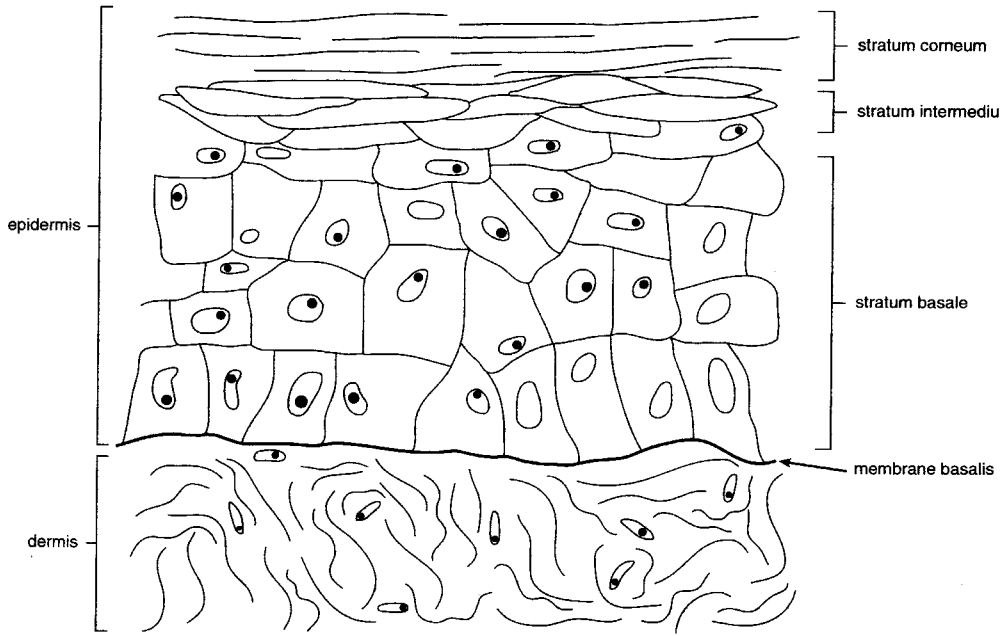

FIG. 2. Diagram illustrating the structure of chicken skin. (Adapted from Clark, 1993.)

and others will float in the water film coating the skin (Thomas and McMeekin, 1980). Large numbers of cells adhere rapidly and attachment increases with time (Lillard, 1985). Cells in the water film may eventually adhere to the skin unless removed by subsequent rinsing (Notermans and Kampelmacher, 1975b; Lillard, 1986a). However, unattached spoilage microflora will grow in this water film (McMeekin *et al.*, 1986). Modification of chicken skin by treatment with papain, lipase, formalin, or trisodium phosphate has no effect on ultimate attachment (after 30 to 60 minutes) although papain treatment increased initial attachment (Kim *et al.*, 1996a). Flagella and fimbriae do not appear to have an important role in adherence to chicken skin (McMeekin and Thomas, 1978; Lillard, 1986b, 1989) though earlier work of Notermans and Kampelmacher (1974) indicated otherwise. Inactivated cells attach to skin as readily as live cells (Kim *et al.*, 1996c). Cells grown under different conditions to produce different surface characteristics (as determined by surface charge) attach equally well (Kim *et al.*, 1996c). The ultimate number attaching depends on the concentration of cells in the suspending medium (McMeekin and Thomas, 1978). For initial attachment to and entrapment in poultry skin, bacteria act as inanimate particles.

Physical entrapment of bacteria on chicken skin is related to water uptake (Thomas and McMeekin, 1984). Water absorption causes capillary-size channels to open in surface layers. Cells can be observed within these crevices and channels after rinsing (McMeekin *et al.*, 1979; Lillard, 1988a; Kim *et al.*, 1996b). Confocal micrographs show *Salmonella* deep within skin crevices, where they are presumed to be protected from removal by rinsing or chemical inactivation (Fig. 3). *Salmonella* on hydrated skin can be seen floating freely deep within feather follicles with other cells apparently adhering to the interior follicle surface (Fig. 4). *Salmonella* can penetrate up to 200 μm into turkey skin (Kim *et al.*, 1993a), and up to 140 μm into chicken skin (Kim *et al.*, 1996b).

2. *Effect of process treatments*

Process treatments can alter microbial attachment by changing surface topography, exposing the underlying dermis, and opening and exposing crevices via hydration. Skin hydration is unaffected by salt or pH (Thomas and McMeekin, 1982). Thomas *et al.* (1987a) found that soft scald process did not cause significant removal of the epidermis, but did cause partial separation of the stratum corneum and damage to the underlying tissue, especially that close to the epidermal–dermal boundary. Steam spray defeathering produced more epidermal damage than conventional scalding (Kim and Doores, 1993a). Lillard (1985) observed that

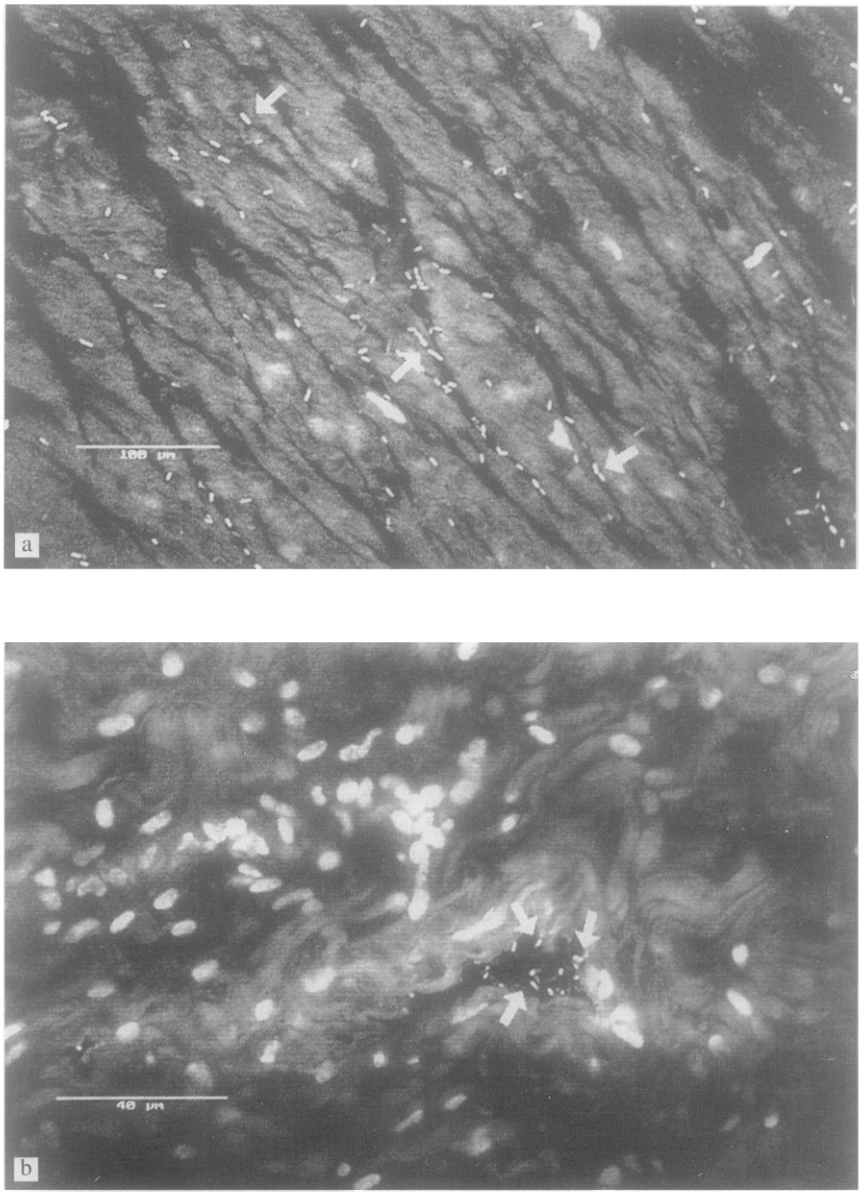

FIG. 3. CSLM micrograph showing *Salmonella typhimurium* on chicken skin. Top micrograph is at the skin surface (bar = 100 µm); bottom micrograph is 30 µm below the skin surface showing *Salmonella* within a feather follicle (bar = 40 µm). Arrows point to *Salmonella* cells. (From Kim *et al.*, 1996b.)

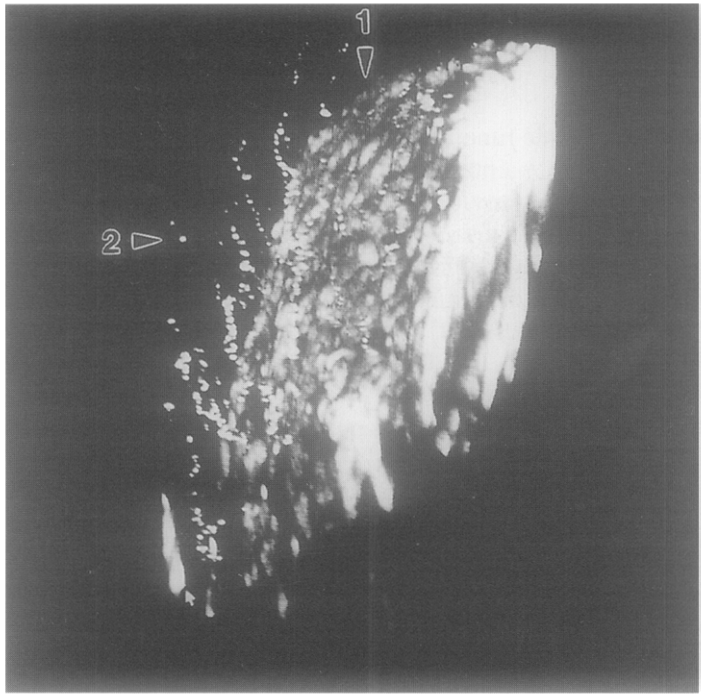

FIG. 4. Three-dimensional volume reconstruction of an interior portion of a chicken feather follicle showing entrapped *Salmonella.* Arrow 1 points to the interior wall of the follicle showing adherent cells; arrow 2 points to cells floating in liquid away from the follicle wall. The image is 142 μm in height. Optical thin sections were collected using a stepping motor increment of 0.3 μm. (From Kim *et al.*, 1996b.)

soft scald (52°C) and hard scald (57°C) treatments did not lead to differences in attachment. Kim *et al.* (1993b) did not observe loss of epidermis due to soft (52°C) or hard (56°C) scald, but noted losses at 60°C. *Salmonella* attachment was 1.1 to 1.3 log higher for the 60°C scald skin as compared to skin treated at lower temperatures. *Salmonella* attach more readily to the dermis than the epidermis. Attachment to the dermis is probably related to exposure of collagen fibers (Kim and Doores, 1993a,b). The *stratum corneum* is more hydrophobic than the underlying tissue and may be imbibed with sebaceous material, but bacteria adhere to it probably because of entrapment within the rough layers (Kim *et al.*, 1993b). Defeathering systems that allow retention of epidermis lead to less bacterial attachment and reduce penetration of bacterial cells into the skin (Kim *et al.*, 1993a,b).

C. PLANT TISSUE

1. Plant surfaces

Plant epidermal tissue functions as protection against infection, insect and physical damage, to maintain turgor pressure within the tissue by preventing water loss, and to provide for gas exchange between internal cells and the environment. An idealized plant surface is diagramed in Fig. 5. The epidermis is typically covered with a multilayered hydrophobic cuticle 1 to 15 μm thick. The main cuticle layer consists of cutin, high molecular weight lipid polyesters of long chain, substituted aliphatic acids (Romberger *et al.*, 1993). The cuticle is embedded with crystalline or amorphous aliphatic wax. This produces a highly water-repellent surface. Gas exchange is accomplished through pores in the epidermis called stomata. Stomata are surrounded by guard cells that open or close the pore in response to environmental stimuli. Trichomes are protuberances from the surface. They have a variety of shapes and functions, and if numerous enough can impart a hairy surface that acts as an antiwetting agent and discourages mold spores and insects from direct contact with the cuticle. Stomata and trichomes are found to different degrees on all plant surfaces, including fruits and leaves. Young fruit have a certain number of stomata which does not increase, so as the fruit enlarges they grow further apart and may become difficult to locate (Blank, 1986). Trichomes on fruit and leaves often break off leaving behind a stump or basal area (Getz *et al.*, 1983). Some trichomes excrete sticky substances which may collect bacteria.

2. Bacterial attachment to plant surfaces

Unlike animal tissue, plant surfaces only sparingly allow microbial attachment. Microscopic investigations of bacterial attachment to plant tissue

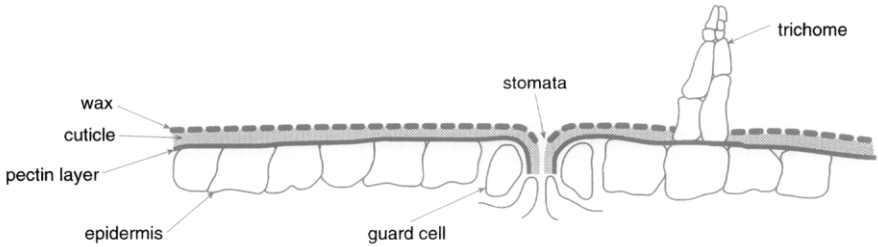

FIG. 5. Diagram illustrating the surface layers of a plant. (Adapted from Fahn, 1990.)

have been mainly concerned with determining how plant pathogens initiate infection. However, the investigation of O'Brien and Lindow (1989) provides evidence that there is little association between attachment ability and phytopathogenicity. If so, then plant pathogen-oriented studies could provide clues as to how human pathogens interact with plant surfaces. SEM shows that the initial attachment of plant pathogens is often at the stomata, broken trichomes, or cracks in the cuticle. *Pseudomonas syringae* enters pear leaves through the open base of trichomes and microscopic fissures in depressions of the cuticle, and to a lesser extent through the stomata (Mansvelt and Hattingh, 1987). Open trichome bases also serve as the point of initial attachment of *P. syringae* pv. *tomato* on tomato fruit (Getz *et al.*, 1983). Royle (1976) observed penetration of *Pseudoperonospora humuli* through closed stomata of the hop plant. Photomicrographs in Fig. 6 provide evidence that nonphytopathogenic *Pseudomonas fluorescens* interacts with plant tissues in a similar manner to that observed for phytopathogenic bacteria. Some microorganisms may infect plant tissue by dissolving the outer waxy layer, but this possibility has been little studied (Juniper, 1992). During storage, spinach leaves became colonized mainly in areas where the waxy cuticle is broken (Babic *et al.*, 1996). Lectins on root hairs may provide specific attachment sites for *Rhizobia* spp. (Matthysse, 1985).

There have been few observations of concern to public health of bacteria attaching to plant tissues. Itoh *et al.* (1998) observed *Escherichia coli* 0157:H7 adhering to the outer surfaces of radish sprouts produced from contaminated seeds. The organism was also internalized during sprout outgrowth. This pathogen also adheres to lettuce leaf, with preferential attachment to cut surfaces, broken trichomes and stomata (Seo and Frank, 1999). *E. coli* 0157:H7 grows on the exterior surface of intact melon rinds (Del Rosario and Beuchat, 1995), but growth could be in the water phase rather than as attached cells. Studies such as that of Zhuang *et al.* (1995), Wei *et al.* (1995) and Rafii *et al.* (1995) demonstrating survival of *Salmonella* and *Shigella* on plant surfaces do not indicate attachment ability, since the surfaces were not rinsed to remove unattached cells after inoculation. The review of Beuchat (1996) indicates numerous pathogenic bacteria have been associated with fresh produce. His conclusion that washing and chlorination do not effectively remove or inactivate pathogens associated with fresh produce is indirect evidence that pathogens adhere to or enter tissues, thus becoming resistant to removal efforts. Bartz and Showalter (1981) demonstrated that if warm tomatoes are immersed in cool water, spoilage bacteria in the water will be taken up into the interior of the fruit, primarily through the stem. Similar observations by Zhuang *et al.* (1995) demonstrated internalization of *Salmonella* on the tomato. This

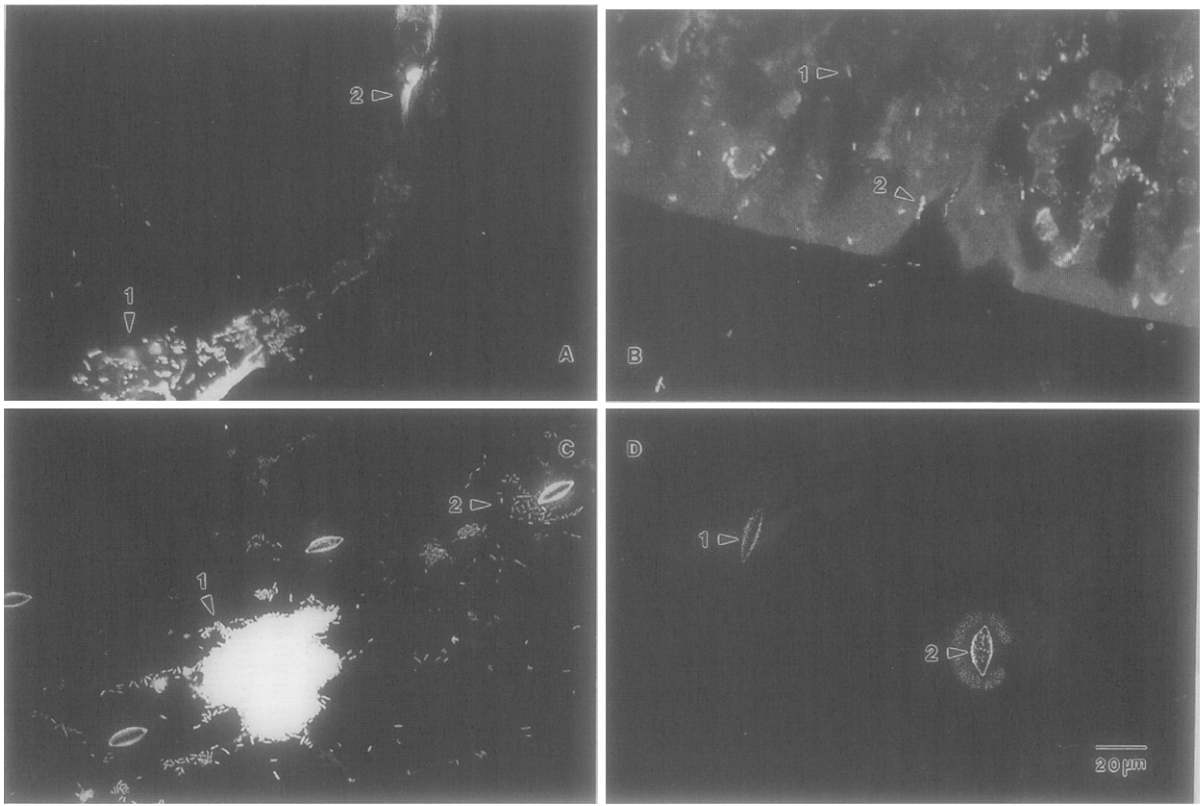

FIG. 6. CSLM micrographs (optical thin sections) showing the interaction of psychotrophic *Pseudomonas fluorescens* with an iceberg lettuce leaf. The leaf was inoculated by immersion in a cell suspension (10^7/ml deionized water) for 48 h at 7°C followed by two water rinses. A. Arrow 1: Bacteria attached to base of broken trichome at leaf surface. Arrow 2: Tip of broken trichome rising above the leaf surface. B. Arrow 1: *Pseudomonas* in leaf interior. Arrow 2: *Pseudomonas* leaving a track as it enters the cut edge of a leaf. *Pseudomonas* cells can also be seen moving freely in the liquid next to the cut edge. C. Arrow 1: A *Pseudomonas* microcolony on the leaf surface. Arrow 2: Cells attached near a stomata. D. Arrow 1: An uninfected stom-

is presumably due to the contraction of gases within the tissue causing fluid uptake. These studies indicate that surface decontamination treatments for fresh produce must be applied in conjunction with procedures to prevent microbial infiltration to the internal tissues.

VI. PROPERTIES OF ATTACHED CELLS

Attached cells often behave differently than their free-living counterparts. Attachment may increase resistance to inactivation treatments, stimulate exopolymer production, and alter metabolism. These effects are of significance to food safety; pathogens attached to food contact surfaces and food tissues are more difficult to inactivate; exopolymer production makes pathogens more difficult to remove; and altered metabolism may influence spoilage rate.

A. SANITIZER RESISTANCE

Increased sanitizer resistance of cells attached to nonporous surfaces is associated with development of the protective glycocalyx associated with biofilms (Wirtanen and Mattila-Sandholm, 1992). DeBeer *et al.* (1994) demonstrated that the penetration of chlorine into a biofilm is limited, and probably responsible for the ability of biofilm cells to survive the treatment. However, the process of attachment by itself may also provide some protection. Attachment to particles is associated with protection from chlorination for *Enterobacter cloacae* (Herson *et al.*, 1987). A four-hour attachment period is sufficient to impart some resistance to quaternary ammonium, acid anionic and chlorine sanitizers for *L. monocytogenes* (Frank and Koffi, 1990; Lee and Frank, 1991). *L. monocytogenes* attached to chitin survived sanitation processes (McCarthy, 1992). Attachment to glass or stainless steel produced only a marginal increase in resistance of *S. enteritidis* to sanitizers (Dhir and Dodd, 1995). The type of underlying surface can influence the effectiveness of a sanitizing treatment on attached cells (Krysinski *et al.*, 1992; Frank and Chmielewski, 1997). The inability of sanitizer treatments to inactivate cells on organic porous surfaces (such as meat or poultry skin) is readily explained by the protection the cells receive by being entrapped within the surface structure limiting their exposure to the sanitizing chemical. However, even sanitizer resistance of cells on relatively smooth nonporous surfaces may be associated with lack of intimate contact, rather than changes in cell physiology. For example, when Frank and Koffi (1990) detached and dispersed adherent cells, the cells regained sanitizer susceptibility.

B. HEAT RESISTANCE

Heat inactivation of microorganisms is affected by the surrounding environment. Since bacteria attached to muscle or skin tissue are in a more protective environment (high in dissolved substances), the results of Notermans and Kampelmacher (1975a) showing increased resistance of *Salmonella* attached to chicken skin are not surprising. Heat inactivation of *Salmonella* on chicken skin is not logarithmic, there being a significant tailing effect. The seemingly different heat resistance of the attached population is probably due to the variety of different microenvironments in which the cells are located. Heat resistance was not associated with exopolymer production (Notermans and Kampelmacher, 1975a). *L. monocytogenes* attached to glass and stainless steel exhibited increased heat resistance (Frank and Koffi, 1990; Lee and Frank, 1991), perhaps a result of tailing caused by adherent clumps or microcolonies. However, Dhir and Dodd (1995) observed that the increased heat resistance of attached *S. enteritidis* was maintained in the detached, dispersed cell population. This observation indicates an adaptive response based upon selective gene expression during attachment.

C. EXOPOLYMER PRODUCTION

Bacteria often respond to attachment by producing exocellular polymeric materials, usually polysaccharides, that produce a stronger bond to the surface. This material becomes the glycocalyx of the resulting biofilm. Production of attachment polymers can result from genes activated as a direct response to attachment (Davies *et al.*, 1993) or as a response to dehydration of the attached cells (Roberson and Firestone, 1992). The presence of a stainless steel attachment surface increased the carbohydrate content of extracellular polymeric substances produced by *Desulfovibrio desulfuricans* but not *P. fluorescens* (Beech *et al.*, 1991). Beech *et al.* (1991) also observed that the type of attachment surface can influence the amount of exocellular polymeric material produced.

D. MICROBIAL ACTIVITY

Since the observations of Zobell (1943) and Heukelekian and Heller (1940), attachment has been observed to produce various effects on microbial activity. These have been reviewed by Fletcher (1985). The reported effects of attachment as summarized by van Loosdrecht *et al.* (1990) include: increased or decreased growth rate, increased or decreased substrate assimilation, increased or decreased respiration, lowered substrate affinity, decreased mortality, increased productivity and changed fermentation

pattern. Van Loosdrecht *et al.* (1990) concluded that attachment can affect microbial activity, but these effects are not predictable with existing knowledge. They also point out that reversibly attached cells (initial adhesion) are too far from the surface for the surface to have a direct effect on the cell. In addition, only a very small portion of the cell surface is in direct contact with the layer of nutrients adsorbed onto the surface. Consequently, it is unlikely that surface attachment has direct influence on cell activity, rather the observed effects of attachment are more likely due to the ability of the surface to affect the growth medium. Once attached cells reproduce, the arguments of van Loosdrecht *et al.* (1990) are less relevant, since the resulting biofilm produces its own environment, different from the underlying surface and the surrounding medium.

VII. DETACHMENT

The life cycle of microorganisms in natural systems involves attachment to a surface, growth on that surface to form a biofilm, and finally detachment with migration to new growth sites (Bar-Or, 1990a). Therefore, the ability to detach under appropriate conditions is an integral part of the survival strategy of many microorganisms. The ability of microorganisms to disperse from surfaces is of concern in food processing plants where products must be protected from microbial contaminants. Detached microorganisms are spread to food or food contact surfaces via aerosol, water, or surface contact (onto gloves, hands, utensils, etc.). Means to induce detachment are required by practical methods for detecting microbial contamination on both food and food contact surfaces. This section will discuss mechanisms involved in cell-directed detachment, and means employed to induce detachment.

A. CELL-DIRECTED

The initial attachment of microorganisms involves weak forces that are readily overcome. Consequently, reversibly attached cells are in a dynamic exchange with suspended cells, as cells constantly adsorb and desorb from the surface (Bar-Or, 1990a). Adsorbed cells that become firmly attached must act to neutralize the adhesive bond when environmental conditions require seeking a new growth site. Detachment is often a response to starvation (Gilbert *et al.* 1993). When *P. fluorescens* is attached to a hydrophilic surface (glass), and subject to starvation, cells actively detach by becoming more hydrophobic (Delaquis *et al.*, 1989). When growing on oil, *Acinetobacter calcoaceticus* produces emulsan, an amphiphilic

polysaccharide. When nutrients in the oil droplet are completely utilized, the organism detaches the emulsan from the cell surface by producing an exocellular esterase. The cell then floats free of the used droplet in search of another (Bar-Or, 1990a; Neu, 1996). *Pseudomonas aeruginosa* produces extracellular alginate, which increases its biofilm-forming ability. Detachment is controlled by the production of alginate lyase (Boyd and Chakrabarty, 1994). *Clostridium thermocellum* adheres to cellulose, but when glucose accumulates (repressing cellulose utilization) the organism detaches (Bar-Or, 1990a). In addition to enzymatic hydrolysis of the binding exopolymer, bacteria can reverse the attachment process by changing the orientation of surface-active molecules excreted to the cell envelope (Neu, 1996) or change the surface-active characteristics of their cell envelope by synthesizing new components (Bar-Or *et al.*, 1985).

Daughter cells of attached bacteria are often released from the surface upon completion of cell division. Gilbert *et al.* (1993) suggest that this is related to changes in the cell surface associated with the division process. The released daughter cells of attached *E. coli* and *P. aeruginosa* are more hydrophilic than their attached counterparts (Allison *et al.*, 1990a,b). Upon release from the surface, daughter cells may return to the original growth surface and attach.

B. EXTERNALLY DIRECTED

1. Enzymatic release

Breaking attachment bonds using enzymes has been employed to detach microorganisms from both biological and inert surfaces. Brisou (1995) authored an extensive report on the use of enzymes to hydrolyze exocellular polymers resulting in the release of attached microorganisms from food tissues and other surfaces. Enzymes employed included cellulase, amylase, glucoronidase, and hyaluronidase. Trypsin and amylase improved the release of bacteria attached to granular activated carbon, but enzymatic release was not greater than that achieved by homogenization (Camper *et al.*, 1985). Release of microorganisms from food tissues by enzyme treatment may be due as much or more to fragmentation and hydrolysis of tissues entrapping the cells as it is to dissolution of attachment polymers.

2. Oxidation of attachment polymer

Strong oxidizing agents such as 5% sodium hypochlorite will dissolve the polymer material surrounding attached cells (Shaw *et al.*, 1985). However, a more realistic treatment with 50 ppm hypochlorite had no effect

(Schwach and Zottola, 1984). Oxidizing agents that inactivate attached microorganisms will not necessarily remove the associated polymers.

3. Physicochemical detachment

Partial detachment of bacteria can be obtained by physicochemical interventions. Mechanical forces from flowing liquid can assist removal of attached bacteria from food tissues and food contact surfaces (Wirtanen *et al.*, 1996). Rinsing of chicken carcasses will only remove a small portion of the total attached microflora (Lillard, 1988b). De Zuniga *et al.* (1991) found that high pressure was no better than low pressure rinses in removing surface microorganisms from meat tissue. Ultrasonic energy will cause detachment, in some cases without damaging the microorganisms (Puleo *et al.*, 1967; Jeng *et al.*, 1990). Surfactants, such as Tween 80, and detergents can also assist removal (Corpe, 1973; Ørstavik, 1977). McEldowney and Fletcher (1988) found that temperature and adsorbed layers of organic molecules had no effect on the detachment of bacteria on food container and food processing surfaces, whereas Lewis *et al.* (1989) reported that increasing temperature and pH increased detachment of *Acinetobacter* from stainless steel. Chelating divalent cations also assists detachment from food contact surfaces, probably by dissociating exocellular polymers (Turakhia *et al.*, 1983; Lewis *et al.*, 1989). Neutralization of electrostatic forces using salt solutions has only limited effectiveness in causing detachment from animal tissues (Thomas and McMeekin, 1981b; Appl and Marshall, 1984; Dickson, 1988; Piette and Idziak, 1992). Attempts at removal of bacteria from meat surfaces by means that preserve the integrity of the tissue have had limited effectiveness (Piette and Idziak, 1992). Treatments utilizing hot water, steam, chlorine, trisodium phosphate, sodium hydroxide and short chain organic acids have been employed with various degrees of success for decontamination of animal carcasses (Dickson, 1988; Dickson and Naderson, 1992; Lillard, 1994; Slavik *et al.*, 1994; Hardin *et al.*, 1995; Dorsa *et al.*, 1996; Fratemico *et al.*, 1996). Although partial removal of bacteria is accomplished by these treatments, the relative importance of removal as opposed to inactivation of attached cells has not been investigated. In addition, treatments that remove or inactivate attached microflora will not necessarily effectively remove or inactivate microorganisms entrapped with tissues.

VIII. DETECTION OF ATTACHED MICROORGANISMS

Since attached microorganisms in a food processing plant can have deleterious effects on product quality and safety, detection of attached

microorganisms is required for effective monitoring of cleaning and sanitation efforts. Conventional methods rely on detaching the microorganisms using a swab or sponge followed by plating the suspension fluid to produce countable colonies (Hickey *et al.*, 1992). For more rapid detection, plating can be replaced by ATP analysis (Bautista *et al.*, 1992, 1995; Siragusa and Cutter, 1995; Siragusa et al., 1995), direct epifluorescent filter technique (Holah *et al.*, 1988, 1989) or polymerase chain reaction to detect specific pathogens (Rafii *et al.*, 1995). Since, under most conditions, an unknown proportion of the surface microflora are tenaciously bound or entrapped, detachment techniques that preserve the underlying surface (whether food or equipment) as well as the viability of the detached microflora, will detach only a portion of the total population. Therefore, rinse methods for food tissues produce lower microbial counts than stomaching or blending (Cox *et al.*, 1976). Effective removal of microflora from inert surfaces (with viability retention) can be achieved by use of ultrasonic energy (Puleo *et al.*, 1967; Jeng *et al.*, 1990), but this application is generally impractical in the industrial setting. Lindsay and von Holy (1997) compared ultrasonication, vortexing, and shaking with beads for detachment of biofilm bacteria from stainless steel and polyurethane. Although the resulting bacteria counts were similar, microscopic analysis indicated that shaking with beads left the least residual cellular material.

Various procedures have been developed for determination of viable attached microflora that do not require cell detachment. Since these procedures involve direct microscopic examination of the surface or direct agar overlays, they are suitable only for use as research tools. Most have been applied only to smooth inert surfaces. Low levels of viable attached microflora can be detected by pouring agar-containing media directly on the test surface, followed by incubation (Angelotti and Foter, 1958). This method was adapted by Frank and Chmielewski (1997) for determining residual attached microflora on various surfaces after sanitizer treatment. However, numbers greater than 10–50 cfu/cm^{-2} are difficult to enumerate due to colony crowding. When sufficient attached microflora is present (about 10,000 cells/cm^{-2} or more) direct epifluorescent microscopic observation can be used. This works best when combined with image analysis techniques (Blackman and Frank, 1996). Physiological responses indicative of viability can be observed in attached populations by using fluorescent probes such as 5-cyano-2,3-ditolyl tetrazolium chloride and rhodamine 123 (Schaule *et al.*, 1993; Yu and McFeters, 1994; Stewart *et al.*, 1994; Huang *et al.*, 1995). Adherence to nonbiological surfaces can be also be determined by using enzymatic markers (Dunne and Burd, 1991).

IX. INHIBITION OF ATTACHMENT

Limited success in inhibiting attachment to nonporous materials has been achieved. Surface coatings can inhibit attachment of some microorganisms, but eventually, in an open system, selected cells will attach and colonize. Dissolved proteins reduced attachment of *Pseudomonas* to polystyrene (Fletcher, 1976) and *Salmonella* and *Listeria* to stainless steel and Buna-N rubber (Helke *et al.*, 1993). However, in other studies, adsorbed proteins have increased microbial attachment (Meadows, 1971; Carballo *et al.*, 1991). Adsorbed proteins can change both the electrostatic and hydrophobic properties of a surface, so the precise nature of the inhibition is not known. Changes in surface hydrophobicity resulting from adsorption of bovine serum albumin and β-lactoglobulin to silica can help explain decreased attachment of *L. monocytogenes* (Al-Makhlafi *et al.*, 1994, 1995a). Basic proteins, protamine and histone did not inhibit attachment to polystyrene, indicating an important role for surface charge (Fletcher, 1976). Electrostatic forces involved in adhesion can also be neutralized by high electrolyte concentrations in the surrounding medium (Gordon and Miller, 1984). Interference with electrostatic attractive force is the basis for using electrical stimulation in salt solutions to inhibit attachment of *Salmonella* to chicken skin (Li *et al.* 1994).

Nonspecific inhibition of microbial attachment has been achieved by using graft co-polymers consisting of outward-pointing polyethylene glycol side chains with charged backbones (Humphries *et al.*, 1987). Glass, stainless steel, and hydroxyapatite were used as substrata and *Pseudomonas, Serratia,* and *Streptococcus mutans* were the test microorganisms.

Surfactants can act as antiadhesive agents in biological systems. Erne *et al.* (1984) used a phospholipid-cholesterol surfactant to inhibit adhesion of *Klebsiella pneumonia* to lung epithelial cells. Velraeds *et al.* (1996) determined that the mechanism by which *Lactobacillus* inhibited attachment of uropathogenic *Enterococcus faecalis* to glass was through production of surfactant. Preconditioning a surface with a surfactant-producing microorganism can result in deposition of a surfactant film which reduces attachment of other microorganisms (Cowan and Busscher, 1993).

Another approach to inhibit attachment is to alter cell surface structures. Chlorine treatment of enterotoxigenic *E. coli* resulting in sublethal injury also reduced attachment to leukocytes, probably by damaging specific adhesins (Walsh and Bissonnette, 1983). Such an approach is not effective when adhesion is mediated by nonspecific mechanisms, such as attachment of *Salmonella* to chicken skin (Kim *et al.* 1996c).

Although various procedures can dramatically reduce attachment of microorganisms, if conditions permit growth, any effects of attachment

inhibition would be rapidly overcome by surface growth of the few cells that overcome the attachment barrier. The approaches of Daeschel *et al.* (1992) and Bower *et al.* (1995) of adsorbing antimicrobial proteins onto a surface, and of Wood *et al.* (1996) of generating biocide at the surface have the potential to overcome the limitations inherent in previous attempts at attachment inhibition.

X. USE OF CONFOCAL SCANNING LASER MICROSCOPY

Attachment of microorganisms to food tissues is a dynamic process, in which, over time, cells suspended in liquid at the tissue surface are taken up into pores and adsorbed onto tissue structures. At any given time there are populations of microorganisms in liquid suspension, reversibly or irreversibly attached, or physically entrapped in tissue structures. Confocal scanning laser microscopy (CSLM) provides a means for the direct observation and characterization of these microbial populations during and after the attachment process. Although, there currently is little published on the use of CSLM to study microbial attachment, the application of this technology has the potential to provide a deeper understanding of this process.

The principles underlying CSLM have been reviewed by Brackenhoff *et al.* (1988). The power of CSLM lies in its potential to image fully hydrated systems in their natural state. This is accomplished by obtaining optical thin sections of the specimen using focused laser light which scans the field, and a pinhole detector to remove out-of-focus light. Since only light emanating from the focal plane is collected, the resulting image has little depth of field, but is highly focused. Depth of image can be achieved by collecting optical sections at different sample depths and using computer software to combine them into a "stacked" image that projects the three-dimensional data in two dimensions. Software is also available for obtaining three-dimensional volumetric projections. Most imaging with CSLM relies on the use of fluorescent molecular probes and dyes. Most often, microorganisms are stained with nucleic acid or protein-specific fluorochromes. Fluorescent antibodies can be used if species specificity is required. Temporal data on microbial interactions with foods can be obtained by the use of nontoxic fluorescent probes. For example, Hassan *et al.* (1995) viewed the pH gradient between the casein and whey fractions of milk as it was converted to yogurt by lactic acid bacteria.

Applications of CSLM in food research have been discussed by Vodovotz *et al.* (1996), Blonk and van Alst (1993), and Heertje *et al.* (1987). Techniques for using CSLM in the study of microbial interactions with the environment have been reviewed by Caldwell *et al.* (1992a, b);

many of these techniques are applicable to food systems. Delaquis *et al.* (1992) were the first to observe microorganisms on food tissue surfaces via CSLM. They found that spoilage of porcine muscle was associated with the formation of microcolonies adhering to tissue surfaces. Microcolonies on leaf surfaces have also been observed using CSLM (Morris *et al.*, 1997; Seo and Frank, 1999). Kim *et al.* (1996b) used CSLM to observe the attachment of *Salmonella* to chicken skin. These data (Figs. 3 and 4) illustrate the ability of CSLM to characterize bacteria–tissue interactions. Cells can be seen adhering to grooves on the skin surface, adhering to internal tissues (inside a feather follicle) and floating freely in fluid within feather follicles. Since micrograph data is collected over a period of seconds, blurred images of bacteria indicate movement during data collection (Fig. 4). A similar characterization of bacteria–tissue interactions is illustrated by the images in Fig. 6. *Pseudomonas* can be seen moving in the water phase surrounding the leaf (Fig. 6), attached to and growing on the surface (Fig. 6a, c), and moving within the stomata (Fig. 6c). *E. coli* 0157:H7 behaves in a similar manner (Seo and Frank, unpublished observation). The importance of maintaining a water phase when observing attachment is illustrated by the study of Wong Liong *et al.* (1997) on the penetration of eggshell membranes by *Salmonella.* When excess water was removed from the membrane, cells that were previously observed free floating within membrane pores (Fig. 7) appeared to be adsorbed onto the surface of the membrane fibers. Since water is a crucial component for movement of cells into surface structures, observation of the attachment process and differentiation of attached from entrapped cells requires a fully hydrated specimen. When adherent cells are removed from tissue surfaces by stomaching or rinsing, information on whether they were attached or entrapped is lost. Distinguishing between entrapped and attached cells may have practical importance for decontamination effectiveness. Entrapment is not associated with specific cell surface structures or specific tissue attachment sites. Attached and entrapped cells are held by different forces and thus removal by rinsing or surfactant treatment may be different. In addition, attachment is known to increase sanitizer resistance, whereas the effects of entrapment have been less well studied. One report on the effectiveness of sanitizers against entrapped cells indicates that bacteria internalized within lettuce stomata are not as readily inactivated by chlorine treatment as those adhering to the leaf surface (Seo and Frank, 1999). Entrapped cells probably attach after a period of time. The amount of time this takes may be crucial to developing effective decontamination processes.

Future applications of CSLM in studying microbial attachment to food tissues will involve the use of viability stains or assays for the differentiation of live and dead cells, and the use of genetic transformants for cell

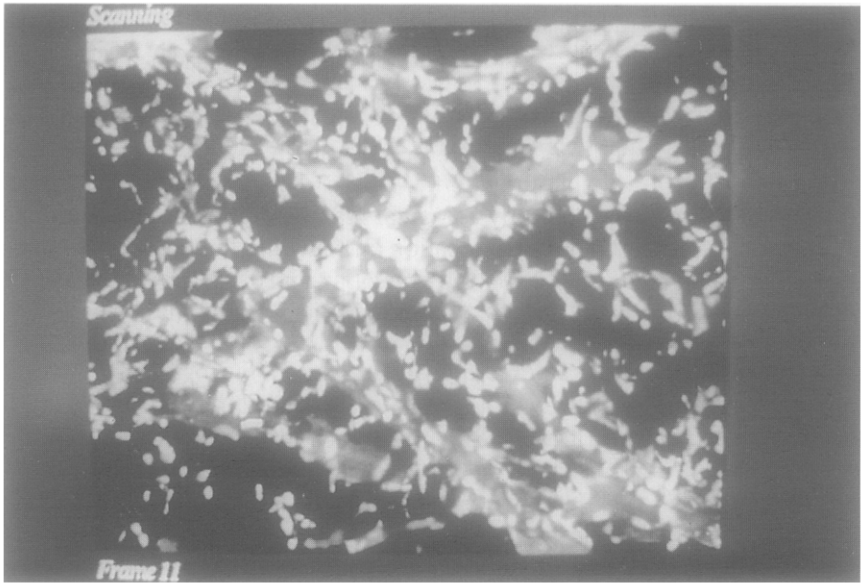

FIG. 7. Three-dimensional volume reconstruction showing *Salmonella* moving between the fibers of an outer eggshell membrane. The image depth is 50 μm. Optical thin sections were collected using a stepping motor increment of 0.3 μm. (From Wong Liong *et al.*, 1997.)

visualization. The current practice of staining adherent cells with nucleic acid binding fluorochromes inactivates the cells, limiting the type of information that can be gained. However, the movement of live pathogenic bacteria through and their association with tissues can be directly observed by using strains transformed to produce green fluorescent protein (Valvida *et al.* 1996). Such strains autofluoresce due to the continuous production of the fluorescent protein. Direct observation of viability or metabolic status can be achieved through the use of fluorochromes that detect membrane integrity (Live/Dead BacLight™ Bacterial Viability Kit, Molecular Probes, Eugene, Oregon, USA). Seo and Frank (1999) modified this technique by using a fluorescent antibody as a counterstain to visualize live cells. Dead cells take up propidium iodide stain. This technique produces intact live and dead cells that fluoresce different colors. Cellular uptake and reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) results in conversion of this compound to its fluorescent form. Nonviable cells are assumed to have lost their reducing potential, and therefore will not fluoresce (Rodriguez *et al.* 1992). The method of Kogure *et al.* (1979) for determining nonculturable but viable cells can also be readily adapted for confocal microscopy. Their

procedure involves incubating the sample with a concentration of naladixic acid that will allow viable cells to increase in size but inhibit division. Nonviable cells remain normal size. Abrishami *et al.* (1994) used this method in combination with scanning electron microscopy to determine that bacteria entrapped in a wood cutting board maintained viability. The application of CSLM in combination with viability assays has the potential to provide insights into the physiological status of an adherent microbial population in respect to physical location on or within the food tissue. Such information will be invaluable for the evaluation and development of surface decontamination treatments for both animal carcasses and fresh produce.

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