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Adhesion and immobilization of bacteria on hydrophobic cloths

by

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements
for the degree of Master of Science

Ottawa-Carleton Institute of Biology

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September 14th, 1998

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the Faculty of Graduate Studies and Research
acceptance of the thesis,

Adhesion and Immobilization of Bacteria on Hydrophobic Cloths

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Chair, Department of Biology

Thesis Supervisor

September 14, 1998

Carleton University

Abstract

Using *Escherichia coli* 3300 as a model bacterium, it was demonstrated that hydrophobic cloths such as polyester provide higher levels of adhesion than hydrophilic cotton, and thus allow for higher rates of bacterial immobilization. Immobilization of *E. coli* 3300 was demonstrated to be a two stage process, which consists of bacterial adhesion followed by bacterial growth inside the cloth structure. All the gram-negative and gram-positive bacteria examined exhibited greater adhesion on hydrophobic cloths. Since coating of the cloths with polyvinyl alcohol (PVA) greatly reduced adhesion levels, it was concluded that hydrophobic interaction is a dominant force involved in bacterial adhesion. The glutaraldehyde treatment of PVA-coated cloth greatly stabilized the PVA coating.

Acknowledgements

I would like to thank Dr. Hiroshi Yamazaki, my supervisor, for his patience and expert guidance throughout the course of this project, and for allowing me to acquire the understanding and knowledge of the practical aspects of research.

I would also like to thank Mrs. Ilona Fiser and Mr. Kouichi Matsumoto for their friendly presence and excellent technical assistance.

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Abbreviations

A_{420}	β -galactosidase activity
A_{500}	absorbancy at 500 nm
B 58	Brij 58
cm	centimetre
dH ₂ O	distilled water
C	Celsius
g	gram(s)
h	hour(s)
M	molar concentration
min	minute(s)
mg	milligram(s)
mL	millilitre(s)
MW	molecular weight
nm	nanometre(s)
o/n	overnight
ONPG	O-nitrophenyl- β -D-galactopyranoside
PVA	polyvinyl alcohol
rpm	rotations per minute
SDS	sodium dodecyl sulphate
sec	second(s)
w/v	weight per volume

v/v	volume per volume
Xgal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
μ L	microlitre(s)

Chapter 1

General Introduction

Bacterial presence and activity have important impacts on the human environment and the quality of our daily human life. Bacteria are usually associated with infectious diseases, food poisoning, and drinking water contamination, all of which have a detrimental effect on the health, economy, and prosperity of our society. At the same time, however, the scientific and technological advancements in the area of biology have allowed us, over the last few decades, to understand, control, and harness the biological activities of the bacterial cells, and put them into use in a variety of commercial applications, including the fermentation industry for the production of foods, pharmaceuticals, and chemicals, the commercial wastewater and industrial waste treatment technology, and the production of biomass (Linko and Linko, 1983; Kennedy and Cabral, 1983; Suzuki and Karube, 1983).

The use of biological catalysts on a commercial scale has been gaining more attention and momentum in the recent decades. The traditional use of non-renewable petroleum energy sources by the present industrial processes is not only the major cause of the pollution of the human environment, but is also susceptible to the eventual depletion of these resources. Therefore, it is of commercial and industrial significance to seek new and improved ways of utilizing the available energy sources for the future development of the nonpetroleum-based industry using solar energy and the renewable biomass for the production of foods, chemicals and fuels.

Enzymes are the most appropriate source of the catalytic activity for transformation of biological compounds. As opposed to traditional chemical conversion processes which often introduce extreme reaction conditions, the enzyme-based transformations can be performed at moderate pressure, temperature, and pH, with less energy input. In addition,

enzymes possess considerable substrate specificity, which ensures high product yield (Chibata and Tosa, 1983). Since enzyme extraction and purification is often a costly process, the recovery of the active enzyme from the reaction mixture is often necessary to allow for the repeated use of the catalyst. In addition, the use of the enzyme in the free form requires its inactivation in order to stop the conversion process. However, if enzymes are stably immobilized (or fixed) on an inert and insoluble support, the products and the enzymes can be separated without the need for extraction of the enzyme from the reaction mixture. This allows for a repeated use of the immobilized enzyme, thus reducing the costs of the enzymic process. Furthermore, there is no need for inactivation, since the contact time of the immobilized enzyme with the reaction mixture can be controlled (Chibata and Tosa, 1983). Another important advantage of the immobilized system is the fact that enzyme stability is often greatly enhanced, [presumably due to restricted access to inactive conformations for the immobilized enzyme, which increases the operational longevity of the enzymic system (Joshi-Sontakke, 1987)].

Since the process of enzyme extraction and purification is often costly, and many desired enzymes, especially intracellular, are often inactive or unstable upon extraction, the use of immobilized cells with the enzymes *in situ*, has been given a considerable amount of attention in the last three decades. The immobilization of whole cells is not only more economical, as the extraction and purification process is not required, but also has several other advantages. Firstly, as mentioned already, the enzyme in its natural intracellular environment is generally more stable than the extracted enzyme. Secondly the living cell is capable of carrying out complex multistep enzyme reactions, utilizing the enzyme pathways

that are already in place, and can regenerate both the enzyme and the often required cofactors, which in the case of the enzyme-based immobilized systems would have to be included in the reaction mixture. In addition, living cells are also capable of reproducing, thus increasing the amount of the biocatalyst and the biomass (Kolot, 1988).

Immobilization of cells can be achieved by a number of currently available methods. The method of covalent coupling involves covalent attachment of cells to a suitable insoluble support and allows for a stable, often permanent, attachment. However, due to the toxic nature of the coupling chemicals, such as glutaraldehyde, this method tends to be toxic to the cells, resulting in the loss of both the viability and the biocatalytic activity of the cells. In addition, the support usually requires chemical activation that introduces the necessary chemical residues for coupling, which makes this method relatively expensive (Bucke, 1986). Another way of immobilizing cells is cross-linking, where the cells are aggregated together by a bifunctional reagent such as glutaraldehyde. Cells immobilized in this way can be molded into pellets, chips or films, allowing for an efficient column operation. Although this method provides highly concentrated form of the immobilized cell catalyst, achieved by the high degree of packing, it introduces diffusional limitations for large-sized substrates and products (Klein and Wagner, 1983). Another disadvantage is some loss of viability upon cross-linking with glutaraldehyde (Bucke, 1986). Cells can be also efficiently immobilized by a physical entrapment within gels such as synthetic polyacrylamide or natural kappa-carrageenan. As in the case of the cross-linking method, this method also allows one to achieve a high concentration of the immobilized cell catalyst, but also suffers from the diffusional limitations, especially in the case of large-sized compounds, introduced by the

gel framework, thus decreasing the rate of catalysis. In addition, the gel networks tend to be unstable, requiring further strengthening chemical treatments, which increases the diffusional limitations and can be toxic to the entrapped cells (Klein and Wagner, 1983; Bucke, 1986). Finally, cells can be immobilized through the natural process of adsorption onto a suitable insoluble support. The degree of the attractive interactions between the cells and the support and therefore the strength of the attachment is dependent upon the surface characteristics of both the support and the cells, as well as on the nature of the environment. Therefore, the physicochemical properties of the support have to be matched with that of the cells to be immobilized to maximize the strength of the attractive interactions (Kolot, 1988). Because of its mildness, this method allows for the maximum retainment of viability and biochemical activity. Another advantage of this method is that the cells are free to reproduce, thus allowing for the continuous biomass generation (Klein and Wagner, 1983). However, depending on the strength of the adsorption and the degree of the shear forces (introduced by the operational conditions such as flow rate) that can remove the cells from the support, cell loss from the support can occur, which is the main disadvantage of the adsorption method.

One major application of the bacterial catalysts is the process of fermentation. Commercial fermentations are commonly carried out by batch fermentation, which require a seed culture that is prepared in 2 to 3 stages of volume scale-up. In order to repeat the batch fermentation the whole procedure has to be repeated, or a part of the fermented culture can be used as an inoculum, although it often contains metabolites that reduce the cell growth and product yield. Although harvested cells can be used instead, on the commercial scale cell

harvest and readdition add to the overall cost and make the whole process less amenable to automation. However, if the original inoculum is immobilized onto a suitable support and is capable of generating free cells, such cell-support system can be permanently placed in a stirred tank as a resident inoculum. Therefore, at the end of each batch fermentation, the fermented culture and its products can be separated from the immobilized inoculum, which is retained in the tank, thus permitting automatic repetition of batch fermentation without the need for preparing the inoculating seed cultures (Joshi and Yamazaki, 1985).

Since the originally immobilized inoculum must be capable of producing free cells that are released into the fermentation medium, the immobilized cells must be free to reproduce on the support. Therefore the attachment and containment of the cells on and/or within the support cannot restrict the ability of the cells to reproduce. In addition, maximum cell viability upon immobilization is also desired, since the progeny generating potential of the immobilized inoculum depends on the number of viable immobilized cells. The natural process of adsorption seems to be the method of choice: it offers high degree of viability upon immobilization, and since the cells are attached through the natural process of adsorption, it does not restrict the capacity of the cells to reproduce and release the free cells into the bathing medium.

In terms of the support for the immobilization of the bacterial inoculum, porous supports are usually preferred over non-porous ones, since they provide larger surface area available for adhesion per volume or weight of the support, therefore effectively increasing the concentration of the adsorbed cell catalyst. Another important and unique advantage of the porous immobilization carriers is that the initially adhered cell population can increase

its size by reproduction inside the porous carrier and subsequent biofilm formation, further increasing the concentration of the immobilized cells (Klein and Wagner, 1983). In addition, the presence of pores, into which the initially adhered inoculum can grow into, prevents washout of the cells under extreme operational conditions, which could result in the loss of cells from the adhesive surface. Therefore, porous carriers should allow for immobilization of a highly concentrated inoculum, thus permitting high progeny-generating potential and fast inoculation and colonization of the fermenter tank by the cells released from the support.

Textile fabrics such as cloths are relatively inexpensive and have high degree of porosity that provides large surface area to volume ratio. The macroporous character of the cloth allows for a free access to nutrients and an unrestricted release of the progeny cells into the fermenter space, which is otherwise restricted in microporous arrangements. The manufacturing process and therefore the final structures are different for woven and non-woven cloths. In woven cloths, such as cotton, two systems of yarns, known as the warp and the filling, are produced from the cellulose fibres and are interlaced to produce a planar sheetlike structure with pores created by the interlacing pattern, the size of the pores being determined by the yarn structure (fibre thickness and arrangement) and dimensions. Such arrangement, however, does not offer particularly strong structure, and woven fabrics are normally deformable and susceptible to fibre loss from the cloth edges due to shearing forces. The sorptive capacity (to absorb fluids) of woven cloths is strongly dependent on the degree of packing or interlocking, or density, of the fibres within the yarn, and decreases with the increasing degree of fibre density. The schematic representation of typical yarns, and a weaving pattern of a woven cloth are demonstrated in Figure 1.1. The sorptive capacity also

(A)

(B)

Figure 1.1. Schematic description of typical multifilament yarns (A) (from Rebenfeld, 1986), and a woven pattern of polypropylene filament yarns in woven polypropylene cloth (B) (from Drelich, 1986).

depends on both the chemical structure of the polymer that makes up the fibre and the physical structure of the fibre. The chemical structure determines the presence of hydrophilic chemical sites that are able to interact with the water, such as hydroxyl or amine groups, while the physical structure determines the ratio of the crystalline and non-crystalline (amorphous) regions in the fibre. Since the polymer molecules interact intermolecularly within the crystalline regions, any chemical sites that are available for interacting with water are present only within the non-crystalline regions of the fibre. Figure 1.2 shows the morphology of polyester fibres containing both crystalline and non-crystalline regions. Non-woven cloths, on the other hand, are produced directly from the fibres by arranging into a web-like structure held together by either entanglement, or thermal, adhesive, or covalent bonding. As in the case of woven cloths, the degree of bonding or interlocking of the fibres has a critical influence on the final properties of the cloth. In general, with increasing degree of bonding or interlocking, physical strength of the cloth increases, and sorptive capacity (to absorb fluids) decreases. Figures 1.3 and 1.4 show typical fibre arrangements for the non-woven cloths. Since in both cases the fibre density controls the sorptive capacity, or the permeability to fluids, the access of bacterial cells to the adhesive surface of the fibre is also most likely to depend on the fibre density and the pore characteristics. Since the degree of fibre bonding or interlocking can be controlled, very compact cloth structures can be obtained, offering high tensile properties and resistance to damage by shearing. In contrast to woven cloths, the loss of fibres from the cloth edges is therefore prevented in the nonwoven cloths.

Cellulose-derived materials have been studied extensively as immobilization supports

Figure 1.2. Morphology of the polyester fibres (from Rebenfeld, 1986).

(A)

(B)

Figure 1.3. Scanning electron microscope photomicrographs of rayon and polyester blend fibres mechanically bonded by entanglement in nonwoven rayon-polyester cloth (A) (from Drelich, 1986), and of polyester fibres in nonwoven polyester cloth (B) (from Rebenfeld, 1986).

(A)

(B)

Figure 1.4. Rayon web with oriented fibre distribution in nonwoven rayon cloth (A), and polyester web with random fibre distribution in nonwoven polyester cloth (B) (from Drelich, 1986).

for both enzymes and microbial cells. The main advantage of cellulosic materials is that the free hydroxyl groups of cellulose can be converted or activated with a variety of chemical groups, thus allowing for the control of the physicochemical surface properties of the cellulosic support, and for covalent attachment of catalysts to the support (Gemeiner, 1993). For example, the negatively charged yeast *Saccharomyces cerevisiae* can be efficiently immobilized through adsorption onto epichlorohydrin-triethanolamine (ECTEOLA) anion exchanger cotton cloth (Leung *et al.*, 1983) and a vast variety of enzymes can be covalently attached to chemically activated cellulosic materials (Gemeiner, 1993).

Cotton cloth has been shown to be a suitable immobilization carrier for a number of bacterial species, and production of two β -lactam antibiotics by immobilized *Streptomyces* strains has been demonstrated (Joshi and Yamazaki, 1985; Joshi and Yamazaki, 1987 a). In addition, the originally immobilized cells were shown to be resistant to replacement by contaminating bacteria, and the plasmid pBR322 (commonly used as a cloning vector for recombinant DNA) was stably maintained in immobilized *Escherichia coli*, indicating a stable retainment of the originally immobilized bacterial population within the cloth (Joshi and Yamazaki, 1987 b; Joshi and Yamazaki, 1987 c). The immobilization process was initiated by allowing the cultured cell suspension to grow on the cloth for 24 h, which was followed by daily incubation of the cloth in the growth medium. The cell generation capacity of the cloth with immobilized bacteria, measured daily, showed a gradual increase in the progeny-generating potential until a stable plateau was reached, indicating a full saturation of the cloth by the immobilized bacteria. It was speculated that the gradual increase in the progeny-generating potential of the bacteria-cloth system was due to the generation of

cellulophilic variants, which initially present as a small fraction of the original culture, were adhering to the cotton cloth and were therefore retained during the washing process. Since the cultured bacteria were afterwards derived from the originally adhered population of cells, their progeny, consisting of the cellulophilic variants and generated continually each day, was binding continually until all available sites were occupied, as demonstrated by a stable plateau in the progeny-generating potential. The nature of the bacterial attachment to cotton cloth was not studied, although it was indicated that the bacterial attachment to cotton might be mediated through hydrophobic interactions, because negatively charged residual carboxyl groups in cotton cannot attract net negative charges of bacteria, and its hydroxyl groups are extensively engaged in the hydrogen bonding of the crystalline cellulose structure and are therefore not available for bonding with exogenous substances (Sontakke-Joshi, 1987).

Recent studies in the area of bacterial adhesion to solid surfaces have indicated that the microbial surface hydrophobicity is the dominant surface characteristic, and the adhesion is more pronounced to hydrophobic, rather than to hydrophilic surfaces (Van Loosdrecht *et al.*, 1987 a, b). The currently accepted model of bacterial adherence treats the adhesion process as a two step event: first the bacterial particles must be transported close to the adhesive surface, which is followed by the actual adhesion that is controlled by the physicochemical interactions and shear forces (Rijnaarts *et al.*, 1993). The spectrum of the physicochemical interactions that controls bacterial adhesion includes hydrophobic and hydrogen-bonding interactions, which are attractive, and electrostatic interactions, which can be either attractive or repulsive, depending on the net charges of the bacterial cell and the support surface. However, since most solids, including bacteria, are negatively charged (thus

introducing repulsive electrostatic interactions), and any surface charges are counterbalanced by oppositely charged ions provided by the dissolved salts in the growth medium, it is generally accepted that the greater hydrophobicity of cells and the attachment surface results in greater attractive forces and higher levels of adhesion (Van Loosdrecht *et al.*, 1987 b; Loder and Liss, 1985; Wood, 1980).

A practical definition of hydrophobicity is elusive. Formally, it is defined in terms of the hydrophobic interactions process, in which two solute molecules are brought together within a solvent from infinity (Duncan-Hewitt, 1990). In terms of the process of bacterial adhesion to surfaces, hydrophobicity is treated as an interfacial phenomenon, that takes place at the interface between the bacterial particle and the surface in an aqueous environment. Thermodynamically, it is described by the surface free energies between the solid surface and the liquid, the particle and the liquid, and the particle and the solid (Duncan-Hewitt, 1990). In practice, the information about the interfacial free energies is derived from tests of wettability, which quantify the surface free energies in terms of surface tensions, and determine the ability of water to spread out over the surface rather than form a discrete droplet by measuring contact angles that a drop of liquid makes with the surface (Figure 1.5) (Duncan-Hewitt, 1990; Tampion and Tampion, 1987; Van Loosdrecht *et al.*, 1987 a). Since the contact angle decreases with the increasing degree of spreading of the water droplet on the surface, the hydrophobic character of the surface increases with the increasing contact angles. The contact angles can be applied to measure hydrophobicity of the bacterial surfaces using dried films of bacteria. Van Loosdrecht *et al.* (1987 b) compared adhesion levels of 23 different bacterial strains to negatively charged polystyrene and concluded that the bacterial

Figure 1.5. Relationship between the contact angle and the interfacial tensions: solid-liquid (γ_{SL}), solid-vapour (γ_{SV}), and liquid-vapour (γ_{LV}) (from Van Loosdrecht *et al.*, 1987 a).

surface hydrophobicity (measured by contact angles of the dried bacterial films) was the dominant characteristic of the adhesion process: at high contact angles complete adhesion was found, irrespective of the electrostatic property of the cell (measured by electrophoretic mobility), indicating that hydrophobicity is the dominant characteristic. For strains of low cell surface hydrophobicity, the electrostatic character of the cells became more important and the adhesion was found to increase with the decreasing negative surface net charge (as expected, since less negative surface charge decreases the magnitude of repulsive electrostatic interactions between the cell and the negatively charged solid) (Figure 1.6). Since hydrophobicity was found to be the dominant characteristic, hydrophobic supports should be most appropriate for adhesion of bacteria. Since no direct correlation between contact angles for Gram-positive and Gram-negative cell walls was observed, and the contact angles were found to deviate strongly even within the same genus, the exposure of the hydrophobic structures on the surface of the cell walls must be strain-specific.

Although the cellulose fibres in cotton exhibit high degree of crystallinity due to the strong and extensive intermolecular hydrogen bonding between the individual cellulose polymers, 15-35% of the fibre constitutes the amorphous regions, where as the absence of extensive hydrogen bonding allows for the contact of the free hydroxyl groups with the water, resulting in cotton being very hydrophilic (Rebenfeld, 1986). Since bacterial adhesion to solid surfaces seems to be mediated largely by the hydrophobic interactions, more hydrophobic cloths, such as polyester or polypropylene, should provide higher adhesion levels, than the hydrophilic cotton, allowing for faster colonization of the cloth by the cells. This in turn would allow for a higher rate of immobilization of bacteria on the cloth, thus

Figure 1.6. Relationship between bacterial adhesion to negatively charged polystyrene and cell surface characteristics as determined by contact angle and electrophoretic mobility measurements. High levels of adhesion (% coverage) can be observed for highly hydrophobic cells (high contact angle), irrespective of the surface net charge (measured by electrophoretic mobility). The net surface charge becomes important for the cells of low hydrophobicity (low contact angle), with the adhesion levels rising with the decreasing net negative surface charge (from Van Loosdrecht *et al.*, 1987 b).

shortening the time needed for preparing a fully immobilized bacterial cloth system. In addition, since the degree of hydrophobicity determines the strength of attachment, immobilization should be more resistant to shear forces, permitting the use of the cloth system at more extreme operational conditions. Another important advantage of using non-cellulosic cloths as immobilization supports is their applicability to cellulose-degrading bacteria.

The purpose of the present investigation was to examine and compare the suitability of the woven hydrophilic cotton cloth and nonwoven hydrophobic cloths, such as polyester, polypropylene, Nylon, and Nomex, for immobilization of bacteria. A model bacterium *Escherichia coli* 3300, which is constitutive for β -galactosidase and permits fast and efficient estimation of the number of adhered bacteria, will be extensively employed to study the mechanism of bacterial adhesion and the immobilization process on the cloths.

Methods and Materials

Bacteria and growth conditions

Escherichia coli (*E. coli*) 3300, GM30, JE3515, H585, and EHL1 were obtained from *E. coli* Genetic Stock Center, Yale University. American Type Culture Collection (ATCC) supplied *E. coli* Crooks (ATCC 8739), *E. coli* (ATCC 25922), *Brevibacterium ammoniagenes* (*Br. ammoniagenes*) (ATCC 6872), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 10145), and *Staphylococcus aureus* (*St. aureus*) (ATCC 12600). *Salmonella typhimurium* (*Sa. typhimurium*) LT2 was obtained from the University of Calgary. *Bacillus megaterium* (*Ba. megaterium*) QMB 1551 (Setlow, 1973) was from this laboratory (Yeung *et al.*, 1976). All bacteria were grown in basal M63 medium (Miller, 1972) supplemented with 0.5% (w/v) glucose, and 0.1% (w/v) yeast extract (full M63) by shaking at 240 rpm at 37°C for 17 h (overnight culture; o/n). The exponential culture was obtained by diluting 5 mL of the o/n culture with 5 mL of the fresh full M63 and incubating at 240 rpm and 37°C for 2 h.

Chemicals

Chloroform, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, KOH, (pellets) and glutaraldehyde (50% v/v) were obtained from The British Drug Houses (BDH, Inc.). Mallinckrodt provided NaOH (pellets) and concentrated H_2SO_4 . Polyoxyethylene 20 cetyl ether (Brij 58), sodium dodecyl sulphate (SDS), and D-(+)-glucose were obtained from Sigma Chemical Co. The Fisher Scientific Co. provided FeSO_4 and Na_2CO_3 . J.T. Baker Chemical Co. supplied KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$. Bacto[®] yeast extract and Bacto-Agar were supplied by Difco. Polyvinyl alcohol (PVA), molecular weight (MW) of 124000-186000 (99+% hydrolyzed), and MW of 13000-

23000 (98% hydrolyzed) were obtained from Aldrich Chemical Co. O-nitrophenyl- β -D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (XGal) were obtained from Diagnostic Chemicals Limited. Ethanol (95% v/v) was obtained from Commercial Alcohols.

Cloths

Woven cotton cloth was obtained locally. All other fabrics obtained for the study were nonwoven. Spunlaced polyester Sontara[®] 8100 and spunlaced polyamide Nomex[®] cloths were obtained from Dupont. Nylon type 31-trilobal (Nylon 1) and spunbonded Cerex[®] Nylon (Nylon 2) cloths were supplied by the James River Corporation. Fiberweb North America, Inc. supplied spunbonded Celestra[®] polypropylene (polypropylene 2), and Aldrich Chemical Co. supplied polypropylene filter cloth (polypropylene 1). The addresses of the cloth manufacturers are listed in Appendix 1. The cloths were cut into 1 cm squares for all experiments. To wet the cloths, cotton, polyester, Nylon, and Nomex[®] cloths were briefly soaked in 20% (v/v) ethanol, washed 10 times with 1 mL distilled water (dH₂O) on the Büchner funnel under suction, and stored in distilled water before testing or modification. In the case of the extremely hydrophobic polypropylene, the cloths were soaked in 50% (v/v) ethanol, the ethanol was replaced with distilled water, and the wet cloths were kept in distilled water before testing or modification.

Solution preparation

All PVA solutions were prepared to 1% (v/w) final concentration by dissolving the

solid through autoclaving in distilled water or basal M63 buffer. Glutaraldehyde solutions were prepared by dissolving the stock solution (50% v/v) with distilled water to 0.1%, 1%, and 10% final concentration (% v/v) and adding 15 μ L/mL of concentrated H₂SO₄. 1% (w/v) SDS was prepared by dissolving 1g of solid in distilled water. Brij 58 solution was prepared to 1% (w/v) concentration by dissolving 1 g of solid in 90°C distilled water or basal M63 buffer.

Dry weight of the cloths

To obtain the dry weight (mg/cm²) of the cloths, the wet cloths were blotted and placed in an oven at 120°C for 30 min before weighing; five independent measurements were performed and the average values with standard deviations were reported.

Relative hydrophobicity of the cloths

An 'immersion' assay was developed to measure the relative hydrophobicity (wettability) of the cloths. Cloth squares were prepared and dried as described, forced with forceps to the bottom of a polystyrene petri plate filled with 50 mL of dH₂O, and the time needed for water to fully penetrate the immersed dried fabric was measured and taken as a measure of the relative hydrophobicity of the cloth. Five independent measurements were performed and the average values with standard deviations were reported.

Adhesion of bacteria to cloths

Exponential phase cultures were diluted with basal M63 to absorbancy at 500 nm

(A_{500}) of 1.0, and used within 1 h. A wet cloth was suspended in 1 mL of the culture and shaken at 240 rpm and 37°C for 10 min. The cloths with adhered bacteria were removed from the culture suspension with sterile forceps, washed 5 times with 2 mL basal M63 medium on the Büchner funnel under suction to remove unbound cells, and blotted. In the case of extremely hydrophobic polypropylene, which could not be washed on the Büchner funnel, cloths were washed by vortexing (vortex obtained from Fisher Scientific Co., cat# 12-612-V1). The washed cloths with adhered *E. coli* 3300 were immediately examined for the β -galactosidase activity. Three independent trials were conducted in each case, and the average values with standard deviations were reported.

β -Galactosidase assay of adhered *E. coli* 3300

A cloth segment containing adhered *E. coli* 3300 was shaken at 320 rpm and 30°C for 10 min in 1 mL of 0.05M sodium phosphate buffer (pH 7.2) containing 20 μ L chloroform and 60 μ L 0.1% (w/v) SDS to permeabilize the cells. One mL β -galactosidase substrate, prepared by dissolving 90 mg ONPG in 100 mL 0.05M sodium phosphate buffer (pH 7.2), was then added, and the mixture was shaken for additional 3 min. The reaction was terminated with the addition of 1 mL 2M Na_2CO_3 . Absorbancy at 420 nm was taken as the measure of β -galactosidase activity (A_{420}).

Standard curve for the β -galactosidase assay

An exponential culture of *E. coli* 3300 was prepared as described. Triplicate samples of 50 μ L, 100 μ L, and 200 μ L were mixed with 950 μ L, 900 μ L, and 800 μ L, respectively,

of 0.05M sodium phosphate buffer (pH 7.2) containing 20 μ L chloroform and 60 μ L 0.1% (w/v) SDS, and shaken at 320 rpm and 30°C for 10 min to permeabilize the cells. One mL β -galactosidase substrate, prepared by dissolving 90 mg ONPG in 100 mL 0.05M sodium phosphate buffer (pH 7.2), was then added, and the mixture was shaken for additional 3 min. The reaction was terminated with the addition of 1 mL 2M Na_2CO_3 . Absorbancy at 420 nm was taken as the measure of β -galactosidase activity (A_{420}). The cell number (colony forming units, CFU) in the assayed samples was estimated by plating triplicate 100 μ L samples of the culture (upon diluting 10^5 fold) on full M63 agar plates and incubating overnight. The colony counts were performed with the New Brunswick colony counter. The A_{420} values were plotted against the CFU values and the linear regression was obtained with the GraphPad Prism software (GraphPad Software, Inc.). The standard curve was used for converting the A_{420} values, obtained for the cloths, to the corresponding cell numbers (CFU). The slope of the line with the value of 0.112 was used in the following equation: $\text{CFU} = A_{420} / (0.112)$ which converts A_{420} values to the corresponding cell number (CFU) values.

Progeny generation assay of adhered bacteria

The washed and blotted cloths with adhered bacteria were suspended in 1 mL of full M63 growth medium and shaken at 240 rpm at 37°C for 30 min. 0.1 mL of the resulting progeny suspension, produced and released from the cloth by the adhered cells, was removed, diluted 1000 fold with basal M63, and 0.1 mL sample plated on full M63 agar plates. The plates were incubated overnight at 37°C, and the colony counts were performed with the New Brunswick colony counter. Three independent measurements were performed and the

average values (with standard deviations) were reported.

Treatment of the cloths with PVA

The cloths, prepared as described above, were blotted, soaked in 1% (w/v) PVA solution for 10 min, washed as described above, and blotted.

Treatment of the PVA-modified polyester cloth with glutaraldehyde

The blotted PVA-modified polyester cloth was soaked in 0.1% to 10% (v/v) glutaraldehyde solution for 10 min, washed as described above, and blotted.

PVA coating of polystyrene dish

The 1% (w/v) PVA solution (20 mL) was poured into the polystyrene petri culture plate to completely cover the inside bottom of the plate. The plate was swirled occasionally for 10 min, and was washed 3 times with 20 mL of distilled water by swirling. The plate was then dried in the oven at 120°C for 10 min. The control plate was treated the same way, distilled water was used instead the modifying PVA solution. To examine the relative hydrophobicity of the control and the PVA-coated plates, 1 mL of distilled water was spotted onto the surface and spread with a hockey stick.

Immobilization of *E. coli* 3300 on to cotton, polyester and PVA-polyester cloths

Triplicate samples of cotton, polyester, and PVA-polyester cloths were incubated

with an exponential culture of *E. coli* 3300 for 30 min at 240 rpm and 37°C, washed, and the initial progeny generating potential was measured with the progeny generation assay. The cloth and the bacterial suspension was then shaken at 240 rpm and 37°C for 24 h. Each day the cloths were removed from the suspension, washed, suspended in the fresh full M63 growth medium, and the potential of the cloth to produce progeny was examined with the progeny generation assay. The process was repeated for 20 days.

Adhesion of the 20th day progeny to cotton, polyester, and PVA-polyester

The overnight progenies released from the cotton, polyester, and PVA-polyester fabrics on the 20th day of the immobilization experiment were collected and transformed to the exponential phase by diluting 1:1 with fresh full M63 medium and shaking at 240 rpm at 37°C for 2 h. The adhesion of the resulting exponential cultures of the progenies (prepared as described above) was examined by the β -galactosidase assay, as described above.

Measurement of the doubling time

One mL of the exponential and overnight cultures, diluted to A_{500} of 1.0, was mixed with 4 mL of fresh full M63 growth medium and then incubated at 240 rpm and 37°C. A portion (1 mL) of the culture was removed every 60 min to measure A_{500} . The doubling time was obtained by calculating the time required for the culture to double its cell mass, measured by A_{500} .

Inhibition of adhesion to polyester cloth in the presence of PVA and Brij 58

Triplicate samples of 0.9 mL of the exponential culture of *E. coli* 3300, containing

polyester cloth were mixed with 0.1 mL of the 1% (w/v) PVA or Brij 58 solution, and the adhesion test was conducted as described above. The cloth was assayed for β -galactosidase activity, and the cell number (CFU) of adhered cells was obtained with the standard curve for the β -galactosidase assay, as described above.

Removal of bound bacteria from polyester cloth by PVA and Brij 58

Polyester cloth with bound bacteria, obtained as described above, was shaken in 1% (v/w) PVA and Brij 58 solutions prepared in basal M63 buffer for 10 min at 320 rpm and 30°C. The resulting cell suspension, released from the cloth, was diluted 10^4 fold and 0.1 mL was plated on the full M63 agar plates. The plates were incubated overnight at 37°C and the colony counts were performed with the New Brunswick colony counter. The cloths were removed, washed as described above, assayed for β -galactosidase activity, and the numbers (CFU) of adhered cells was obtained with the standard curve for the β -galactosidase assay. To account for the mechanical loss of cells due to shaking and washing, a control test was performed in which the cloth with bound bacteria was treated by shaking and washing in the absence of PVA or Brij 58.

Blending of bacterial cells

Five mL samples of exponential cultures, prepared as described above, were placed in an ice bath to decrease the temperature down to 1-2°C, and were blended at 25000-30000 rpm with the tissue homogenizer (tissue tearor™ by Biospec Products, Inc., cat# 985-370) for 5 min while in the ice bath. The culture samples were equilibrated to 37°C, before measuring the adhesion by the β -galactosidase or the progeny generation assay.

Chapter 2

Adhesion of *E. coli* 3300 to cotton cloth and hydrophobic cloths

Abstract

Adhesion of a model bacterium *Escherichia coli* 3300 to cloths was more efficient to hydrophobic polyester, Nylon, Nomex, and polypropylene, than to hydrophilic cotton, indicating hydrophobicity as the dominant force involved in adhesion of this bacterium to cloths. The 'immersion' assay, developed to assess wettability (relative hydrophobicity) of cloths by measuring the time of water migration through a dry cloth, was strongly dependent on the cloth structure, indicating that the transfer of bacterial particles to the fibre surface through aqueous medium is likely to be controlled by the cloth structure.

Introduction

It has been indicated by a number of recent studies that the adhesion behaviour of bacteria to solid surfaces is mediated by hydrophobic interactions. Higher hydrophobicity of the support surface was found to stimulate bacterial attachment, resulting in higher levels of adhesion (the number of adhering bacteria) and greater attractive interactions between the bacterial cells and the support (Van Loosdrecht *et al.*, 1987 a, b). The model of bacterial adhesion treats this process as a two-step event: first the bacterial particles must be transported close to the adhesive surface, which is immediately followed by the actual adhesion process largely controlled by the hydrophobic interactions (Rijnaarts *et al.*, 1993; Van Loosdrecht *et al.*, 1987 b).

In the case of porous supports, such as cloths, the actual levels of adhesion should be controlled by both the hydrophobicity of the surface and the pore characteristics (size, density, and distribution) of the support which controls the total surface area available for

bacteria to attach to. A relatively hydrophilic cotton cloth has been demonstrated as a suitable carrier for bacterial immobilization (Joshi and Yamazaki, 1985, 1987 a, 1987 b, 1987 c). Since hydrophobicity of the support primarily mediates bacterial adhesion, cloths that are more hydrophobic than cotton cloth should provide a more suitable surface for bacterial attachment, resulting in higher levels of adhesion.

The purpose of the investigation presented in this chapter is to compare the adhesion of the model bacterium *Escherichia coli* 3300 to the hydrophilic woven cotton cloth and a number of nonwoven hydrophobic cloths of different chemical and structural character, including polyester, polypropylene, Nylon, and Nomex in order to determine the suitability of hydrophobic cloths as potential bacterial immobilization supports. *E. coli* 3300 is constitutive for β -galactosidase due to a *lacI* mutation (Pardee et al., 1959), and allows for an efficient estimation of the number of cells adhering to cloths by measuring the activity of β -galactosidase on the cloths.

Results

Chemical and physical properties of cloths

The chemical structure of the cellulose fibres is shown in Figure 2.1, and the chemical formulas of the polymers that constitute the fibres of the hydrophobic cloths are shown in Figure 2.2. The cotton fibre is known to be very hydrophilic due to the presence of the noncrystalline regions that constitute 15-35% of the fibre and contain free hydroxyl groups available for contact with water via hydrogen bonding. Nylon and Nomex fibres are more hydrophobic than cotton, due to the fact that the only sites available for contact with

Cellulose: poly(β -1 $\rightarrow$ 4-D-anhydroglucopyranose)

(A)

(B)

Figure 2.1. The chemical formula of the cellulose polymer (A) (from Rebenfeld, 1986), and the schematic description of the extensive intermolecular hydrogen bonding between the cellulose polymers in the crystalline regions of the cotton fibre (B) (from Holum, 1968).

Polyester: poly(ethylene terephthalate) (PET)

Nylon 6,6: polyhexamethylenedipamide

Nomex: poly(m-phenyleneisophthalamide)

Polypropylene

Figure 2.2. The chemical formulas of the polymers that make up the fibres of the hydrophobic cloths used in the study.

water are the free amine groups which are located at the ends of the polymeric chains (the internal amide groups are extensively involved in the intermolecular chain interactions), and whose fraction within the fibre is much lower than that of the free hydroxyl content in the cotton fibre. Polyester fibre exhibits an even higher degree of hydrophobicity, which is due to lack of hydrophilic sites and chain stiffness, that results from the chemical nature of the polymer and strong interactive forces between the polymeric chains of the fibres. In the case of polypropylene fibre, the lack of hydrophilic sites, high crystallinity, and the general chemical inertness of polypropylene, makes the fibre extremely hydrophobic and essentially inert to aqueous chemical systems, and is therefore expected to be most hydrophobic. (Rebenfeld, 1986).

The thickness of the cloths and their structural appearance varied, indicating different cloth structures, and therefore different fibre arrangement. The physical characteristics of the cloths, such as the fibre density and the dry weight (mg/cm^2) of the cloths, which approximates the overall surface area and volume of the cloth per cm^2 , are shown in Table 2.1. Since the fibre density determines the pore characteristics (size, density and distribution), all cloths are expected to have varying surface areas available for contact with water and the bacterial cells.

Cloth hydrophobicity

In order to correlate the adhesion of *E. coli* 3300 to cloths with the cloth hydrophobicity, the relative hydrophobic character of the cloths was examined by measuring the wettability (ability to absorb water) of the cloths by water. All cloths were cut into 1 cm

Table 2.1. Dry weight and wettability of the cloths as measured by the ‘immersion’ assay.

Fabric	Fibre density^(a)	Weight/area (mg/cm²)	Wettability (sec.)
Cotton	medium-low	14.4±0.7	1±0
Polyester	medium	16.0±1.6	551±229
Nylon 1	high	3.4±0.5	5±2
Nylon 2	high	6.4±0.3	570±42
Nomex 1	medium	6.5±0.3	370±194
Nomex 2	medium	26.9±0.9	97±35
Polypropylene 1	very low	22.5±2.7	12±3
Polypropylene 2	very high	6.8±0.6	>24 hr

^(a) Fibre density is based on the visual examination of the cloths. In the case of very low fibre density (polypropylene 1), the individual fibres were observed; in the case of very high fibre density (polypropylene 2), no individual fibres could be observed.

squares to ensure equal washing conditions. To remove residual moisture before weighing the cloths and measuring the relative hydrophobic character of the cloths, all cloths, except for polypropylene, were wetted with 20% ethanol, washed abundantly with water under suction to allow the water to fully penetrate the cloth, and dried at 120 °C for 30 min. In the case of the polypropylene cloth, which was found to be extremely hydrophobic, 50% ethanol was necessary to wet the cloth. Three attempts were made at developing a suitable assay for measuring the wettability of the cloths. The first involved depositing a drop of water on the surface of the dry cloth and measuring the time required for the drop to be absorbed by the cloth. However, although the cotton cloth absorbed the drop instantaneously, indicating a very low degree of hydrophobicity (or high degree of hydrophilicity), for all hydrophobic cloths the drop evaporated before being absorbed, which was indicative of a high degree of hydrophobicity for these cloths, but was unsuitable for quantitatively comparing the hydrophobicity of the hydrophobic cloths. In the second assay, the dried cloths were placed on the surface of distilled water and the time required for the water to wet the cloths was measured. However, this assay was found to be extremely dependent on the cloth density (weight/cm²), making less dense Nylon and Nomex 1 cloths float in the dry state for more than 24 h. In addition, this assay was found to be extremely sensitive to air moisture content, resulting in poor reproducibility. In order to account for the different densities of the cloths, the immersion assay was developed, which proved more suitable for estimating the hydrophobic character of the cloths. In this assay, a standard volume of distilled water (50 mL) was placed in the plastic petri dish and the dried cloths were forced to the bottom of the dish with forceps. The standard amount of distilled water and the placement of the cloths at

the bottom of the dish ensured equal hydrostatic pressures between the measurements. The time required for water to penetrate the cloth was measured and taken as the measure of the relative hydrophobicity of the cloth. Table 2.1 shows the relative hydrophobicity (wettability) of all the cloths as measured by the 'immersion' assay. All hydrophobic cloths were found to be much more hydrophobic than cotton cloth, while the cotton cloth was being wetted by water within 1 sec, the times required to wet the hydrophobic cloths ranged from 5 sec for Nylon 1 to more than 24 h for polypropylene 1. Substantial differences in the wettability values were observed for the cloths of the same chemical character, but different physical structure. For the polypropylene cloths, the values of relative hydrophobicity were very different: only 12 sec for the thicker low fibre density polypropylene 1, and more than 24 h for the thinner high fibre density polypropylene 2. The same was observed for the Nylon cloths: 5 sec for Nylon 1 and 570 sec for Nylon 2. Similarly, the relative value of hydrophobicity for the thinner Nomex 1 was almost 4-fold higher than for the over 4 fold thicker Nomex 2, which indicates that the relative hydrophobicity of cloths as measured by the 'immersion' assay depends on the physical structure of the cloth. Since the immersion assay relies on the ability of water to penetrate the fibre network of the cloth, the rate at which water penetrates the cloth (the measure of the relative hydrophobicity) is affected by the fibre density inside the cloth. This would account for the observation that cloths of equivalent chemical character were found to have very different values of relative hydrophobicity. In the case of polypropylene 1, with a low fibre density, a large pore size and relaxed fibre arrangement most likely allowed water to quickly penetrate the cloth, resulting in the very low value of the relative hydrophobicity of only 12 sec. In contrast, for

polypropylene 2, with a much higher fibre density, the water was forced to migrate through a dense network of extremely hydrophobic and water-repelling polypropylene fibres, greatly decreasing the rate of water migration through the cloth, resulting in an extremely high relative hydrophobicity value of more than 24 h.

Construction of the β -galactosidase standard

E. coli 3300 is constitutive for β -galactosidase and this enzymic activity can be used to estimate the number of bacteria adhering to cloths, if β -galactosidase activity per cell is known. If β -galactosidase activity of a known number of free cells can be measured, a standard relating the cell number and the resulting β -galactosidase activity can be constructed, and used to determine the number of cells adhered to a cloth by measuring the activity of β -galactosidase obtained from the cloth and converting it to the cell number using the standard. Figure 2.3 shows the β -galactosidase standard obtained by measuring the β -galactosidase activity of a set of culture samples containing different numbers of cells. Therefore, any measurable β -galactosidase activity from the cloth containing adhered *E. coli* 3300 cells can be directly converted to the cell number using the standard.

Adhesion of *E. coli* 3300 cells to cloths

Adhesion of *E. coli* 3300 cells to cloths was assayed by the β -galactosidase assay (A_{420}) and the A_{420} values were converted to the cell number (colony forming units, CFU) using the β -galactosidase standard, as described in the Methods. To ensure that the cloths were wet during the adhesion process, all cloths, except polypropylene, were soaked in 20%

Figure 2.3. The β -galactosidase assay (A_{420}) standard obtained with the GraphPad Prism Software for converting A_{420} values to CFU (colony forming units) values. The slope of 0.112 was used for converting the A_{420} values to the CFU x 10^{-7} , as described in Methods.

ethanol, washed with distilled water, and suspended in distilled water before the adhesion test. For the extremely hydrophobic polypropylene, 50% ethanol was required to wet the cloth. Since the polypropylene cloths could not be resuspended in the buffer after washing on the suction apparatus (suction removed all water during washing from the water-repelling polypropylene, which prevented maintaining of the wet state), the cloths were maintained in the wet state by replacement of ethanol by water, followed by suspending in the distilled water. To measure the initial adhesion of *E. coli* 3300 to the cloths, which determines the affinity of the *E. coli* 3300 cells for the surface of the cloth, the cloths were mixed with the *E. coli* 3300 culture for 10 min, after which they were washed with the M63 buffer on the suction apparatus to remove nonadhered cells, and examined by the β -galactosidase assay. Since the suction wash was inapplicable to polypropylene, the cloths containing the adhered cells were washed by vortexing. To ensure that the polypropylene cloths received the same degree of washing by vortexing, as other cloths did on the suction apparatus, the strength of vortexing was adjusted to a level where polyester cloth containing adsorbed bacteria produced equal β -galactosidase activities after being washed by suction and vortex. Table 2.2 shows the summary of both the A_{420} and the CFU values representing the initial adhesion of *E. coli* 3300 to cloths. All hydrophobic cloths adsorbed more *E. coli* 3300 cells than the cotton cloth, indicating that the hydrophobic cloth surface has stronger affinity for the cells of *E. coli* 3300 than the hydrophilic cotton cloth. For the hydrophobic cloths, the thicker cloths adsorbed more cells, indicating that the thickness correlates with the surface area of the cloth that is available for bacterial cell attachment. Because the adhesion levels of *E. coli* 3300 to cloths expressed per cm^2 do not account for the different thickness of the cloths, a

Table 2.2. Adhesion of *E. coli* 3300 to the cloths as measured with the β -galactosidase assay.

Cloth	Weight/area (mg/cm ²)	Adhesion of <i>E. coli</i> 3300	
		A ₄₂₀	CFU x 10 ⁻⁷ ^(a)
Cotton	14.4±0.7	0.027±0.003	0.24±0.03
Polyester	16.0±1.6	0.612±0.106	5.46±1.19
Nylon 1	3.4±0.5	0.030±0.012	0.27±0.11
Nylon 2	6.4±0.3	0.130±0.048	1.16±0.43
Nomex 1	6.5±0.3	0.593±0.132	5.29±1.18
Nomex 2	26.9±0.9	0.973±0.243	8.68±2.17
Polypropylene 1	22.5±2.7	0.718±0.134	6.67±1.19
Polypropylene 2	6.8±0.6	0.260±0.096	2.32±0.86

^(a) The CFU x 10⁻⁷ values were obtained from the A₄₂₀ values upon conversion with the β -galactosidase standard, as described in the Methods.

more representative way of estimating the binding capacity of the cloths is to express the adhesion levels per weight of the cloth. Table 2.3 shows the adhesion levels of *E. coli* 3300 to cloths per mg of cloth. This shows that all hydrophobic cloths have shown much higher potential to bind *E. coli* 3300 than the hydrophilic cotton, and the Nomex 1 cloth appeared to be superior in binding *E. coli* 3300. The adhesion levels did not, however, correlated well with the values of the relative hydrophobicity for the hydrophobic cloths. For example, polypropylene cloths, which had very different wettability values (>24 h and 12 sec., Table 2.1), exhibited almost the same capacity to bind the cells. For Nylon cloths the much more hydrophobic Nylon 2 adhered only twice as many cells as its counterpart Nylon 1. Since the wettability of the cloths, as measured by the 'immersion' assay, was found to depend on the cloth structure, which determines the pore characteristics and therefore the surface area available for the contact with water molecules and bacterial cells, one could expect the adhesion of bacterial cells to be influenced by the cloth structure. If the fibre density were such that it prevented the penetration of the cloth by the cells, attachment would be possible only on the surface of the cloth, resulting in the cells being extremely susceptible to shear forces present during the washing. This would cause low adhesion levels for such cloths as polypropylene 2, where the high fibre density might prevent the cells from penetrating the internal part of the cloth. On the other hand, the very low fibre density of polypropylene 1 could limit the available surface area, in comparison with cloths of higher fibre density, such as Nomex 1.

Table 2.3. The adhesion of *E. coli* 3300 to the cloths in CFU/mg cloth^(a).

Cloth	CFU x 10⁻⁹ / mg cloth
Cotton	1.7
Polyester	34.1
Nylon 1	7.94
Nylon 2	18.1
Nomex 1	81.4
Nomex 2	32.2
Polypropylene 1	29.6
Polypropylene 2	34.0

^(a) The CFU x 10⁻⁹ / mg were obtained from the CFU x 10⁻⁷ (per cm² of the cloth) values (presented in Table 2.2) by dividing by the dry cloth weight (mg).

Discussion

All hydrophobic cloths showed much higher potential to bind *E. coli* 3300 than the hydrophilic cotton cloth, indicating that hydrophobicity of the support mediates adhesion of this bacterium to cloths, and that hydrophobic cloths should be more suitable as immobilization carriers for bacteria. The fact that the immersion assay was found to be dependent on the physical structure of the cloth, and that the level of correlation between the relative hydrophobicity of the cloths and the adhesion levels was not high, strongly suggests that the initial adhesion process is driven by both the hydrophobic character of the cloth and its physical structure. Since the adhesion process is a two-step event, first requiring the transport of the bacterial particle close to the adhesive surface (Rijnaarts *et al.*, 1993), which is followed by the actual adhesion process that is largely driven by the hydrophobic interactions (Van Loosdrecht *et al.*, 1987a, b), the structure of the cloth, which determines the size, distribution, and density of the pores, and therefore the total surface area available for bacterial attachment, plays a large role in determining the number of adhering bacteria. Thus the lower levels of adhesion for the extremely hydrophobic polypropylene cloths, compared with the less hydrophobic Nomex 1 cloth, might be caused by the low overall surface area of the cloth that is available to the cells. In addition, the transport of the bacterial particles to the adhesive surface of the fibre through the aqueous environment, which is the first step of the adhesion process, might be severely inhibited for extremely hydrophobic surfaces such as polypropylene cloth, resulting in lower than expected adhesion levels. This suggests that some retainment of hydrophilicity of the cloth might be a desired characteristics of cloth-based immobilization supports, allowing for an efficient transport of the bacterial

particles to the adhesive surface. The extremely high levels of adhesion exhibited by Nomex 1 cloth are likely due to an extensive surface area offered by this cloth and an efficient transport of the bacterial cells throughout the cloth during the 10 min contact time, thus allowing for adsorption of high number of cells.

Chapter 3

Hydrophobic nature of *E. coli* 3300 adhesion to cloths

Abstract

Polyvinyl alcohol (PVA) binding to hydrophobic cloths resulted in a significant reduction of adhesion of *Escherichia coli* 3300 to the PVA-coated cloths. The reduction of adhesion of *E. coli* 3300 to PVA-coated cloths was generally correlated with the decreased hydrophobicity (wettability) of the PVA-coated cloth, strongly indicating hydrophobicity of the cloth as the dominant force mediating adhesion of *E. coli* 3300 to cloths. The involvement of hydrophobicity in the adhesion process was further substantiated by demonstrating that both PVA and the detergent Brij 58 inhibited adhesion, when added to the bacterial suspension during the adhesion process, and stimulated removal of bound cells from the polyester cloth.

Introduction

Examination of the adhesion potential of *E. coli* 3300 to hydrophilic cotton and a number of more hydrophobic cloths, presented in Chapter 2, have shown that the hydrophobic cloths offer much higher adsorption potential than the hydrophilic cotton, suggesting that the hydrophobicity of the cloth plays a major role in the adhesion of *E. coli* 3300 to cloths. In the present chapter, the involvement of hydrophobicity in the adhesion of this bacterium to cloths will be further substantiated by comparing the adhesion levels to the untreated hydrophobic cloths and their counterparts that were modified with polyvinyl alcohol (PVA) polymer to increase their surface hydrophilicity. If the adsorption of *E. coli* 3300 is mediated by hydrophobicity, increased wettability of the modified hydrophobic cloths should result in a decrease in adhesion levels.

PVA was previously demonstrated as an efficient blocking agent in polyester cloth-based enzyme immunoassay capable of reducing non-specific binding of immunoreagents on the hydrophobic polyester cloth and polystyrene microtiter plate wells (Boyd and Yamazaki, 1995; Rodda and Yamazaki, 1994). It was speculated that binding of the PVA polymer to hydrophobic polystyrene must involve an attractive interaction between its hydrophobic hydrocarbon backbone and the hydrophobic surface of the polystyrene wells, resulting in the exposure of the hydroxyl substituents of PVA on the surface. The binding was shown most effective for the highest molecular weight PVA, indicating that the attraction between PVA and the hydrophobic surface increases with the length of the hydrocarbon backbone (Rodda and Yamazaki, 1994). The exposure of the hydrophilic hydroxyl groups on the surface of the hydrophobic cloths should therefore provide the sites for the contact with water via hydrogen bonding, resulting in the alteration of the cloth surface from hydrophobic to relatively hydrophilic. The chemical formula of the PVA polymer is presented in Figure 3.1 a. The mechanism of PVA binding to hydrophobic fibre surface is pictured in Figure 3.2. In addition, since amphipathic agents are known to disrupt hydrophobic interactions (Absolom *et al.*, 1983), the adhesion process in the presence and absence of the amphipathic nonionic surfactant polyoxyethylene 20 cetyl ether (Brij 58) was also examined. The chemical formula of Brij 58 is shown in Figure 3.1 b.

Step 1: transport of the PVA molecule towards the surface of the polyester fiber

Hydrophobic polyester fiber

Step 2: adsorption of the PVA molecule to the polyester fiber is initiated by the attractive hydrophobic interactions

Step 3: PVA adsorbs to the surface of the polyester fiber exposing the hydroxyl substituents

PVA-coated polyester cloth

Figure 3.2. Mechanism of PVA adsorption onto polyester cloth.

Figure 3.1. The chemical structures of the PVA polymer (a) and the surfactant Brij 58 (b).

Results

Modification of the cloths with polyvinyl alcohol (PVA)

To investigate the effect of the PVA treatment on the hydrophobicity of polyester cloth, the cloth was treated with the highest molecular weight (MW) (124000-186000 MW) PVA available. Table 3.1 shows that the 10 min treatment of the polyester and other hydrophobic cloths resulted in the significant decrease of the hydrophobic character (wettability) of all the hydrophobic cloths, except Nylon 1, as measured by the ‘immersion’ assay (described in Chapter 2). For the already extremely hydrophilic cotton cloth, no change in the value of wettability was observed, indicating that PVA does not bind efficiently to hydrophilic surfaces. The fact that PVA treatment of Nylon 1 did not result in the decrease of the hydrophobicity is most likely due to the fact that the ‘immersion’ assay is dependent on the cloth structure and might not be capable of detecting chemical modifications for some cloths.

Table 3.1. Dry weight and wettability of the untreated and the PVA-coated cloths as measured by the ‘immersion’ assay.

Fabric	Weight/area (mg/cm²)	Wettability (sec.)	Wettability after PVA coating (sec.)
Cotton	14.4±0.7	1±0	1±0
Polyester	16.0±1.6	551±229	14±4
Nylon 1	3.4±0.5	5±2	5±2
Nylon 2	6.4±0.3	570±42	19±2
Nomex 1	6.5±0.3	370±194	30±22
Nomex 2	26.9±0.9	97±35	16±12
Polypropylene 1	22.5±2.7	12±3	6±4
Polypropylene 2	6.8±0.6	>24 h	720±85

Adhesion of *E. coli* 3300 to PVA-modified cloths

Since the hydrophobicity of the hydrophobic cloths was generally decreased by the PVA treatment, the adhesion of *E. coli* 3300 to the PVA-coated cloths, if indeed mediated by hydrophobic interactions, should also be decreased. Table 3.2 shows that the adhesion levels to all the PVA-coated hydrophobic cloths were markedly decreased, indicating that the hydrophobic character of the cloths is responsible for the adhesion of *E. coli* 3300 to cloths. It also indicates that the presence of *E. coli* 3300 on the cloths after washing is due to a genuine attachment and not just a physical trapping of the cells within the pores of the cloth. If the cell retainment on the cloth was due to the physical trapping inside the cloth, the modification of the fibre surface by PVA would not cause the observed reduction in β -galactosidase levels. The levels of adhesion to the PVA-treated hydrophilic cotton cloth were not significantly decreased compared with the untreated control, which indicates that PVA does not adhere efficiently to the hydrophilic cloth, suggesting a hydrophobic nature of the PVA attachment to the cloths. Although the PVA modification of the Nylon 1 cloth did not appear to have any effect on the hydrophobicity of the cloth, as measured by the immersion assay, it did decrease the adhesion levels, thus indicating that the PVA binding to Nylon 1 cloth did occur.

PVA treatment of *E. coli* 3300

Since there were strong indications that adhesion of *E. coli* 3300 to cloths is mediated by the hydrophobic interactions between the hydrophobic cloth surface and hydrophobic cell, PVA may be also capable of binding to hydrophobic surface of *E. coli* 3300 cells, thus

Table 3.2. Adhesion of *E. coli* 3300 to the untreated and PVA-coated cloths.

Cloth	Adhesion of <i>E. coli</i> 3300 (CFU x 10 ⁻⁷) ^(a)	
	untreated	PVA-coated
Cotton	0.24±0.03	0.17±0.02
Polyester	5.46±1.19	0.04±0.02
Nylon 1	0.27±0.11	0.02±0.01
Nylon 2	1.16±0.43	0.03±0.02
Nomex 1	5.29±1.18	0.04±0.03
Nomex 2	8.68±2.17	0.27±0.14
Polypropylene 1	6.67±1.19	0.07±0.04
Polypropylene 2	2.32±0.86	0.03±0.01

^(a) The CFU x 10⁻⁷ values were obtained from A₄₂₀ values upon conversion with β -galactosidase standard, as described in the Methods.

preventing or decreasing bacterial adhesion by making the cells less hydrophobic and thus decreasing their affinity for the hydrophobic surface of the cloth. Polyester cloth was chosen as a model hydrophobic cloth. For the treatment of the cells with PVA, PVA was dissolved in basal M63 buffer, instead of distilled water, to prevent any damage to the cells that might occur by suspending the cells in distilled water. Table 3.3 shows that for the PVA-treated *E. coli* 3300 which were washed only once following treatment, there was a significant decrease in the adhesion to polyester cloth. However, the cells that were washed three times following the PVA treatment were equally capable of adhering to polyester as the untreated cells. This suggests that, either the PVA binding to the cells is very weak and the PVA is progressively removed from the bacterial surface with each wash, or that the single wash was inadequate enough to remove all the traces of PVA from the bacterial suspension that is subsequently used to examine the adhesion. In such a case, the contaminating amounts of PVA present at the time of the adhesion in the bacterial suspension would have to induce an inhibitory effect on the adhesion.

Inhibition of adhesion of *E. coli* 3300 to polyester in the presence of PVA

To account for the possibility that PVA, present in the bacterial suspension during the contact time of the cells with the polyester cloth, prevents the adhesion process, PVA was added to the mixture of cells and the polyester cloth at a low 0.1% (w/v) final concentration during the 10 min adhesion time. Table 3.4 demonstrates that a complete inhibition of adhesion of the *E. coli* 3300 cells to the polyester cloth in the presence of 0.1% PVA occurred, which indicates that PVA might compete with the cells to bind to the surface of

Table 3.3. Adhesion of *E. coli* 3300 treated with PVA and Brij 58 to polyester.

	CFU x 10 ⁻⁷ ^(a)
Control	5.68±0.46
Control, washed ^(b)	5.59±0.78
PVA-polyester	0.03±0.02
Brij 58-polyester	3.69±0.21
PVA-cells, 1x wash	0.89±0.29
PVA-cells, 3x wash	5.86±0.73
Brij 58-cells, 1x wash	0.88±0.30
Brij 58-cells, 3x wash	5.61±0.95

^(a) The CFU x 10⁻⁷ values were obtained from A₄₂₀ upon conversion with the β-galactosidase standard, as described in the Methods.

^(b) To account for any loss of adhesion capacity of the cells due to washing by resuspending and centrifugation, control cells that were subjected to washing by centrifugation and resuspending in the absence of PVA or Brij 58 treatment were assayed .

Table 3.4. Inhibition of adhesion of *E. coli* 3300 to the cloths in the presence of 0.1% (w/v) PVA or Brij 58.

Agent added	Adhesion (CFU x 10⁻⁷)^(a)
control (no agent added)	5.68±0.84
PVA (186000 MW)	0.04±0.01
Brij 58	0.04±0.02

^(a) The CFU x 10⁻⁷ values were obtained from A₄₂₀ upon conversion with the β-galactosidase standard, as described in the Methods.

polyester. The observation that PVA was found to be stably adsorbed on the polyester surface, but did not bind efficiently to the cells, suggests that the affinity of PVA for the hydrophobic surface of polyester is greater than that of the cells. Since the low 0.1% concentration of the free PVA in solution during the adhesion process was found to significantly reduce the adhesion levels of *E. coli* 3300 to polyester, it is hard to speculate if PVA is capable of weakly binding to the cells: washing the cells only once might have been inefficient enough to remove all the traces of the free PVA from the washed bacterial suspension, resulting in the reduction of the adhesion levels not because the cells were coated with PVA, but because there was a sufficient amount of the free PVA in the inadequately washed bacterial suspension that prevented the adhesion of the cells to the polyester cloth. On the other hand, PVA binding to the cells might have taken place, but the strength of binding was very low, resulting in PVA being completely removed from the cell surface after three washes.

Inhibition of adhesion of *E. coli* 3300 to polyester in the presence of Brij 58 surfactant

Since surfactants are known to interfere with the hydrophobic interactions between bacterial cells and the substrate through reduction of the surface tension of the suspending medium caused by the surfactant (Absolom *et al.*, 1993), the effect of the addition of the nonionic surfactant Brij 58 during the 10 min adhesion period was investigated. Brij 58 was chosen because of its non-lethal effects on *E. coli* 3300 (the effect of Brij 58 on the cell viability was examined by exposing the cells to 1% Brij 58 for the period of 1 h, after which

the viability of the treated cells and the untreated control cells was examined by plating and colony counts; data not shown). In contrast, both sodium dodecyl sulphate and sodium cholate were found to decrease the viable counts over a 1 h period (data not shown). Table 3.4 shows that the presence of 0.1% (w/v) Brij 58 in the bacterial suspension during the contact time with the polyester cloth significantly reduced adhesion levels, strongly indicating the involvement of hydrophobic interactions in the adhesion of *E. coli* 3300 to polyester cloth.

Treatment of *E. coli* 3300 and polyester cloth with Brij 58

It was observed that both PVA and Brij 58 were equally efficient at inhibiting the adhesion of *E. coli* 3300 to polyester cloth (Table 3.4). If Brij 58 is also capable of binding to polyester cloth and coating the cells, then the mechanism of the interaction of these two molecules with the hydrophobic surfaces may be the same. For treatment of the cells with Brij 58, the detergent was dissolved in basal M63 buffer, instead of distilled water, to prevent any damage to the cells that might occur by suspending the cells in distilled water. Table 3.3 shows that Brij 58 was equally efficient at coating the cells of *E. coli* 3300 as in the case of PVA: significant reduction in the adhesion of the Brij 58-treated cells to polyester was observed upon washing the cells only once, but the capability of the cells to bind to polyester that were washed three times was equal to that of the untreated control cells. In addition, treatment of the polyester cloth with Brij 58 was found to decrease the adhesion levels to some degree, indicating that Brij 58 weakly binds to polyester cloth. The similar behaviour and effect on the adhesion process of PVA and Brij 58 is suggestive of the same or similar

mechanism that these molecules employ in interfering with the adhesion process. Since the strength of the adhesion of PVA to polyester cloth was found to increase with the length of the PVA polymeric chain, the lower efficiency of binding of Brij 58 to the polyester cloth is most likely due to a much shorter length of the hydrocarbon chain in the Brij 58 molecule, as compared to the PVA polymer molecule.

Release of adhered *E. coli* 3300 from polyester by PVA and Brij 58

Both PVA and Brij 58 were found to reduce the adhesion levels of *E. coli* 3300 to polyester through coating of the hydrophobic cloth and by interfering with the adhesion process. If the adhesion of the cells to the polyester cloth is mediated by the hydrophobic interaction, then one could argue that PVA and Brij 58 should be capable of removing the cells from the hydrophobic surface of the polyester cloth. To account for this possibility, the polyester cloth containing the adhered bacteria was treated by shaking in 1% (w/v) PVA and Brij 58 solutions (both PVA and Brij 58 were dissolved in basal M63 buffer instead of distilled water, in order to prevent any damage to the cells that might occur by suspending the cells in distilled water) for the period of 10 min, after which the cloths were washed to remove PVA and Brij 58, before assaying for the presence of the cells both on the cloth and in the PVA and Brij 58 solutions. To account for the purely mechanical removal of the cells from the cloth due to shaking and washing, a control cloth was also assayed that was shaken in the M63 buffer without the presence of PVA or Brij 58. Table 3.5 shows both the number of cells that were retained on the cloth, and were removed into the PVA or Brij 58 solution after shaking the cloths containing the adhered cells, in the presence or the absence of PVA

Table 3.5. Release of *E. coli* 3300 cells from polyester cloth by PVA and Brij 58^(a).

Cloth treatment	Total cells released (x 10⁻⁷)	Residual cells (CFU x 10⁻⁷)
control (no treatment)	0.00±0.00	5.80±0.54
control (shaking and washing) ^(b)	0.52±0.09	4.70±0.45
shaking (Brij 58)	3.25±0.39	2.43±0.34
shaking (PVA)	2.80±0.23	2.32±0.28

^(a) Cells released from the cloths upon treatment by shaking in the presence of PVA or Brij 58 were assayed by colony counts. The number of cells retained by the cloth after the treatment with PVA or Brij 58 (CFU) was obtained from A₄₂₀ upon conversion with the β -galactosidase standard, as described in the Methods.

^(b) To account for any non-PVA or non-Brij 58 induced cell release from the cloth due to the shaking and subsequent washing before assaying the cloths by the β -galactosidase assay, a control cloth was assayed that was shaken in the M63 buffer and washed afterwards.

and Brij 58. Although there was some loss of the cells from the control cloth that was shaken in the absence of PVA or Brij 58 and washed afterwards, due to mechanical removal of the cells by the 10 min shaking and washing, substantially higher fractions of cells were removed from the polyester cloths that were shaken in the presence of PVA or Brij 58, indicating that these molecules stimulated the removal of the cells from the hydrophobic surface of the polyester cloth. Since a substantial number of cells were still retained on the cloth after the 10 min PVA or Brij 58 shaking treatment, the strength of adhesion of the cells to the cloth must be quite substantial. This experiment further substantiates the notion that the adhesion of *E. coli* 3300 to polyester cloth is mediated by hydrophobic interactions.

Discussion

The set of experiments presented in this chapter strongly indicates hydrophobic interactions between the *E. coli* 3300 cells and the cloth as the driving force of the adhesion process. The PVA modification of the cloths was found to be extremely efficient at reducing both the hydrophobic character of the hydrophobic cloths and the levels of adhesion of *E. coli* 3300, indicating that the introduction of the hydrophilic hydroxyl groups to the hydrophobic surface of the cloth via PVA adsorption has an inhibitory effect on the adhesion process, and therefore hydrophobic surface of the cloth is necessary for the process of adhesion to take place.

The fact that the nonionic surfactant Brij 58 exhibited similar effects on the adhesion of *E. coli* 3300 to polyester cloth, as PVA, indicates that the mechanism of action for PVA and Brij 58 molecules is similar: both were found to adhere to polyester cloth, although the

adsorption of Brij 58 onto polyester cloth was much less efficient, both molecules were found to be equally efficient at inhibiting adhesion when added at 0.1% concentration during the adhesion process. In addition, both PVA and Brij 58 were almost equally efficient at removing bound cells of *E. coli* 3300 from the polyester cloth. Since both molecules contain both hydrophobic and hydrophilic regions, and the mechanism of PVA adhesion involves the hydrophobic backbone of the PVA polymer that binds to the surface, and the hydrophilic hydroxyl substituents that inhibit the adhesion process (Figure 3.2), the binding of the Brij 58 molecule to the hydrophobic surface of the polyester cloth most likely takes place via the hydrophobic part of the molecule, while the hydrophilic part of the molecule inhibits the adhesion process. In fact, the anti-adhesive effects of alcohol ethoxylate surfactants, such as Brij 58, on the adhesion of bacteria to hydrophobic polystyrene were demonstrated previously. It was proposed that adsorption of the surfactant takes place via the hydrocarbon moiety, resulting in a surface covered by a high density of hydrophilic (poly)ethylene oxides, thus preventing the contact of bacteria with the hydrophobic surface (Humphries *et al.*, 1987). The hydrophobic and hydrophilic moieties of the Brij 58 molecule are presented in Figure 3.3.

Figure 3.3. Hydrophobic and hydrophilic parts of the Brij 58 molecule.

This accounts for the observation that Brij 58 was much less efficient at binding to the polyester cloth, as compared with PVA. Since binding is mediated by the hydrophobic part of the molecule, and binding strength increases with the chain length of the polymer, as demonstrated for PVA (Rodda and Yamazaki, 1994), the much shorter hydrophobic part of Brij 58 is much less efficient at binding to hydrophobic surface, than the much longer hydrocarbon backbone of PVA.

It is hard to speculate on the adhesion of PVA and Brij 58 to the *E. coli* 3300 cells, since in both cases low 0.1% concentrations of these molecules present during the adhesion process were extremely efficient at preventing adhesion. If low concentrations of these molecules are able to inhibit adhesion so efficiently, therefore it is possible that the single wash of the cells before testing adhesion was inefficient and resulted in retainment of low concentrations of PVA and Brij 58, which was the primary reason for the reduction of the adhesion levels to polyester, to some degree. In fact, the addition of nonionic surfactants at concentrations as low as 10 ppm to bacterial suspensions during the adhesion process was demonstrated to have significant inhibitory effects on the adhesion process (Humphries *et al.*, 1987). On the other hand, the strength of binding of these molecules to *E. coli* 3300 cells might be very weak, in which case the triple washing that included centrifuging would result in the removal of PVA and Brij 58 from the surface of the *E. coli* 3300 cells. Since the surface of the cells is not as organized as the crystalline hydrophobic surface of polyester fibres, the binding of PVA or Brij 58 to the cell surface might be in fact occurring, but at significantly reduced strength, resulting in a complete removal of these molecules from the cell surface after the triple washing involving centrifuging treatment.

Chapter 4

Immobilization of *E. coli* 3300 onto cotton and polyester cloths

Abstract

The immobilization of *Escherichia coli* 3300 on cotton, polyester, and PVA-coated polyester cloths was demonstrated to be a two-stage process, consisting of initial bacterial adhesion followed by bacterial growth inside the porous structure of the cloth. Since the initial adhesion levels were higher to hydrophobic polyester cloth, as compared with the hydrophilic cotton and PVA-coated polyester, the rate of growth of the cells within the pores of the cloth was also higher for the hydrophobic polyester, resulting in the completion of the immobilization process in 5 days for polyester, 11 days for cotton, and 17 days for PVA-coated polyester.

Introduction

Cotton cloth, although very hydrophilic, was demonstrated as a suitable support for immobilization of a number of bacterial species, and the cotton cloth-*Streptomyces* system was successfully employed in the production of two β -lactam antibiotics through a repeated batch fermentation process utilizing the system as the 'resident' inoculum (Joshi and Yamazaki, 1985; Joshi and Yamazaki 1987 a). Furthermore, the immobilization was found to be stable and resistant to replacement by other bacteria (Joshi and Yamazaki, 1987 b; Joshi and Yamazaki, 1987 c). However, the process of immobilization was found to take place over a period of days, until the cloth became fully saturated with bacteria, suggesting a progressive colonization of the cloth by cellulophilic variants.

As demonstrated in the previous two chapters, the initial adhesion levels of *E. coli* 3300 were found to be much higher to hydrophobic cloths, including polyester, than to the

hydrophilic cotton cloth. In addition, it was demonstrated in Chapter 3 that the adhesion of *E. coli* 3300 to polyester cloth is largely mediated by the hydrophobic interactions. Since the initial coverage of the hydrophobic polyester by the *E. coli* 3300 cells was much higher than for the hydrophilic cotton, the process of full saturation of the cloth, which was observed to take place over the period of days for the cotton cloth, should occur at a higher rate for the hydrophobic polyester cloth.

The present chapter examines the immobilization process on the cloths by comparing the rate of the immobilization for the model bacterium *E. coli* 3300 on cotton, polyester, and PVA-modified polyester cloths. Polyester cloth will be employed as a model hydrophobic cloth, due to its similar macroporosity as that of the cotton cloth, thus ensuring that the cloths being compared have approximately similar surface areas.

Results

Adhesion of *E. coli* 3300 to cotton, polyester, and PVA-modified polyester cloths

The measurements of the initial adhesion levels performed in the previous two chapters were based on the 10 min contact time of the cells with the cloth. Since the 10 min period might be not sufficient to allow the cells to reach all available binding sites on the cloth, the adhesion potential of the cells was monitored for a period of 120 min to account for any possible additional adhesion that might take place after the 10 min period. Figure 4.1 shows that the adhesion levels for polyester continued to increase within the 30 min period, after which no initial adhesion was observed, indicating that the surface area of the polyester

Figure 4.1. Kinetics of *E. coli* 3300 adhesion to cotton cloth, polyester cloth, and PVA-coated polyester cloth. The number of adhering bacteria (CFU x 10⁻⁷) was obtained from the A₄₂₀ values with the A₄₂₀ standard, as described in the Methods.

cloth available for the cell adsorption was fully occupied. In contrast, the levels of adhesion to the hydrophilic cotton and PVA-modified polyester cloths remained at extremely low levels throughout the 120 min period, indicating that the adhesion to hydrophilic surfaces is very minimal and occurs at an extremely slow rate.

Development of the 'progeny generation' assay

Since the β -galactosidase assay is unsuitable for a repeated measurement of the *E. coli* 3300 cell numbers that adhere to cloths (cells are ruptured and released from the cloth during the vigorous shaking in the presence of chloroform and SDS, which are utilized by the β -galactosidase assay), a more suitable assay was employed allowing for a repeatable assessment of the development of the adhered/immobilized cells on the cloth. The 'progeny generation' assay employed measures the number of the progeny cells released from the cloth due to cell division of the adhered cells on the cloth and the subsequent release of the free cells into the bathing growth medium, which are accounted for through colony counts. To minimize the cells being produced by the already released progeny cells from being counted as the primary progeny cells, the cloths were exposed to the growth medium for a period of 30 min, which is less than the doubling time of *E. coli* 3300 (58 ± 6 min). Since not all initially adhered cells are expected to produce progeny within the 30 min period that would be released to the bathing medium and accounted for by the colony counts, this assay does not estimate the number of the adhered cells, it only measures the capacity of the adhered cell population to produce progeny. Since the progeny production is directly proportional to the size of the adhered/immobilized cell population, the progeny generation assay should allow

for the measurement and comparison of the rate of the immobilization of the cells on the cotton, polyester, and PVA-polyester cloths.

To measure the immobilization rate of *E. coli* 3300 on the cloths, the exponential *E. coli* 3300 was allowed to adhere to the cloths for a 30 min period (initial adhesion is completed within the 30 min period), and the ability of the initially adhered cells to generate progeny cells was measured. Afterwards, the cloths were incubated for a period of 24 h, washed, suspended in fresh growth medium, and incubated for a period of 30 min to allow for the production of progeny cells. This process was repeated for a period of 20 days. Since the cells used as the initial inoculum for binding to the cloths were in the exponential phase, and each day the immobilized population was after approximately 24 h incubation period (close to the stationary growth phase), the ability of the exponential and the 24 h old (overnight) *E. coli* 3300 to generate progeny was compared by measuring their doubling times. Since it is well known that the age of bacterial cells has an influence on their adhesion behaviour (Van Loosdrecht *et al.*, 1987 b), the ability of the exponential and overnight *E. coli* 3300 cells to adhere to cotton, polyester, and PVA-polyester cloths was compared and measured by the β -galactosidase assay. Table 4.1 shows that the doubling time of the overnight culture was only 15% lower than that of the exponential culture, indicating that the overnight cells still maintained a high capacity to generate progeny. Therefore, the rate of the progeny production from the cloth containing overnight cells was expected to be 15% lower than that of the cloth containing exponential cells. Table 4.1 also shows that the adhesion capacity of the overnight *E. coli* 3300 to all cloths was lower than that of the exponential culture, which indicates that the overnight cells are not expected to adhere to cloths as

Table 4.1. The doubling times (D.T.) of the exponential and the overnight (24 h old) cultures of *E. coli* 3300, and the adhesion of both cultures to polyester (P), cotton (C), and PVA-modified polyester (P-PVA) as determined by the initial adhesion (10 min) β -galactosidase assay.

Cells		D.T. (min)	Adhesion to	CFU x 10 ⁻⁷ ^(a)
Exponential		58±6	P	5.95±0.70
			C	0.21±0.02
			P-PVA	0.06±0.02
Overnight	unwashed	68±5	P	3.42±0.28
			C	0.09±0.02
			P-PVA	0.01±0.01
	washed	not measured	P	3.09±0.50
			C	0.13±0.04
			P-PVA	0.03±0.02

^(a) The CFU x 10⁻⁷ values were obtained from the A₄₂₀ values upon conversion with the β -galactosidase standard, as described in the Methods. Since the β -galactosidase level of the overnight culture was lower than that of the exponential culture, the A₄₂₀ values for the overnight cultures were corrected for by multiplying by the ratio of the β -galactosidase levels of the exponential to overnight cultures.

strongly as the exponential cells. To account for the possible presence of metabolites accumulated in the overnight culture, which might interfere with the adhesion process (Paul, 1984), the adhesion of washed and unwashed overnight cells to the three cloths was compared. Table 4.1 shows that both the washed and unwashed cells were equally capable of adhering to cloths, indicating that the overnight suspension did not contain any metabolites capable of interfering with the adhesion of *E. coli* 3300 cells to cloths.

Immobilization of *E. coli* 3300 on cotton, polyester, and PVA-modified polyester cloth

The rates of development of the immobilized *E. coli* 3300 population on cotton, polyester, and PVA-modified polyester were compared with the 'progeny generation' assay over a period of 20 days. Figure 4.2 shows the progressive increase in the capacity to generate progeny for all the cloths, which was previously demonstrated for the cotton cloth. As shown by the Figure 4.1, the initial capacity to produce progeny, measured on the first day, was substantially higher for polyester, than for cotton or PVA-polyester cloths, which is in agreement with the results presented in Figure 4.1. By comparing Figures 4.1 and 4.2, one can observe that the number of progeny cells measured with the progeny generation assay, was only a fraction of the adhered cell population, which was measured by the β -galactosidase assay (2.1×10^7 CFU for progeny vs 9.6×10^7 CFU for adhered cells). This was expected since the 30 min period during which the immobilized population was allowed to generate progeny, was only approximately half of the doubling time for *E. coli* 3300. In addition, the low fraction of the progeny cells released from the cloth into the bathing growth

Figure 4.2. Development of immobilized bacteria on polyester, cotton, and PVA-coated polyester cloth. The rates of progeny generation from the cloth segments (1 cm squares) are shown.

medium is likely partly due to the physical retainment of some of the progeny cells within the pores of the cloth.

Figure 4.2 demonstrates that the rate of the immobilization of *E. coli* 3300 on the polyester cloth was much greater than that observed for the cotton and the PVA-coated polyester: the plateau in the progeny generation potential was reached in approximately 5 days for the polyester cloth, 11 days for the cotton cloth, and 17 days for the PVA-coated polyester. Since the initial adhesion levels for both cotton and PVA-coated polyester cloths were minimal, yet the final capacities to produce progeny were equal for all three cloths, an additional mechanism (other than adhesion), must be involved in the immobilization of *E. coli* 3300 on these cloths. In addition, equal final capacity to generate progeny for cotton and polyester suggests that the surface areas of these two cloths are quite similar. Since a visible increase in the potential to produce progeny was also observed for polyester cloth (over the period of 5 days from 2.1×10^7 CFU/mL/30 min to 3.5×10^7 CFU/mL/30 min), an additional immobilization of cells must have taken place through means other than adhesion (Figure 4.1 showed that the adhesion to polyester was completed within 30 min), such as growth of the initially adhered cells within the pores of the cloth. The ability of the cells to reproduce and grow inside the cloth was demonstrated for polyester cloth by transferring a very dilute cell suspension (10-50 CFU/mL) of *E. coli* 3300 onto the cloth, which was followed by incubating the cloth on the surface of agar containing chromogenic X-gal for 24 h. Since *E. coli* 3300 produces high levels of β -galactosidase that breaks down X-gal substrate into a blue product, the growing colonies of *E. coli* 3300 inside the cloth could be easily visualized. Figure 4.3 shows that the single cells spotted onto the cloth produced visible blue single

colonies, showing that the cells are free to multiply within the cloth.

Adhesion of the *E. coli* 3300 progeny

Previous work on immobilization of bacteria on cotton cloth (Joshi and Yamazaki, 1985) has speculated that the progressive colonization of cotton by bacteria was due to the generation of cellulophilic variants that were capable of adhering to cotton. Since the initial adhesion to cotton and PVA-coated polyester cloths was very minimal (Figures 4.1 and 4.2), in comparison to the polyester cloth, it is possible that the immobilization of *E. coli* 3300 on cotton and PVA-coated polyester cloths was due to the generation of such variants (or surface mutants) within the normal wild-type cell population, which had higher affinity for the hydrophilic cotton and PVA-modified polyester. This would lead to a progressive colonization of the hydrophilic cloths by such variants, eventually leading to the saturation of the cloths. In order to account for this possibility, the progeny released on the 20th day of the immobilization experiment (Figure 4.2) was collected, grown until the exponential phase, and the capacity to adhere to fresh cotton, polyester, and PVA-coated polyester was assessed with the β -galactosidase assay. If the colonization of the hydrophilic cotton and PVA-modified polyester cloths was indeed due to the presence of variants with increased affinity for hydrophilic surfaces, than the progeny released from the cloths on the 20th day of the experiment, after the cloths were fully saturated with the immobilized bacteria, should also have such an altered affinity for the hydrophilic surfaces, as compared with the original wild-type *E. coli* 3300 used initially as the inoculum for the immobilization experiment. Table 4.2 shows that the progenies released from the cotton, polyester, and PVA-modified polyester

Figure 4.3. X-Gal agar plate with three samples of polyester cloth that received approximately 50 CFU of *E. coli* 3300, after 24 hr incubation period. Since *E. coli* 3300 produces high levels of β -galactosidase which breaks down the X-gal substrate to a blue-coloured product, the colonies growing inside the cloth can be easily visualized. The dark spots on the cloths are the bacterial colonies, indicating that the cells are not inhibited from growing inside the cloth.

on the 20th day of the immobilization experiment had exactly the same adhesion behaviour as the original wild-type cells of *E. coli* 3300, indicating that the proposed existence of variants with altered affinity for hydrophilic surfaces was incorrect. Therefore, the immobilization of *E. coli* 3300 on the hydrophilic cloths must be taking place through a progressive colonization and growth of very weakly adhered cells inside the cloth.

Discussion

The rate of immobilization of *E. coli* 3300 was found to be greater for the hydrophobic polyester cloth than for the more hydrophilic cotton and PVA-coated polyester cloths: the plateau in the potential to generate progeny was reached within 5 days for polyester, 11 days for cotton, and 17 days for PVA-polyester cloth (Figure 4.2). Because the initial adhesion levels to the hydrophobic polyester cloth were much greater than to hydrophilic cotton and PVA-coated polyester cloths (Figure 4.1), the initially adhered cells had a greater potential to reproduce inside the cloth and colonize the total surface area and volume of the cloth available for immobilization. In addition, since the strength of the cell attachment to the more hydrophobic polyester is expected to be much greater than to the hydrophilic cotton or PVA-coated polyester, daily washing resulted in the greater loss of the cells from the hydrophilic cotton and PVA-modified polyester cloths, than from the hydrophobic polyester. Thus, the rate of cloth colonization by the cells for hydrophobic polyester was greatly increased, as compared with the hydrophilic cotton and PVA-coated polyester cloths.

Since the initial adhesion levels, monitored for the three cloths over the period of 120

Table 4.2. Adhesion of *E. coli* 3300 progeny generated from polyester, cotton, and PVA-coated polyester cloths on the 20th day of the immobilization experiment^(a).

Adhesion to	Adhesion (CFU x 10 ⁻⁷) of <i>E. coli</i> 3300 generated from			
	polyester	cotton	PVA-polyester	slant
polyester	5.34±0.60	5.13±0.40	5.86±0.60	5.44±0.40
cotton	0.51±0.10	0.40±0.05	0.35±0.13	0.30±0.10
PVA-polyester	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

^(a) *E. coli* 3300 progenies generated from cotton, PVA-coated polyester, and polyester cloth on the 20th day (Figure 2) were allowed to adhere to polyester, cotton, and PVA-coated polyester cloth for 10 min, and were assayed by the A₄₂₀ assay. The CFU values were obtained upon conversion of the A₄₂₀ data with the β -galactosidase standard as described in Methods. The culture obtained from the slant was provided as a control.

min, showed no significant increase in the adhesion levels after the 30 min period (Figure 4.1), the observed increase in the progeny generation potential for the cloths over the period of 5 days for polyester, 11 for cotton, and 17 for PVA-coated polyester cloth, must be a result of progressive growth of the initially adhered bacteria inside the pores of the cloth, that takes place during the daily 24 h incubation period between washings that remove weakly adhering cells. The potential of the cells to freely grow within the polyester cloth was demonstrated (Figure 4.3), indicating that the normal colony formation inside the cloth is not inhibited. Since the daily washing of the cloths, prior to the examination of the potential to generate progeny by the progeny generation assay, was efficient enough to remove the non-adhering cells of *E. coli* 3300 from cotton and PVA-coated polyester cloth, the growth of the cells inside the cloths, that results in the progressive increase in the progeny generation potential each day, must result in the formation of a stable cell network inside the cloth, which is resistant to removal from the cloths by the daily washing process. Since the number of the immobilized cells was identical for all three cloths on the 20th day of the experiment, the porosity of the cotton and polyester cloth, and therefore the capacity to allow for the cell network formation must be very similar.

The formation of cell networks by bacteria upon immobilization takes place during the natural process of biofilm formation. The natural process of biofilm formation consists of two separate steps. The first involves the formation of a primary cell monolayer, and is governed by the physicochemical properties (hydrophobicity, electrostatic interactions) of the support surface and the adhering cell. The initial adhesion is subsequently followed by the formation of other layers of cells that accumulate on top of each other, and is governed

by the attractive cell-cell interactions (Mozes and Rouxhet, 1990). The similar process must be taking place during the immobilization of *E. coli* 3300 on the cloths. In this case, the formation of the initial monolayer takes place for the hydrophobic polyester, but is very limited for the hydrophilic cotton and PVA-coated polyester cloths (Figure 4.1). However, during the daily 24 h incubation periods, in between the washings, the cells are free to migrate throughout the cloths, reproduce, and grow inside and on the surface of the cloth, effectively colonizing the pores of the cloth and forming colonies of cells that are retained during the washing process. The growth and migration of the expanding colonies each day into the pores that might be initially inaccessible to the adhering cells, results in a further increase of the immobilized bacteria within the cloth. The formation of the colonies is most likely mediated by the cell-cell interactions that are likely to be more efficient than the cell-cloth interactions in the case of the hydrophilic cotton and PVA-polyester, because the cells are expected to be more hydrophobic than the hydrophilic cloths (the adhesion to hydrophobic polyester was much more effective than to hydrophilic cotton and PVA-coated polyester). In addition, stabilization of the colonies inside the pores against washout might also be due to the production of external polysaccharides that coat the cells and form the matrix for the cells in the colony. Evidence for the presence of extracellular polysaccharide matrices within the bacterial surface films (Figure 4.4 A) and colonies (Figure 4.4 B) has been documented for various gram-positive and gram-negative bacteria (Fletcher and Floodgate, 1976; Tetz *et al.*, 1993). In addition, the presence of carbohydrate filaments connecting individual cells within bacterial films has been demonstrated as well (Figure 4.5) (Brooker and Fuller, 1976).

(A)

(B)

Figure 4.4. Transmission electron microscope micrographs of sections from bacterial film of *Pseudomonas* sp. showing the bacterial cells and their associated polysaccharides (from Fletcher and Floodgate, 1976) (A), and surface film of 1 day old colonies of *E. coli* K12 showing bacteria embedded in the polysaccharide matrix; arrows indicate the boundary of the surface film (from Tetz *et al.*, 1993) (B).

Figure 4.5. Transmission electron microscope micrograph of section from bacterial film of *Lactobacillus* cells showing surface carbohydrate material extending between the individual cells (from Brooker and Fuller, 1976).

The increase in the number of immobilized cells for the polyester cloth over the 20 day period was not as substantial as expected from the assumption that the cells divide inside the cloth and effectively increase the number of immobilized bacteria. Figure 4.2 shows that the increase was only approximately two fold (2.1×10^7 to 3.5×10^7 CFU/cloth/30 min). This low increase in the capacity to generate progeny over the 20 day period, as compared with day 1, is due to a number of reasons. First, the washing likely removes the cells that are immobilized close to the surface of the cloth. Secondly, since the cells form colonies inside the pores, the release of the progeny cells is most likely to be slowed down as the size of the immobilized colonies increases, because the cells inside a colony are expected to be growing more slowly due to limited diffusion of the nutrients and the accumulation of waste products, compared with the cells on the outside of the colony. In addition, the release of the progeny cells from the colonies immobilized in less accessible pores is likely to be minimal within the 30 min period. Finally, the capacity of the 24 h old cells to generate progeny was demonstrated to be lower than that of the exponential cells, indicating that measurement of the potential to generate progeny from the cloths after the first day was based on cells with decreased potential to generate progeny, thus underestimating the number of immobilized cells with respect to the first day which was based on the exponential cells.

It was demonstrated that the progeny cells released on the 20th day of the immobilization experiment (Figure 4.2) from the three cloths displayed the same adhesion characteristics to fresh polyester, cotton, and PVA-modified polyester cloths, as did the original *E. coli* 3300 used for the immobilization experiment. This indicates that the immobilization of the cells on the hydrophilic cotton and PVA-coated polyester cloths does

not occur due to the generation of cell surface mutants of *E. coli* 3300, which bind more effectively to hydrophilic surfaces than the wild-type cells, thus eventually occupying the hydrophilic cloths. This further supports the idea that the immobilization process on the cloths occurs due to the growth of the cells inside the cloth.

Since the experimental procedures involved daily washing of the cloths, which would not be necessary during the fermentation process, the colonization of the cloths by bacteria in the absence of washing, is most likely to occur at a much higher rate. However, since the retainment of bacteria inside the cloth is dependent on the operational conditions of the fermentation process, such as stirring, which introduces shear forces and therefore removes weakly attached cells, the hydrophobic cloths, binding cells stronger than the hydrophilic cloths, should offer more stable retainment of the immobilized cells within the cloth at high rates of stirring.

Chapter 5

Adhesion of bacteria to cotton and polyester cloth

Abstract

Adhesion of five strains of gram-negative bacteria of three genera and three strains of gram-positive bacteria of three genera was found to be more efficient to hydrophobic polyester, than to hydrophilic cotton and PVA-coated polyester, indicating general usefulness of polyester cloth for bacterial immobilization as well as the involvement of hydrophobicity in the process of bacterial adhesion. In addition, blending of the bacterial cultures, which is known to damage bacterial cell surface appendages without affecting cell viability, resulted in significant reduction in adhesion for 6 out of 8 strains examined, suggesting that cell surface appendages may play an important role in the process of bacterial adhesion to cloths.

Introduction

In Chapter 4, hydrophobic polyester cloth was demonstrated to be superior for immobilization of the model bacterium *E. coli* 3300, offering higher rate of immobilization than the hydrophilic cotton and PVA-coated polyester cloths. Since higher immobilization rates shorten the time required for the development of the fully immobilized bacterial cloth system, it is necessary to investigate the applicability of the polyester cloth to other bacteria, if the polyester cloth is to be applied as an universal cloth-based bacterial immobilization support.

In the present chapter the adhesion of five strains of gram-negative bacteria of three genera and three strains of gram-positive bacteria of three genera to cotton, polyester, and PVA-coated polyester cloths were compared in order to examine the general usefulness of the hydrophobic polyester cloth for immobilization of different bacteria.

In addition, the significance of the presence of bacterial cell wall appendages, that are susceptible to shearing, on the adhesion process to cloths, will be studied by comparing the adhesion capacity of the untreated cells with the cells that were exposed to blending.

Results

Adhesion of bacteria to cotton, polyester, and PVA-coated polyester

To measure the capacity of polyester, cotton, and PVA-coated polyester to adsorb various strains of bacteria, the initial adhesion (10 min contact time) of the bacteria to the cloths was measured with the 'progeny generation' assay (described in Chapter 4), as the β -galactosidase assay was only applicable to *E. coli* 3300 that expressed high levels of β -galactosidase. Table 5.1 shows that the potential of adhering cells to generate progeny, and therefore that the number of adhering bacteria, was greater to the hydrophobic polyester than to hydrophilic cotton and PVA-coated polyester cloths for all bacteria examined, indicating that cloth hydrophobicity determines the adhesion potential to cloths not only for *E. coli* 3300, but for other bacteria as well. Since both gram-positive and gram-negative bacteria adhered more efficiently to hydrophobic polyester, hydrophobic interaction must be the dominant force involved in the adhesion process of both types of bacteria. Therefore, the more hydrophobic polyester should be considered as a more efficient support for immobilization of both gram-negative and gram-positive bacteria, than the hydrophilic cotton.

Table 5.1. Adhesion of various bacteria to polyester, cotton, and PVA-coated polyester cloth.

Bacterium	Doubling time (min)	No. of progenies x 10 ⁻⁴ generated from ^(a)		
		polyester	cotton	PVA-polyester
<i>E. coli</i> 3300	58±6	468±96	22±7	13±9
<i>E. coli</i> Crooks	60±2	58±10	4±1	5±2
<i>E. coli</i> ATCC 25922	61±5	47±4	6±1	3±2
<i>Ba. megaterium</i> QMB 1551	43±2	26±6	3±2	2±1
<i>Br. ammoniagenes</i> ATCC 6872	75±3	26±4	4±1	1±1
<i>St. aureus</i> ATCC 12600	63±1	30±5	15±4	5±1
<i>Sa. typhimurium</i> LT2	55±2	203±52	41±16	9±6
<i>P. aeruginosa</i> ATCC 10145	75±3	442±44	90±20	65±20

^(a) The 'progeny generation' assay was described in Chapter 4.

Effect of blending treatment on the adhesion capacity to polyester

Bacterial cell surface appendages, flagella and fimbriae, have been implicated as contributing factors in the adhesion process, and can be easily removed by blending a cell suspension without affecting the viability of the cells (Brock, 1974). To examine the significance of the presence of bacterial surface appendages on the adhesion of bacteria to polyester cloth, bacterial cell suspensions were blended at 25000-30000 rpm for 5 min prior to the adhesion test. The initial (10 min contact time of the cells with the cloth) adhesion capacity to polyester cloth was measured and compared by the 'progeny generation' assay for untreated control cells and cells that were exposed to blending. To correlate the adhesion levels for the untreated control and blended cells with the presence of flagella, microscopic hanging drop test for bacterial motility was performed (described in Wistreich and Lechtman, 1969). However, of the eight strains examined (Table 5.1), positive motility was only observed for *P. aeruginosa* ATCC 10145, and the fraction of motile cells was found to be substantially lower following the blending, indicating the removal of flagella due to shearing. All other strains, however, were not observed to be motile, although very low fractions of cells were observed as motile for *Sa. typhimurium* LT2. Since the presence of fimbriae has been widely demonstrated for *E. coli* and *P. aeruginosa* (Irvin, 1990), *E. coli* 3300 and *E. coli* ATCC 25922, *P. aureginosa* ATCC 10145, and *Sa. typhimurium* LT2 were chosen for the blending experiment. In addition, a collection of *E. coli* mutants, which included the wild type parent strain GM30, flagellar mutants H585 and EHL1, and a double flagellar-fimbrial mutant JE3515, was employed to study the effect of mutations that abolish the synthesis of flagella and/or fimbriae on the adhesion process to polyester cloth. Although the microscopic

examination confirmed the nonmotile character of the mutants, only very low fraction of cells were identified as motile for the wild type GM30 strain, indicating that the production of flagella or its functionality were minimal at the exponential phase of growth in full M63 medium for all the strains examined, except for *P. aeruginosa* ATCC 10145. Since the 'progeny generation' assay relies upon the ability of the adhered cells to divide and release the progeny cells, which in terms depends upon the cell viability, the doubling times, which measure the ability of the cells to divide, were measured for both the untreated control and the treated cells. Table 5.2 shows the summary of the adhesion tests, as measured by the 'progeny generation' assay, and the doubling times for the control and the blended bacteria. The doubling times were identical for the control and the treated cells, indicating that the blending treatment did not affect the capacity to generate progeny for any of the tested bacteria. Significant reductions in the adhesion levels upon blending were observed for the majority of the strains: 52% for *E. coli* 3300 (which correlates well with the 41% reduction detected by the β -galactosidase assay, as shown in Table 5.3), 74% for *Sa. typhimurium* LT2, 56% for *P. aeruginosa* ATCC 10145, 83% for the wild type parent *E. coli* GM30, and 85% for the flagellar mutant *E. coli* H585. No reductions were observed for *E. coli* ATCC 25922, the flagellar mutant *E. coli* EHL1, and the double flagellar-fimbrial mutant *E. coli* JE3515. All three mutants displayed lower adhesion levels than the parent wild type strain (at similar doubling times), indicating that the ability to express flagella and/or fimbriae might stimulate adhesion. The observation that the adhesion levels for the majority of the tested strains were significantly lowered following the blending treatment, strongly indicates that the cell surface structures that are susceptible to destruction by shearing forces mediate the process of

Table 5.2. Effect of blending treatment on the adhesion to polyester, as measured by the progeny generation assay, and viability of bacteria, as measured by the doubling time.

Bacterium	Progeny generation (CFU x 10 ⁻⁴) ^(a)		Doubling time (min)	
	control	blended	control	blended
<i>E. coli</i> 3300	458±62	222±30	58±6	59±4
<i>E. coli</i> ATCC 25922	48±3	46±7	61±5	61±2
<i>E. coli</i> GM30	216±19	36±7	51±3	51±3
<i>E. coli</i> H585	129±21	19±7	52±4	51±3
<i>E. coli</i> EHL1	70±27	54±11	57±3	57±4
<i>E. coli</i> JE3515	59±11	66±15	56±3	52±3
<i>Sa. typhimurium</i> LT2	203±39	52±16	55±3	55±2
<i>P. aeruginosa</i> ATCC 10145	446±42	196±19	75±3	73±2

^(a) The 'progeny generation' assay was described in Chapter 4.

Table 5.3. Adhesion (CFU x 10⁻⁷) of untreated and blended *E. coli* 3300 to polyester, as examined by the β -galactosidase assay^(a).

Untreated	Blended
5.57±0.40	3.32±0.41

^(a) The CFU x 10⁻⁷ values were obtained from A₄₂₀ upon conversion with the β -galactosidase standard, as described in the Methods.

bacterial attachment to polyester cloth.

Discussion

The eight strains of bacteria, which included five strains of gram-negative bacteria of three genera and three strains of gram-positive bacteria of three genera, were found to adhere in higher numbers to the hydrophobic polyester cloth, as compared with the hydrophilic cotton and PVA-coated polyester cloth, which indicates that hydrophobicity mediates the attachment of bacteria to polyester cloth, which is in agreement with the previous studies indicating that hydrophobicity is the dominant interaction involved in the bacterial adhesion to solid surfaces (Van Loosdrecht *et al.*, 1987 a, b). Since, as demonstrated in Chapter 4, the rate of immobilization of *E. coli* 3300 on the cloths was found to be dependent on the number of initially adhered bacteria, the higher levels of adhesion of bacteria to the hydrophobic polyester should allow for higher rates of immobilization of bacteria on this cloth, compared with the hydrophilic cotton and PVA-coated polyester that were less efficient in adsorbing bacteria.

The adhesion of five of the eight strains to polyester cloth was found to be reduced following the blending treatment, indicating that the presence of bacterial cell surface appendages that are susceptible to destruction by the blending treatment is important in the adhesion process of bacteria to polyester cloth.

The bacterial cell surface appendages, flagella and fimbriae (also called pili), have been indicated as important factors involved in the adhesion of bacteria to surfaces. Figures 5.1, 5.2, and 5.3 show the appearance of flagella and fimbriae on the surface of bacterial

Figure 5.1. Electron micrograph of a dividing cell of *Salmonella typhi*, showing flagella and fimbriae (from Brock *et al.*, 1994).

(A)

(B)

Figure 5.2. Electron micrographs of *Pseudomonas coronafaciens* (A) (from Brock, 1974) and *Bordetella bronchiseptica* (B) (from Salle, 1973).

(A)

(B)

Figure 5.3. Electron micrographs of piliated *Escherichia coli* (A) (from Boyd, 1988) and (B) (from Salle, 1973).

cells. The significance of flagella on the process of surface colonization was demonstrated to have a two-fold significance. A study, testing the efficiency of surface colonization by motile and nonmotile mutants of *Pseudomonas fluorescens*, concluded that bacterial motility conveyed a selective advantage in the surface colonization due to an increased transport of the motile cells to the hydrodynamic boundary layer between the cell and support surfaces where the adhesion process takes place (Korber *et al.*, 1994). In another study, adsorption of *Azospirillum brasilense* to wheat roots was also found to be increased for the motile strains, but in this case the binding of the isolated and purified flagellum to wheat roots was demonstrated, indicating that the flagellum was the principal adhesive factor stimulating the attachment of the cells to the wheat root surface (Croes *et al.*, 1993). In the case of cloths, where the cells have to penetrate the fibre network, bacterial motility might offer a similar advantage in colonization of the fibre network of the cloth by the bacterial cells. Since the adhesion process is a two-step event, first requiring the transport of the bacterial particle close to the adhesive surface, where the adhesion is initiated (Rijnaarts *et al.*, 1993), the ability of the motile cells to actively migrate through the fibre network might increase the rate of the transport of the bacterial cells towards the adhesive surface and the frequency of attachment to the fibre surface of polyester cloth, therefore resulting in higher adhesion levels for the motile cells.

The nonflagellar bacterial cell surface appendages, fimbriae, have been generally associated with adhesive functions for a wide variety of bacterial species and a wide variety of surfaces (Irvin, 1990). The fact that fimbriae have a widespread distribution, are exposed at the surface of the cell and extend beyond the capsule, and are quite hydrophobic (Irvin,

1990), indicate that they might be involved in the process of bacterial adhesion via hydrophobic interaction with the surfaces. The proteins that constitute fimbriae, pillins, were found to contain approximately 30% hydrophobic amino acids, and their expression was linked to increased cell surface hydrophobicity (McConnell *et al.*, 1987). In addition, isolated and purified pili of *P. aeruginosa* were shown capable of binding to hydrophobic polystyrene, which indicates that pili are capable of mediating attachment to hydrophobic surfaces (Irvin, 1990).

The inability to identify strains that displayed lower adhesion levels due to shearing as motile, except for *P. aeruginosa* ATCC 10145, either indicates the absence of flagella altogether, or the presence of a nonfunctional flagella. In the case of the absence of the flagellar structure, the involvement of fimbriae in the attachment to polyester cloth for these strains would be a plausible possibility. In the other case, either the presence of a nonfunctional flagella or fimbriae, or both would be responsible for stimulating the attachment of the cells to the polyester fibres. The fact that the flagellar mutants of *E. coli* were found to adhere at much lower levels than the wildtype parent strain GM30, that was not found to be motile, indicates that the presence of a nonfunctional flagellae might be involved in the adhesion of GM30. On the other hand, the fact that the flagellar mutant *E. coli* H585 displayed a significant (85%) reduction in adhesion upon blending, indicates a substantial involvement of fimbriae (or some other structures that are susceptible to blending) in the adhesion process. In addition, adhesion levels of the double flagellar-fimbrial mutant strain *E. coli* JE3515, not expected to express either flagella or fimbriae, were unaffected by blending, strongly indicating that the inability to produce flagella and

fimbriae was the primary reason for the reduced adhesion levels of this mutant, compared with the wildtype GM30. The fact that the flagellar mutant strain EHL1 displayed lower adhesion levels than that of H585 and was only slightly affected by the blending treatment (in contrast to H585), suggests that the production of fimbriae by this mutant had to be less significant than in the case of H585. In the case of *P. aeruginosa* ATCC 10145, the involvement of functional flagella on the adhesion process can be anticipated, since the reduced adhesion levels were correlated with the reduced fraction of the motile cells upon blending. For *Sa. typhimurium* LT2 and *E. coli* 3300, which were found to be nonmotile, the involvement of fimbriae in adhesion would be the most likely explanation. Finally, in the case of the nonmotile *E. coli* ATCC 25922, where the adhesion levels were unaffected by blending, the production of any structures that would be susceptible to blending must have been insignificant, or the adhesion mechanism employed by this strain did not involve any surface appendages. In fact, the synthesis of fimbriae has been shown to be tightly regulated and responsive to specific environmental signals (Irvin, 1990), which suggests that some of the strains used in the study did not produce any fimbriae in the exponential phase. However, in order to rule out the possible existence of a nonfunctional flagella and fimbriae, a thorough microscopic examination of the examined bacteria would have to be conducted to conclusively determine the involvement of flagella and fimbriae on the adhesion of the examined bacteria to polyester cloth.

The interesting observation that bacterial motility stimulates adhesion through increasing the rate of transport of the cells to the adhesive surface (Korber *et al.*, 1994) might be of significance to the immobilization systems utilizing cloths as the supports: since the

bacterial cells have to migrate through the fibre network of the cloth to colonize the surface, motile strains would be preferred as the immobilized bacterial catalysts, offering higher rate of surface colonization, and therefore immobilization, and most likely a higher rate of release of progeny cells from the cloth.

Chapter 6

Stability of the PVA coating on the polyester cloth

Abstract

The PVA coating of polyester cloth was found to be stable to autoclaving, but unstable in the presence of detergents, indicating hydrophobic nature of PVA binding to polyester cloth. The treatment of the PVA-coated cloth with the glutaraldehyde cross linking agent stabilized the coating, making it resistant to removal by detergents under autoclaving conditions. In addition, PVA binding to hydrophobic polystyrene Petri culture plates was demonstrated and was correlated with the increased hydrophilic character of the PVA-coated plate, suggesting that PVA may bind to a variety of hydrophobic surfaces.

Introduction

It was demonstrated in Chapters 3, 4, and 5 that polyvinyl alcohol (PVA) modification of the polyester cloth and other hydrophobic fabrics resulted in significant decreases in bacterial adhesion to cloths. The mechanism of the PVA binding to the hydrophobic surface of the polyester cloth, proposed in Chapter 3, involved adhesion of the hydrophobic hydrocarbon backbone of the PVA molecule to the hydrophobic surface of the cloths, thus efficiently exposing the hydrophilic hydroxyl substituents outward, which in turn resulted in the conversion of the relatively hydrophobic surface to a more hydrophilic one, resulting in the reduction of the bacterial adhesion. If the PVA coating is sufficiently stable, PVA-coated polyester, and other cloths, might be employed as components of medical dressings that efficiently prevent bacteria from binding to them. In addition, if PVA is capable of binding to other hydrophobic surfaces, the PVA coating might be a simple inexpensive surface modification for a variety of materials that have the ability to prevent or

significantly reduce bacterial adhesion.

The present chapter will examine the stability of PVA coating at high temperatures in the absence or the presence of the sodium dodecyl sulphate (SDS) and polyoxyethylene 20 cetyl ether (Brij 58) detergents. Since PVA binding to hydrophobic surfaces is dependent upon hydrophobic interactions, the PVA coating might not be resistant to the action of detergents, resulting in the coating being removed. If the coating turns out to be stable at the sterilizing conditions of an autoclave in the presence of detergents, it might allow for the PVA-coated materials to be reusable. Since the adsorption of PVA to hydrophobic surface was found to be dependent on the chain length of the PVA polymer, the stability of adsorption for PVA of different molecular weights will be also examined. To test if the PVA has an affinity for materials other than cloths, the efficiency of the PVA coating to reduce the hydrophobic character will be tested on a hydrophobic polystyrene culture plate.

Results

Stability of the PVA coating

PVA coating of hydrophobic cloths was found to significantly reduce levels of bacterial adhesion. If the PVA coating is sufficiently stable on the surface of the cloth, it might permit its use as a component of medical dressings. The reusability of the PVA-coated cloths could be achieved if the coating was stable to harsh sterilizing treatments such as autoclaving in the presence of detergents. Since the PVA modification of the polyester cloth was proposed to be based on the hydrophobic interactions between the hydrocarbon backbone of the PVA molecule and the hydrophobic surface of the cloth, the coating might

be susceptible to removal from the hydrophobic surface by detergents. To examine the stability of the PVA coating, the PVA-coated polyester cloth was subjected to extreme temperatures by autoclaving at 120°C in the presence or the absence of 1% sodium dodecyl sulphate (SDS) and polyoxyethylene 20 cetyl ether (Brij 58). The length of the PVA polymer was shown to determine the efficiency of binding to hydrophobic surfaces (Rodda and Yamazaki, 1994). To examine the effect of the length of the PVA polymer on its adsorption stability to polyester, PVA of different molecular weight (MW) were compared: 13000-23000 MW and 124000-186000 MW. *E. coli* 3300 was employed to test for the presence of the PVA coating on the polyester cloth by the β -galactosidase assay. To account for any possible damaging effect to the cloth induced by the autoclaving treatment in the presence of detergents, which could interfere with the testing for the presence of the PVA coating by the β -galactosidase assay, the control (no PVA coating) polyester cloth was exposed to the same treatments that the PVA-coated cloths were subjected to. Table 6.1 shows that autoclaving had no effect on the capacity of the polyester cloth to adsorb *E. coli* 3300. Treatment of the cloth with 1% SDS or Brij 58 at room temperature and autoclaving in 1% SDS or Brij 58 did not have any significant effect either. Brij 58 (1% w/v) decreased the adhesion levels, which was expected (the potential of Brij 58 to hydrophobic polyester cloth was discussed in Chapter 3). Autoclaving in 1% Brij 58 had no effect on the cloth, indicating that Brij 58 although capable of weakly binding to polyester cloth, is removed from the cloth at high temperatures. The length of the PVA polymer had an important effect on the stability of PVA binding to polyester: while both the short and the long PVA were equally capable of repelling the cells, indicating equally stable binding, the shorter PVA was less resistant

Table 6.1. The adhesion of *E. coli* 3300 to untreated and PVA-coated polyester cloth that was subjected to SDS and Brij 58 detergent treatment and autoclaving in the presence and the absence of SDS and Brij 58 detergents.

Treatment	Adhesion of <i>E. coli</i> 3300 to (CFU x 10 ⁻⁷) ^(a)		
	Control (no PVA)	PVA (124-186000 MW)	PVA (13-23000 MW)
No treatment	5.91±0.17	0.01±0.01	0.02±0.01
Autoclaved	5.69±0.85	0.02±0.01	2.24±0.03
Brij 58	3.87±0.51	0.39±0.05	1.17±0.17
SDS	5.93±0.53	0.56±0.07	1.36±0.25
Autoclaved/Brij 58	6.09±0.69	3.69±0.36	-
Autoclaved/SDS	6.42±0.31	5.17±0.18	-

^(a) The CFU x 10⁻⁷ values were obtained from the A₄₂₀ values upon conversion with the β-galactosidase standard, as described in the Methods.

to autoclaving and the detergent treatments. The long PVA maintained its binding on the cloth after being exposed to autoclaving, however, the binding was not resistant to detergents, and was almost completely removed after autoclaving in the presence of detergents. Therefore, the 124000-186000 MW PVA coating of the polyester cloth maintains stability at high temperatures, but is not resistant to the detergent treatment, even at room temperatures, which strongly indicates the hydrophobic nature of the PVA binding to polyester.

Glutaraldehyde modification of the PVA-coated polyester cloth

The 124000-186000 MW PVA coating of the polyester cloth was found to be stable at high temperature, but susceptible to removal by detergents. Since PVA is readily cross linked with low molecular weight dialdehydes such as glutaraldehyde, glutaraldehyde treatment of the PVA coating adsorbed on the surface of the polyester fibre might stabilize the PVA coating and make it more resistant to the removal by detergents. The reaction of glutaraldehyde with PVA is based on the hemiacetal mechanism (Carey and Sundberg, 1977): the hydroxyl group of alcohol attacks the carbonyl carbon of glutaraldehyde, forming a tetrahedral intermediate, and the presence of an acid catalyst is necessary to remove the water from the intermediate, which reacts with the second molecule of alcohol, thus completing the cross linking reaction (Figure 6.1). The schematic representation of the glutaraldehyde bridge cross linking two PVA polymer molecules is presented in Figure 6.2. However, since the cross linking reaction takes place via the free hydroxyl groups of the PVA, which were demonstrated to be responsible for the inhibition of the bacterial adhesion

Figure 6.1. Reaction mechanism of the PVA cross linking reaction by glutaraldehyde.

Figure 6.2. Glutaraldehyde bridge cross linking two PVA polymer molecules.

through the increase in the surface hydrophilicity, the degree of cross linking would have to be found that maintains enough free hydroxyl groups. To find an appropriate concentration of the glutaraldehyde for the cross linking reaction with the PVA-coated cloth, 0.1%, 1%, and 10% (v/v) solutions were first tested on the unmodified polyester cloth, to account for any effect of the glutaraldehyde on the polyester cloth. Table 6.2 shows that the 10 min treatment of the polyester cloth with the solutions did not have any effect on the capacity of the polyester cloth to bind *E. coli* 3300. The PVA-coated cloth was then exposed to the 10 min treatment with the glutaraldehyde solutions. Both 0.1% and 1% glutaraldehyde concentrations had no effect on the ability of the PVA-coated polyester cloth to decrease bacterial adhesion, however the 10% concentration resulted in some increase in adhesion of *E. coli* 3300, indicating that a significant fraction of the hydroxyl groups of PVA had reacted with the glutaraldehyde. To examine the effect of the glutaraldehyde treatment on the stability of the PVA coating at high temperature in the presence of detergent, the PVA-coated polyester cloth, treated with the three concentrations of glutaraldehyde, was exposed to autoclaving in 1% SDS solution for 10 min. Table 6.2 shows that the treatment of the PVA-coated polyester cloth with 0.1% glutaraldehyde concentrations did not stabilize the PVA coating to removal by SDS at high temperature: the adhesion levels of *E. coli* 3300 were significant after the SDS treatment, indicating removal of PVA from the cloth. However, both 1% and 10% glutaraldehyde concentrations did have a stabilizing effect on the coating: the PVA-coated polyester cloth that was treated with either 1% or 10% glutaraldehyde maintained its capacity to inhibit adhesion after the SDS treatment, indicating that the glutaraldehyde cross linking at a concentration of 1% is efficient enough to prevent the

Table 6.2. Stabilization of the PVA coating against removal by detergents at high temperatures by glutaraldehyde cross linking.

Modification and treatment^(b)	Adhesion of <i>E. coli</i> 3300 (CFU x 10⁻⁷)^(a)
control (no modification or treatment)	5.60±0.20
10 % GH	5.75±0.13
1% GH	5.98±0.34
0.1% GH	5.75±0.12
PVA, 10% GH	0.46±0.09
PVA, 1% GH	0.02±0.01
PVA, 0.1% GH	0.02±0.01
PVA, 10% GH, SA	0.31±0.08
PVA, 1% GH, SA	0.04±0.02
PVA, 0.1% GH, SA	3.98±0.11

^(a) The CFU x 10⁻⁷ values were obtained from A₄₂₀ upon conversion with the A₄₂₀ standard, as described in Chapter 1.

^(b) GH - glutaraldehyde modification, PVA - 124000-186000 MW PVA coating, SA - autoclaving in 1% SDS solution.

removal of the cross linked PVA network from the surface of polyester by SDS during autoclaving.

PVA coating of the polystyrene petri dish

If PVA is also capable of binding to other hydrophobic surfaces, its use could be extended to a variety of other materials where bacterial adhesion is undesirable. To examine the ability of PVA to bind to another hydrophobic surface, hydrophobic polystyrene surface of a culture plate was treated with the 1% PVA solution for the period of 10 min. After washing the treated plate abundantly with distilled water to remove the traces of the PVA solution and drying to remove residual moisture, the wettability of the PVA-treated plate was compared with an untreated control by spotting 0.1 mL droplets on the surface and spreading the droplets over the plate surface with a hockey stick. While the droplets on the control plate were unspreadable, staying intact or breaking into smaller droplets, the droplets were easily spreadable on the surface of the PVA-treated plates, strongly indicating that the binding of PVA to the hydrophobic surface of polystyrene has occurred.

Discussion

PVA coating of polyester and other hydrophobic cloths was found to significantly reduce bacterial adhesion (Chapter 1), suggesting that it could be employed as a component of medical dressings. Since the coating was demonstrated to be stable after the autoclaving treatment, the PVA-coated polyester cloth can be sterilized and reused. The retainment of the PVA coating after the autoclaving treatment indicates a stable binding of PVA to polyester,

and its removal from the cloth by SDS and Brij 58 is a strong indication that the binding of the PVA polymer to hydrophobic polyester is based on hydrophobic interactions. More efficient binding was demonstrated for the high 124000-186000 MW PVA, than for the 13000-23000 MW, indicating that strength of binding increases with the length of the PVA polymer.

The glutaraldehyde cross linking reaction provided further stability to the coating by making it resistant to the removal by detergents. Cross linking with 1% (v/v) glutaraldehyde was found to be efficient enough to render the PVA-coating resistant to removal after autoclaving in 1% SDS. The observation that the treatment of the PVA-coated polyester cloth with the 10% glutaraldehyde concentration increased levels of adhesion of *E. coli* 3300, compared with the 1% glutaraldehyde treatment, is a strong indication that the loss of hydroxyl substituents of the PVA adsorbed on the cloth was responsible for the increased levels of adhesion, and therefore the hydroxyl substituents are responsible for the inhibition of adhesion. The availability of the hydroxyl groups of the PVA bound to polyester cloth to glutaraldehyde supports the proposed mechanism of the PVA binding to polyester via the hydrocarbon backbone, which results in the exposure of the hydroxyl substituents.

The demonstrated binding of PVA to hydrophobic polystyrene surface of a culture plate, which resulted in the increase in the wettability of the culture plate, is a strong indication that the binding of PVA most likely takes place to a variety of hydrophobic surfaces. The PVA coating, therefore, could be applied to a variety of surfaces where bacterial adhesion is of potential concern: medical implant and prosthetic devices (Klotz, 1990), water supply pipe systems (Characklis, 1990) and the food industry (Marin *et al.*,

1997). The demonstrated resistance of the PVA coating to the removal by detergents at high temperature upon glutaraldehyde cross linking, makes this surface modification a potential surface modification for the use on a variety of surfaces.

General Conclusions

I have demonstrated that the hydrophobic cloths, such as polyester, polypropylene, Nylon, and Nomex, have much higher capacity to adsorb a model bacterium *Escherichia coli* 3300, than the hydrophilic cotton cloth. Using polyester as a model hydrophobic cloth, I have shown that the adhesion of *E. coli* 3300 is based on hydrophobic interactions, and that the capacity of the more hydrophobic polyester to adsorb both Gram-negative and Gram-positive bacteria is much higher than that of the hydrophilic cotton cloth. Since the less hydrophobic PVA-coated polyester cloth showed significantly lower capacity to adsorb bacteria, as compared with the hydrophobic unmodified polyester cloth, the process of bacterial adhesion to cloths must be based on hydrophobic interactions.

Employing *E. coli* 3300 as a model bacterium, I have demonstrated that the process of immobilization is initiated with the adhesion of bacteria to the cloth, which is followed by the growth of the reproducing cells inside the cloth. Since the adhesion levels to the hydrophobic polyester were higher than to more hydrophilic cotton and PVA-coated polyester, the rate of cell growth and immobilization was higher for polyester cloth, resulting in the completion of the immobilization process in 5 days for polyester, as compared with 11 days for cotton and 17 days for PVA-coated polyester.

The hydrophilic woven cotton cloth has been already demonstrated as a suitable support for immobilization of bacteria, and was proposed for use as a 'resident' inoculum for batch fermentations. Since conventional commercial fermentation processes are commonly carried out by batch fermentation, normally requiring the preparation of seed cultures, which has to be repeated for another batch fermentation, the use of such 'resident' inocula, capable of efficiently releasing progeny, allows for the retainment of the

immobilized cells in the fermentation tank at the end of each fermentation, thus permitting their repeated use as the inoculum. The present research demonstrated that hydrophobic cloths, in comparison with cotton, offer a higher capacity to adsorb bacteria, and, as demonstrated for polyester cloth, shorten the time required for the preparation of the 'resident' inoculum consisting of the bacteria immobilized on a cloth. An additional advantage of hydrophobic cloths in the form of nonwoven fabrics, in contrast to woven cotton cloth, is their resistance to the loss of fibre from the edges of the cloth, thus offering higher longevity of the immobilized system. The use of noncellulosic cloths also permits their applicability to immobilization of cellulose-degrading bacteria.

PVA coating of hydrophobic cloths resulted in a significant decrease in bacterial adhesion of *E. coli* 3300, and, as demonstrated for polyester cloth, was efficient at significantly decreasing adhesion levels for all bacteria examined. Although the PVA binding to polyester cloth was stable and resistant to removal after autoclaving, it was susceptible to detergents, indicating the hydrophobic nature of PVA binding to polyester. However, treatment of the PVA-coated polyester cloth with glutaraldehyde, which is capable of cross linking PVA, resulted in a significant increase in the stability of the PVA coating, making it resistant to the removal by autoclaving in the presence of the detergent. In addition, I have demonstrated that PVA is also capable of binding to the hydrophobic surfaces of polystyrene petri culture plates, increasing their hydrophilicity.

The ability of PVA to bind to hydrophobic surfaces and to increase their hydrophilic character could be employed in surface modification of materials that are relatively hydrophobic, and may be used in industries where bacterial contamination is of significant

problem, such as water supply systems and food industry. The design of future medical materials, such as dressings, implants, and prosthetic devices, could also benefit from the PVA modification.

Appendix 1

James River Corporation (Nonwoven Division - Cerex® Products)

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610 Old Chemstrand Rd., Cantonment, Fl 32533

Fiberweb North America, Inc.

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Washougal, WA 98671

E.I. Dupont de Nemours & Company (Inc.)

Textile Fibres Department

Engineered Nonwoven Structures Division

Fibres Marketing Center

Wilmington, DE 19898

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