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Development of engineered biofilms on poly-L-lysine patterned surfaces

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Abstract

A technique has been developed to selectively attach bacteria to solid supports using poly-L-lysine. The patterned biofilms were labeled with green fluorescent protein (GFP) or a nucleic acid stain and imaged using both confocal microscopy and GFP stereomicroscopy. *E. coli* DH10B, *E. coli* MC1061, and *Pseudomonas* sp. GJ1 were selectively attached to regions coated with poly-L-lysine but not to uncoated regions. In contrast, *E. coli* DH5α, W3110 and 33456 attached indiscriminately to the coated and uncoated regions of the surface. Those organisms that selectively attached to the poly-L-lysine coated regions formed biofilms twice as thick as the organisms that attached indiscriminately to the surface. This technique can be used for selectively patterning surfaces with genetically engineered microorganisms for biosynthesis of secondary metabolites and biodegradation or for developing a bacterial-based microscale medical diagnostic tool.

Introduction

The ability to selectively attach microorganisms to solid surfaces could be a valuable engineering tool. For example, bacteria selectively attached to micromachined devices could be used to rapidly diagnose diseases or to detect toxic compounds (such as nerve agents and heavy metals) in the environment. The increased cell densities that result from selective attachment would allow for increased signal amplification. It may also be possible to selectively pattern surfaces with microorganisms that could degrade toxic compounds. These 'man-made biofilms' would have high cell densities that would promote rapid degradation of the contaminant with minimal cell mass. Finally, this technique could also be used to promote selective attachment of beneficial bacteria to a surface, which may prevent attachment of pathogenic or otherwise destructive bacteria.

OmpR, as well as several other proteins, promote bacterial attachment to surfaces (Vidal *et al.* 1998). Unfortunately, most laboratory strains of *E. coli*, which are most easily engineered to express a heterologous function needed for detection or biodegradation of molecules, do not have the ability to attach to surfaces because they do not express the *ompR* gene and are thus non-adherent. The inability to attach to surfaces prevents the incorporation of genetically engineered *E. coli* into medical devices and mixed culture biofilms.

It is widely known that poly-L-lysine (PLL) can be used to enhance mammalian cell adhesion to solid surfaces. Poly-L-lysine has been used extensively to culture neural cells on solid supports (Banker & Goslin 1991). A few studies have been done on the use of PLL for enhanced microbial adhesion. PLL has been shown to increase microbial adhesion to polystyrene (McEachran & Irvin 1986, Verschoor *et al.* 1990) and to increase microbial cell hydrophobicity (Goldberg *et al.* 1990).

Methods for selectively attaching strains, such as the popular cloning strain *E. coli* DH10B, to solid surfaces have not yet been developed and could prove to be valuable for biosynthesis of secondary metabolites and biodegradation. In this study, we have shown that PLL can be used to pattern surfaces with bacte-

ria and create engineered microbial biofilms. We used either green fluorescent protein or a nucleic acid stain (SYTO 59) to image the resulting biofilms with both a confocal microscope and a GFP stereomicroscope.

Materials and methods

Strains, plasmids, and media

The strains and plasmids used are listed in Table 1. Plasmids were introduced by electroporation with a Bio-Rad *E. coli* Pulser (Hercules, CA; Cho *et al.* 1995). All plasmids were prepared using a QIAprep Spin Isolation Kit (Qiagen Inc.-USA, Valencia, CA). All strains were grown in MOPS minimal medium (Wanner *et al.* 1977) with 0.4% glycerol and either 100 μg ampicillin ml^{-1} or 50 μg kanamycin ml^{-1}. Cultures grown on MOPS were supplemented with a mixture of 19 amino acids (except for cysteine) at concentrations suggested by Neidhardt (1987). *E. coli* and *Pseudomonas* strains were cultured at 37 °C and 30 °C, respectively. All strains had doubling times between 50 and 70 min. Cultures containing pCSAK50 or pARTH2 were induced with 0.02% arabinose. The *gfpmut3* gene from pGFPmut3 (Cormack *et al.* 1996) was inserted into pBAD18-Kan using *Sma*I and *Sph*I to produce pARTH2.

Bench-scale flow cell

Biofilms were prepared in bench-scale parallel plate flow cells (reactor volume of 0.35 ml; Cowan *et al.* 2000). A cover glass was glued to the plastic frame using General Electric Silicone Rubber Adhesive Sealant RTV 102 (GE Silicones, Waterford, NY). Reactors were operated in recirculating batch mode at a constant flow rate (0.862 ml min^{-1}) using a peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL).

*Preparation of cover glass patterned with poly-*L*-lysine (PLL)*

Stripes of Gel-Pak WF-45-X4 film (5 mm by 24 mm, Gel-Pak, Sunnyvale, CA) were placed 5 mm apart on the surface of Fisher brand Microscope Cover Glass (Type 24 × 60-1; Fisher Scientific, Pittsburgh, PA); see Figure 1. Approximately 1 ml 0.025% PLL (Sigma) was placed on the surface of each cover glass which was then dried overnight at room temperature. The Gel-Pak stripes were removed from the cover glass leaving PLL-free regions and the cover glass was subsequently glued to the biofilm reactor with the PLL surface facing the interior of the reaction chamber.

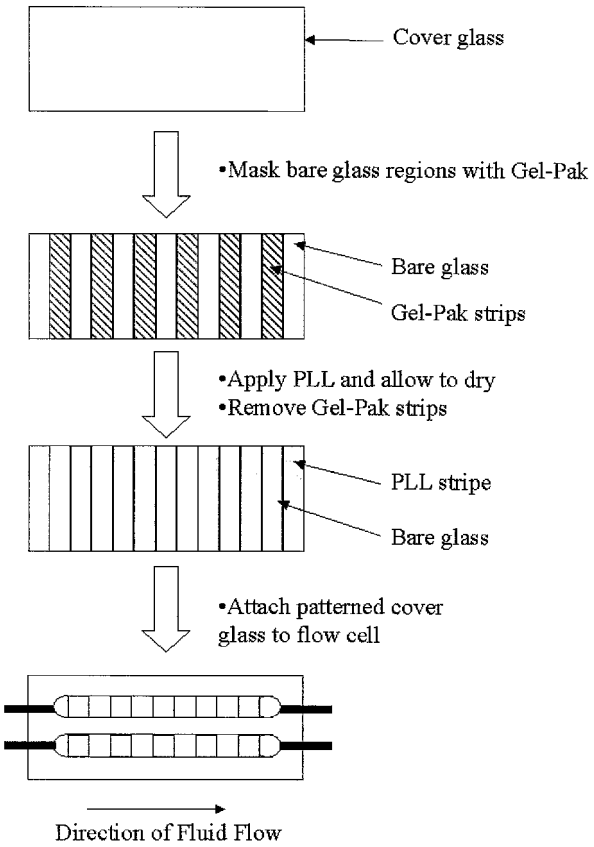

Fig. 1. Cover glass patterned with poly-L-lysine. Stripes of Gel-Pak WF-45-X4 film were placed 5 mm apart on the surface of Fisher brand Microscope Cover Glass (second slide from top). Approximately 1 ml 0.025% PLL (Sigma) was placed on the surface of each cover glass. The PLL was dried onto the cover glass overnight at room temperature. The Gel-Pak stripes were removed from the cover glass leaving PLL-free regions (third slide from top) and the cover glass was subsequently glued to the biofilm reactor with the PLL surface facing the interior of the reaction chamber (bottom slide). The arrow indicates the direction of flow in the apparatus.

Staining E. coli *W3110 with SYTO 59*

After rinsing with MOPS, flow cells containing *E. coli* W3110 or *E. coli* MC1061 were stained with 20 μM SYTO 59 (Molecular Probes Inc., Eugene, OR), a soluble nucleic acid dye that emits in the red region. The stained flow cells were imaged immediately using confocal microscopy.

Table 1. Strains and plasmids.

Strain or plasmid	Description[a]	Reference or source
Strains		
E. coli DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ*(ara, leu)7697 gal*U *gal*K1 *rps*L *nup*G	Life Technologies, Inc. (Gaithersburg, MD)
E. coli W3110	F$^-$ *mcr*A *mcr*B IN (*rrn*D, rrnE)1 λ-	
E. coli DH5α	F-ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Life Technologies, Inc. (Gaithersburg, MD)
E. coli MC1061	F$^-$ *ara*D139 Δ*(ara-leu)7697* Δ(*cod*B-*lac*I)3 *gal*K16 λ$^-$ *mcr*A$^-$ *rel*A1 *rps*L150(str^r) *spo*T1 *mcr*B$^-$ *hsd*R2	Casadaban & Cohen 1980
E. coli 33456		Shen & Wang 1995
Pseudomonas sp. GJ1		Janssen *et al.* 1984
Plasmids		
pARTH2	pBAD18-Km, P_{BAD}, *gfpmut3*, Km^r	This work
pCSAK50	pBAD18, P_{BAD}, *gfpuv*, Ap^r	Khlebnikov *et al.* 2000
pSMC21[b]	pGFPmut2 containing 1.8-kb stabilizing fragment from pUCP181.8, *gfpmut2*, Ap^r, Cb^r, km^r	Bloemberg *et al.* 1997

[a]Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Str^r, streptomycin resistance.
[b]pSMC21 is identical to pSMC2 but it has an additional Km^r gene.

Confocal microscopy

Confocal microscopy was performed using a Bio-Rad MRC-1024 laser scanning confocal imaging system (Bio-Rad Microsciences, Cambridge, MA) equipped with a Nikon Diaphot 200 inverted microscope (Nikon, Inc., Tokyo, Japan). All images were obtained with a 10× lens (numerical aperture = 0.5). The 488 line from a Kr/Ar laser was used to excite both GFP and SYTO 59. Emissions from all green fluorescent proteins were detected with a standard fluorescein filter set (522/35 band pass filter). Emissions from SYTO 59 were collected using a 605/32 band pass filter. The resulting images were processed using Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA) software.

GFP stereomicroscopy

Images were also obtained using a Leica MZ Apo GFP Stereomicroscope connected to a charge-coupled device (CCD) camera. All images were obtained with a 12.5× lens. Image processing was done using Adobe Photoshop 5.0 software.

Patterning surfaces with E. coli *DH10B*

E. coli DH10B harboring pARTH2 was grown to mid-exponential phase in a shake flask and was then recirculated in a flow cell for 8 h at 37 °C. The stock cultures that were perfused through the flow cell were replaced with new mid-exponential phase cultures every 2 h to ensure that the cells remained in the exponential growth phase. MOPS (15 ml) was then pumped through each flow cell to remove cells that had not adhered to the surface of the cover glass. The attached cells were imaged immediately. Control experiments were also performed; all experimental conditions were identical except flow cells with bare cover glass were used.

Patterning surfaces with other bacteria

Exponentially growing cultures of *E. coli* DH5α with pARTH2, *E. coli* 33456 with pCSAK50, *E. coli*

Table 2. Summary of stripe formation by various *E. coli* strains and *Pseudomonas*.

Strains that form stripes	Strains that attach indiscriminately
E. coli DH10B	*E. coli* W3110
E. coli MC1061	*E. coli* 33456
Pseudomonas sp. GJ1	*E. coli* DH5α

W3110, *E. coli* MC1061, and *Pseudomonas* sp. GJ1 with pSMC21 were each recirculated in flow cells as described above. The samples were then rinsed and imaged as described above. Surfaces colonized by *E. coli* W3110 and *E. coli* MC1061 were stained with SYTO 59 prior to imaging.

Determination of biofilm thickness

The thickness of the biofilm was measured by using the stepper motor attached to the confocal microscope to determine the location of the substratum and the top layer of the biofilm. Thickness measurements were made in both the PLL-coated and PLL-free regions. Measurements were made on *E. coli* DH10B and *E. coli* W3110 samples. Four measurements were taken at representative locations in each sample and were then averaged.

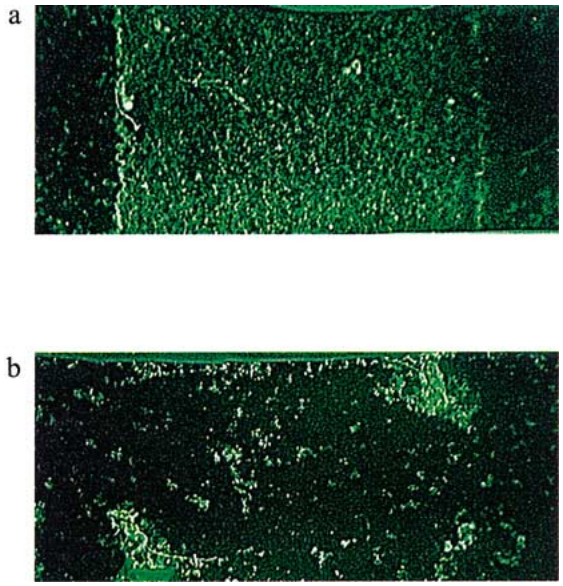

Fig. 2. Green fluorescent protein (GFP)-stereomicroscope images at 12.5× magnification. *E. coli* DH10B harboring pARTH2 was grown to mid-exponential phase in a shake flask and was then recirculated in a flow cell for 8 h at 37 °C. The stock cultures that were perfused through the flow cell were replaced with new mid-exponential phase cultures every 2 h to ensure that the cells remained in the exponential growth phase. MOPS (15 ml) was then pumped through each flow cell to remove cells that had not adhered to the surface of the cover glass. The attached cells were imaged immediately. (a) Stripe of DH10B with pARTH2 (green) on a poly-L-lysine patterned cover glass. (b) Random attachment of DH10B with pARTH2 (green) to a bare cover glass.

Results

Patterning surfaces with E. coli

E. coli DH10B with pARTH2 selectively attached to the PLL-coated regions of the cover glass, while there was only minimal attachment to the uncoated glass (Figure 2). Confocal microscopy was used to obtain a close up view of the edge of a stripe formed by strain DH10B (Figure 3a). Strain DH10B formed distinct stripes on the patterned cover glass with only a few clumps of cells randomly attached to the bare cover glass. *E. coli* MC1061, a distant ancestor of *E. coli* DH10B, was also found to form stripes on PLL patterned surfaces.

In contrast, *E. coli* 33456, *E. coli* DH5α, and *E. coli* W3110 have equal affinity for the PLL coated regions and the bare glass regions. These strains did not form stripes (Table 2), instead they colonized the entire cover glass, including patterned and PLL-free regions (Figure 3c).

As a control, a PLL patterned cover glass that had not been colonized with bacteria was viewed with the confocal microscope and no output signal was obtained. This indicated that all output signal was due to expression of *gfp*, rather than background signal from the PLL coating.

Patterning surfaces with other bacteria

Besides *E. coli* DH10B, *Pseudomonas* sp. GJ1 with pSMC21 selectively attached to the PLL-coated regions to form stripes (Figure 3b). GJ1 formed stripes within 2 h from when the culture was introduced into the flow cell. Unlike *E. coli* DH10B, GJ1 was found to attach extensively to bare glass surfaces after the culture had recirculated for 8 h.

Biofilm thickness

The *E. coli* DH10B formed biofilms on PLL-coated regions were 125 μm thick (Table 3). In the PLL-free regions, microcolonies of 40 μm thick covered only

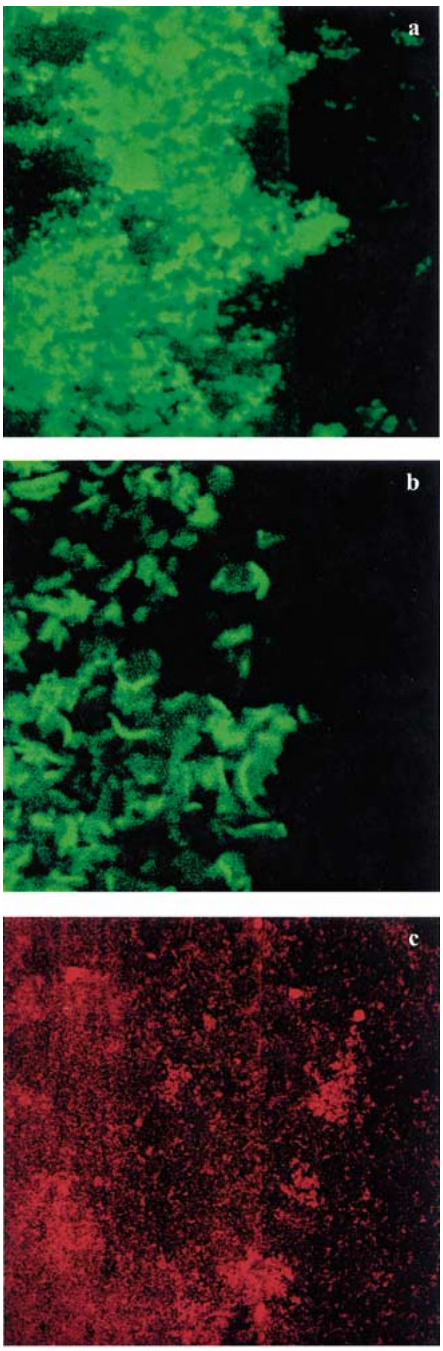

Fig. 3. Close up (100× magnification) view of the edge of a poly-L-lysine region colonized by (a) DH10B with pARTH2 (green), (b) GJ1 with pSMC21 (green), and (c) W3110 stained with SYTO 59 (red). Exponentially-growing cultures were recirculated in a flow cell for 8 h at 37 °C. The stock cultures that were perfused through the flow cell were replaced with new mid-exponential phase cultures every 2 h to ensure that the cells remained in the exponential growth phase. MOPS (15 ml) was then pumped through each flow cell to remove cells that had not adhered to the surface of the cover glass. Surfaces colonized by *E. coli* W3110 and *E. coli* MC1061 were stained with SYTO 59 prior to imaging.

8% of the surface. In contrast, *E. coli* W3110 formed biofilms of uniform thickness (40 μm) on both the PLL-coated and PLL-free regions.

Discussion

The technique discussed in this paper is novel in that we have developed a method for generating microbial biofilms on select regions of a surface. In previous work, surfaces have been patterned with proteins and mammalian cells (Bhatia *et al.* 1993, Craighead *et al.* 1998), but not with bacteria. In addition to being novel, this technique could potentially be used in numerous environmental and diagnostic applications.

We have found that PLL can be used to selectively attach *E. coli* DH10B or *E. coli* MC1061 to glass surfaces. DH10B formed distinct stripes on the patterned cover glass by attaching to the PLL regions. Since DH10B is commonly used for genetic manipulations and does not effectively attach to bare glass surfaces on its own, this technique could be used for incorporating genetically engineered microorganisms (GEMs) onto the surfaces of medical devices and into multispecies biofilms. The high cell density found in biofilms and the strong bacterial attachment could lead to enhanced performance of the GEMs and a prolonged lifetime in a natural environment.

This technique would be particularly valuable for use with a mutualistic consortium that contains a genetically engineered laboratory strain. In mutualism, the species are dependent on each other; each species requires that another member provide a cofactor or other nutrient to survive. The genetically engineered strain could be attached to the surface with PLL in a pattern that would yield optimal exchange of nutrients between the consortium members, which could lead to enhanced biodegradation or biosynthesis.

An ideal GEM for attachment to the PLL regions might be an *E. coli* expressing membrane-associated organophosphorus hydrolase (Richins *et al.* 1997). These cells are capable of degrading hydrolyzing pesticides, such as parathion and paraoxon, and nerve agents. An effective biofilm reactor for pesticide or nerve agent degradation could be created by coating the reactor surface with PLL. The uniform attachment of bacteria in a PLL-coated reactor would allow more uniform and predictable degradation in all areas of the reactor.

The ability to selectively attach microorganisms to surfaces could be useful as a medical diagnostic. PLL

Table 3. Biofilm thickness in poly-L-lysine (PLL)-coated and PLL-free regions.

Strain	Biofilm thickness in PLL-coated region (μm)	Biofilm thickness in PLL-free regions (μm)
E. coli DH10B	125 ± 11	0[a]
E. coli W3110	45 ± 10	45 ± 4

[a]The PLL-free regions were relatively free of cells. Approximately 8% of the surface area of the PLL-free regions had been colonized (this was determined using NIH Image 1.62). Colonies were 40 ± 10 μm thick.

patterned surfaces could be used to rapidly screen for diseases or test antimicrobial agents. Furthermore, it has been demonstrated that surfaces can be patterned with PLL features as small as 10 microns using microcontact printing (James *et al.* 1998). This would allow for a MEMs (micro-electromechanical system) based diagnostic tool. This MEMs diagnostic tool could be coupled with microscale pumps and valves (Krusemark *et al.* 1998) to create an on-line diagnostic system.

The data in Table 3 show that PLL significantly enhances adhesion of non-adherent strains, such as DH10B and MC1061. On the other hand, *E. coli* W3110 has equal affinity for PLL and PLL-free regions. Patterning surfaces with PLL could be used to rapidly create engineered biofilms. For example, biofilms that were more than 100 μm thick were formed in only 8 h. This technique might be used to decrease the start up time of biofilm reactors.

It is an interesting finding that most of the other strains of *E. coli* (33456, DH5α, and W3110) did not exhibit selective attachment to PLL. DH10B and MC1061, two related strains commonly used in genetic manipulations, were both unable to colonize bare glass surfaces. Similarly, both of these strains readily colonized PLL-coated surfaces. Thus, exposure of either of these strains to a PLL-patterned cover glass resulted in a surface patterned with DH10B or MC1061. This suggests that one of the mutations present in both DH10B and MC1061 is responsible for the inability of these strains to colonize a bare glass surface. A mutation present in both DH10B and MC1061 may have resulted in changes to the outer membrane. It is possible that these strains acquired a deletion or mutation in the *ompR* gene resulting in non-adherent organisms. However, we have not evidence that this mutation is responsible for the change in bacterial adherence.

Finally, GFP stereomicroscopy is a valuable tool for imaging GFP-containing cells attached to patterned surfaces. Previously GFP stereomicroscopy has only been used to detect gene expression in transgenic medaka (Hamada *et al.* 1998) and as a reporter gene for detecting the initial stages of *Agrobacterium*-mediated transformation of apple cultivars (Maximova *et al.* 1998). This study shows that GFP stereomicroscopy could be useful for exploring the landscape of large surface areas covered with biofilms. For example, GFP stereomicroscopy could be used to determine the fraction of the surface that has been colonized by microorganisms.

Conclusions

In this study, we have shown that engineered biofilms can be created on PLL patterned surfaces. This technique could be a valuable tool for creating engineered biofilms that contain GEMs or for attaching cells to a microscale medical diagnostic tool. In addition, we have found that GFP stereomicroscopy is a useful technique for imaging large regions of both natural and engineered biofilms.

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