Anal. Chem. **2009,** *81,* 7571–7578

Antibody Selection for Immobilizing Living Bacteria

Zhiyong Suo,[†] Xinghong Yang,[‡] Recep Avci,*[,†] Muhammedin Deliorman,[†] Paul Rugheimer,[†,§] David W. Pascual,[‡] and Yves Idzerda[†,§]

Department of Physics, Veterinary Molecular Biology, and Center for Bioinspired Nanomaterials, Montana State University, Bozeman, Montana 59717

We report a comparative study of the efficacy of immobilizing living bacteria by means of seven antibodies against bacterial surface antigens associated with *Salmonella enterica* Serovar Typhimurium. The targeted bacterial antigens were CFA/I fimbriae, flagella, lipopolysaccharides (LPS), and capsular F1 antigen. The best immobilization of S. Typhimurium was achieved with the antibody against CFA/I fimbriae. The immobilization of bacteria using antiflagellin showed significant enhancement if the flagella rotary motion was paralyzed. Of the four antibodies targeting LPS structures, only one, the antibody against the O-antigen polysaccharides, showed a relatively efficient bacterial immobilization. No bacterial immobilization was achieved using the antibody against F1 antigen, presumably because F1 protein can detach from the bacterial surface easily. The results suggest that an antibody for bacterial immunoimmobilization should target a surface antigen which extends out from the bacterial surface and is tightly attached to the bacterial cell wall. The microarrays of living S. Typhimurium cells immobilized in this manner remained viable and effective for at least 2 weeks in growth medium before a thick biofilm covered the whole surface.

Microarrays using bacterial cells as sensors have found many uses in such areas as high-throughput assays of gene expression[1,2] detection of toxins,[3−5] and determination of the bioavailability of chemicals in soil.[6] Bacterial sensor microarrays benefit from the low cost, fast growth, and easy handling of bacteria.[7] The advances in genetic engineering of the past decades have enabled the tailoring of bacterial functionalities for microarray applications. Currently, one of the major challenges in the development of bacterial cell microarrays is the detachment of bacterial cells from the substrate causing sensor failure. Reliable, facile, and efficient immobilization methods are critical to the success of bacterial cell microarrays. Such immobilization methods will also have wide applications in biomedical fields and the food industry as a means to detect and isolate pathogenic bacteria. For example, contamination by *Salmonella* spp. is a major concern in the food industry, and the detection protocol using differential growth media usually takes days to weeks.[8,9] This process, however, can be shortened and reduced to hours by using sensors based on microarrays with a high degree of specificity. Recently we reported a method for efficient immobilization and patterning of living bacteria facilitated by the interaction between bacterial fimbriae and the corresponding antibody[10] for possible applications in biosensors, bacterial sorting, and microarrays. The core requirement in all of these applications is the efficient immobilization of the microorganisms into some sort of pattern which can be easily monitored by an optical microscope. The focus of this paper is to evaluate the efficacy of a number of antibody−antigen pairs in the immobilization of living bacteria on flat surfaces for microarray applications.

A majority of the documented methods for bacterial immobilization utilize either the physical entrapment of bacteria or the nonspecific binding of bacterial cells (by means of electrostatic forces) on chemically modified surfaces.[4,6,11] The entrapment methods suffer from slow response times, low loading rates into the microwells, and easy detachment from the surfaces. Substrates modified with chemicals, such as polylysine,[12,13] polyethyleneimmine,[14] gelatin,[15] and alginate,[16] offer limited success for reliable and reproducible immobilization under physiological conditions.

* To whom correspondence should be addressed. Recep Avci, Department of Physics, Montana State University, Bozeman, MT 59717. Phone: 406-994-6461. Fax: 406-994-6040. E-mail: avci@physics.montana.edu.

[†] Department of Physics.

[‡] Veterinary Molecular Biology.

[§] Center for Bioinspired Nanomaterials.

(1) Van Dyk, T. K.; DeRose, E. J.; Gonye, G. E. *J. Bacteriol.* **2001**, *183*, 5496–5505.

(2) Kuang, Y.; Biran, I.; Walt, D. R. *Anal. Chem.* **2004**, *76*, 6282–6286.

(3) Brogan, K. L.; Walt, D. R. *Curr. Opin. Chem. Biol.* **2005**, *9*, 494–500.

(4) Kuang, Y.; Biran, I.; Walt, D. R. *Anal. Chem.* **2004**, *76*, 2902–2909.

(5) Lee, J. H.; Mitchell, R. J.; Kim, B. C.; Cullen, D. C.; Gu, M. B. *Biosens. Bioelectron.* **2005**, *21*, 500–507.

(6) Mbeunkui, F.; Richaud, C.; Etienne, A. L.; Schmid, R. D.; Bachmann, T. T. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 306–312.

(7) Belkin, S. *Curr. Opin. Microbiol.* **2003**, *6*, 206–212.

(8) Amaguafia, R. M.; Andrews, W. H. In *Encyclopedia of Food Microbiology*; Robinson, R. K., Batt, C. A., Patel, P. D., Eds.; Academic Press: San Diego, CA, 2000; Vol. 3, pp 1948−1951.

(9) Eaton, A. D.; Clesceri, L. S.; Rice, E. W.; Greenberg, A. E. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, 2005.

(10) Suo, Z. Y.; Avci, R.; Yang, X. H.; Pascual, D. W. *Langmuir* **2008**, *24*, 4161–4167.

(11) Premkumar, J. R.; Rosen, R.; Belkin, S.; Lev, O. *Anal. Chim. Acta* **2002**, *462*, 11–23.

(12) Rozhok, S.; Fan, Z. F.; Nyamjav, D.; Liu, C.; Mirkin, C. A.; Holz, R. C. *Langmuir* **2006**, *22*, 11251–11254.

(13) Rozhok, S.; Shen, C. K. F.; Littler, P. L. H.; Fan, Z. F.; Liu, C.; Mirkin, C. A.; Holz, R. C. *Small* **2005**, *1*, 445–451.

(14) Razatos, A.; Ong, Y. L.; Sharma, M. M.; Georgiou, G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11059–11064.

(15) Doktycz, M. J.; Sullivan, C. J.; Hoyt, P. R.; Pelletier, D. A.; Wu, S.; Allison, D. P. *Ultramicroscopy* **2003**, *97*, 209–216.

(16) Polyak, B.; Bassis, E.; Novodvorets, A.; Belkin, S.; Marks, R. S. *Sens. Actuators, B* **2001**, *74*, 18–26.

10.1021/ac9014484 CCC: $40.75
Published on Web 08/14/2009

This is mostly because bacterial cells have very small contact areas with the substrate surface relative to eukaryotic cells, preventing the bacteria from adhering to the surface effectively. Additionally, many bacterial species have a layer of capsular extracellular polymeric substances (EPS) covering their outer surface, which further weakens nonspecific adhesion.

Here we refer to the immobilization of bacteria based on antibody−antigen (ab−ag) interactions as immunoimmobilization. It takes advantage of the specific interaction between an antibody and the target antigen on the bacterial cell surface. The large variety of bacterial surface antigens and corresponding antibodies offers a variety of choices for immunoimmobilization, which could be highly specific for a given species. This specificity can be used to our advantage in selecting a particular species from a mixed culture of species without the need for preseparation. Antibody-modified gold chips have already been employed as a sensor to detect *S.* Typhimurium and *Pseudomonas aeruginosa* using surface plasmon resonance.[17−19] Similarly, antibody-modified magnetic particles have been used in immunomagnetic separation (IMS).[20] Previous work using antibodies against whole bacterial cells often resulted in low immobilization efficiency.[13,21] A systematic evaluation and comparison of the immobilization efficiencies of selected antibody−antigen pairs associated with common bacterial surface antigens is lacking. Such work will shed light on bacterial immobilization and could have wide applications in such areas as pathogen detection in the food industry.

In this article we evaluate the immobilization efficiencies of seven antibodies, corresponding to four different types of surface antigens: CFA/I fimbriae, flagella, LPS, and capsular F1 antigen. The results show that, with the exception of capsular F1, all the surface antigens listed above can in principle be targeted to achieve some degree of immobilization efficiency.

EXPERIMENTAL SECTION

Bacteria. Four *S.* Typhimurium strains were used as model species in immobilization experiments: one expressing CFA/I fimbriae (Δ*asd*::*kan*^{R} H71-pHC),[22] one with the motion of its flagella paralyzed (*motA*3::*cat* H683-pTP2fliC), one expressing F1 antigen (Δ*asd*::*kan*^{R} H71-pF1)[23] and with strain H647[23,24] as a control. For strain *motA*3::*cat* H683-pTP2fliC, we inserted the *cat* cassette (encoding chloramphenicol acetyltransferase) into the *motA* to arrest the motion of the flagella motor since MotA, together with MotB, constitutes the stator of the motor.[25] We also elevated the flagellin expression for this strain by cloning the flagella major subunit gene *fliC* under the regulation of the chimeric promoter P*tetA*∼P*phoP*;[23] the related plasmid is termed pTP2fliC. An *E. coli* strain (H681-pBBScfa) was also used, to test the immobilization efficiency of anti-CFA/I for bacterial species other than *Salmonella*. All the bacterial species, *S.* Typhimurium and *E. coli*, were inoculated from frozen bacteria stock at −80 °C onto a LB plate and incubated at 37 °C overnight. The bacteria were then inoculated into an LB liquid medium without antibiotics and shaken at 125 rpm at 37 °C. All the bacterial cells were harvested in their exponential phase when the culture optical density at 600 nm (OD600) reached 0.5−0.6, which corresponds to a colony-forming units (CFU) value of $\sim 1 \times 10^8$ per mL.

Materials. PBS buffer salt, 3-aminopropyltriethoxylsilane (APTES), and 16-mercaptohexadecanoic acid (MHA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-[β-Maleimidopropyloxy]-succinimide ester (BMPS) was purchased from Pierce Biotechnology (Rockford, IL).

All the antibodies used in this work are IgG antibodies. The polyclonal anti-CFA/I was prepared by immunizing a rabbit intramuscularly (im) with purified CFA/I fimbriae proteins. Postimmunization (4 weeks), the rabbit was bled to check serum anti-CFA/I titers using enzyme-linked immunosorbent assay (ELISA). The serum was further purified using a protein G column to remove the nonimmunoglobulin serum protein. The polyclonal anti-F1 was prepared similarly.

Monoclonal antibody antiflagellin was purchased from Inotek Pharmaceuticals (Beverly, MA). Monoclonal antibodies against *S.* Typhimurium lipopolysaccharides (LPS), O4-antigen (sc-52223, sc-52224), O-antigen (sc-52221), and core antigen (sc-52219) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Anticytochrome *c* extracted from horse hearts, purchased from Sigma-Aldrich (St. Louis, MO), was used as an irrelevant antibody in control experiments. Antibodies were diluted to the following concentrations with PBS buffer (pH = 7.4) before use: 10 μg/mL for sc-52223, sc-52224, sc-52221, and sc-52219 and 25 μg/mL for anti-CFA/I, antiflagellin, anti-F1, and anticytochrome *c*.

Linking of Antibodies to the Substrate. The antibody molecules were covalently linked to the amino-terminated silicon substrates through a short succinimide cross-linker (BMPS), which reacts with the thiol groups on the antibody surface. Details of substrate modification and antibody linking have been reported previously.[10] Briefly, precleaned silicon chips were soaked in a solution of APTES in methanol (2%) for 15 min, followed by further incubation in a solution of BMPS in anhydrous acetonitrile (10 mM) for 30 min. Antibody solutions were deposited onto such activated substrates as small droplets (each droplet ∼1 μL in volume). These chips with antibody droplets were kept in a humid chamber for ∼1 h at room temperature to allow the covalent linking of the antibody to the substrate. The excess antibody molecules on the chip surface were washed off with copious amount of PBS buffer before incubation with bacteria.

Fabrication of Antibody Microarray with a Microplotter. Antibody arrays were fabricated with a microplotter[26] (Sonoplot Inc., Madison, WI) consisting of three robotic axes which moves a micropipet tip over a large volume (∼1 m^3) with micrometer-

(17) Jenkins, A. T. A.; Ffrench-Constant, R.; Buckling, A.; Clarke, D. J.; Jarvis, K. *Biotechnol. Prog.* **2004**, *20*, 1233–1236.
(18) Oh, B. K.; Kim, Y. K.; Park, K. W.; Lee, W. H.; Choi, J. W. *Biosens. Bioelectron.* **2004**, *19*, 1497–1504.
(19) Mantzila, A. G.; Maipa, V.; Prodromidis, M. I. *Anal. Chem.* **2008**, *80*, 1169–1175.
(20) Cudjoe, K. S. In *Encyclopedia of Food Microbiology*; Robinson, R. K., Batt, C. A., Patel, P. D., Eds.; Academic Press: San Diego, CA, 2000; Vol. 3, pp 1968−1975.
(21) Premkumar, J. R.; Lev, O.; Marks, R. S.; Polyak, B.; Rosen, R.; Belkin, S. *Talanta* **2001**, *55*, 1029–1038.
(22) Suo, Z. Y.; Avci, R.; Deliorman, M.; Yang, X. H.; Pascual, D. W. *Langmuir* **2009**, *25*, 4588–4594.
(23) Yang, X. Y.; Hinnebusch, B. J.; Trunkle, T.; Bosio, C. M.; Suo, Z. Y.; Tighe, M.; Harmsen, A.; Becker, T.; Crist, K.; Walters, N.; Avci, R.; Pascual, D. W. *J. Immunol.* **2007**, *178*, 1059–1067.
(24) Suo, Z. Y.; Yang, X. Y.; Avci, R.; Kellerman, L.; Pascual, D. W.; Fries, M.; Steele, A. *Langmuir* **2007**, *23*, 1365–1374.
(25) Kim, E. A.; Price-Carter, M.; Carlquist, W. C.; Blair, D. F. *Biochemistry* **2008**, *47*, 11332–11339.
(26) Larson, B. J.; Gillmor, S. D.; Lagally, M. G. *Rev. Sci. Instrum.* **2004**, *75*, 832–836.

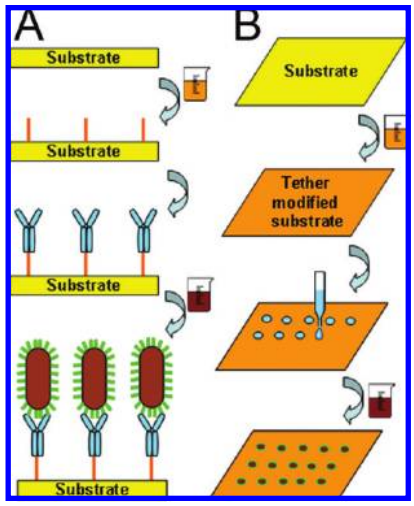


Figure 1. Immobilization of living bacteria and fabrication of the bacterial cell microarray. (A) The substrate was modified with tether molecules (orange bars), followed by the covalent linking of antibody molecules (cyan Y shapes). The antibody-modified substrate was then incubated with a bacterial culture (brown ovals) to form a bacterial monolayer. The bacterial surface antigens are indicated as radial green bars. (B) The pattern of the microarray was achieved by plotting on the tether-modified substrate using a microplotter and antibody solution as "ink." When the substrate is incubated with the bacterial culture, the bacterial cells adhere only to the antibody-modified areas and thus form a bacterial cell microarray. The beakers with orange or brown solution represent incubation with tether solution or bacterial culture, respectively.

length positional precision. The micropipet tip dispenses fluid by means of a patented technique in which a piezo-electric slab is attached to the micropipet and driven at the mechanical resonance of the slab. The resonant vibrations result in fluid dispensing from the tip. This technique allows for the plotting of a wide variety of solutions, colloids, and fluids of nearly any viscosity. In our experiment we used the microplotter in spot mode to produce an array of spots with picoliter volume (Figure 1). The solution of anti-CFA/I in PBS diluted to 100 times was plotted on a substrate in a grid pattern. The substrates with the antibody pattern were kept inside a chamber with 100% humidity for ~1 h before incubation with bacterial culture.

Fabrication of Antibody Microarray by Dip-Pen Nanolithography. Small spots of the anti-CFA/1 were patterned on gold substrates using a previously reported dip-pen nanolithography (DPN) procedure.[27] Briefly, a micropattern of MHA on gold was drawn (by bringing the AFM tip into brief contact (~100 ms) with the gold substrate) using an MHA-modified atomic force microscopy (AFM) tip. Then the unpatterned area of gold substrate was passivated, the MHA pattern was activated, and the substrate was incubated with antibody solution to allow the cross-linking of antibody molecules to the patterned MHA spots. The size of each antibody spot of the micropattern was ~0.8 μm; this could be adjusted to anywhere from ~50 nm to ~1 μm by fine-tuning the tip−substrate contact time.

Immobilization of Living Cells. The method for immobilizing living bacteria and preparing microarrays is shown in Figure 1. The substrate surface was first modified with tether molecules, as described above. These tethers served as the cross-linkers connecting antibodies to the substrate surface covalently. Antibody attachment was achieved by incubating the tether-modified substrate directly in a solution of an antibody against a bacterial surface antigen (Figure 1A), by patterning the antibody on the flat surface using a microplotter with the antibody solution as the "ink" (Figure 1B), or through the DPN technique. After PBS buffer was used to remove the excess antibody, the antibody-modified substrates were incubated with bacterial culture in half LB growth medium (typtone 5 g/L; yeast extract 2.5 g/L; NaCl 5 g/L) at room temperature for ~0.5 h to form a monolayer or a microarray of living bacterial cells. Because bacterial cells only recognize the antibody-modified regions of a substrate surface, these areas become covered with one monolayer of viable bacteria. After incubation, the samples were gently rinsed with a copious amount of PBS buffer to remove the planktonic or loosely attached cells. The rinsed samples were kept in PBS buffer at 4 °C for further analysis. Substrates without antibody linkage were also used in parallel experiments as controls. At least three samples were investigated to determine the immobilization efficacy of each antibody.

Imaging Bacteria Using AFM. Sample preparation for AFM imaging follows the procedure reported previously.[24] Tapping mode imaging of bacterial cells was carried out in air using a Multimode V system from Veeco (Santa Barbara, CA). This system was also used in the DPN experiments.

Field Emission Scanning Electron Microscopy. Immobilized flagella (Figure 4) and the micropatterns of bacteria (Figure 5B) on silicon wafers were imaged using a Zeiss SUPRA 55 VP field emission scanning electron microscopy (FESEM) system (Carl Zeiss, Germany).

Optical Microscopy. Optical images were obtained using an Olympus BX61 microscope. The images were recorded in reflection bright field mode (Figures 3, 6, and 7) and in fluorescence mode (Figure 5A). Reflection mode images were taken along the edges of antibody-modified circular areas so that we could see the sharp boundary of the immobilized cells (Figure 3A−C,E). For fluorescence mode imaging, the cells were stained using Live/Dead *Bac*Light (Invitrogen, Carlsbad, CA) before imaging. A green color attests that the immobilized cells are alive.

RESULTS AND DISCUSSION

Confirmation of Surface Antigens by AFM. The expression of some antigens can be confirmed by AFM imaging. For example, CFA/I fimbriae can be clearly imaged for *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC and *E. coli* H681-pBBScfa though not for other strains, as seen in Figure 2. Similarly, flagella can easily be seen for all *S.* Typhimurium strains. It is, however, difficult to image F1 antigen or LPS on a bacterial cell surface because of their amorphous topography. The expression of F1 was confirmed by immunogold labeling and immunoblotting.[23,28]

Four *S.* Typhimurium strains, expressing CFA/I fimbriae, flagella, LPS, and F1 antigen, as detailed in Table 1, together with an *E. coli* strain genetically modified to express CFA/I fimbriae, were subjected to immobilization by ab−ag coupling. All the *S.*

(27) Lee, K. B.; Park, S. J.; Mirkin, C. A.; Smith, J. C.; Mrksich, M. *Science* **2002**, *295*, 1702–1705.

(28) Chanteau, S.; Rahalison, L.; Ratsitorahina, M.; Mahafaly; Rasolomaharo, M.; Boisier, P.; O'Brien, T.; Aldrich, J.; Keleher, A.; Morgan, C.; Burans, J. *Int. J. Med. Microbiol.* **2000**, *290*, 279–283.

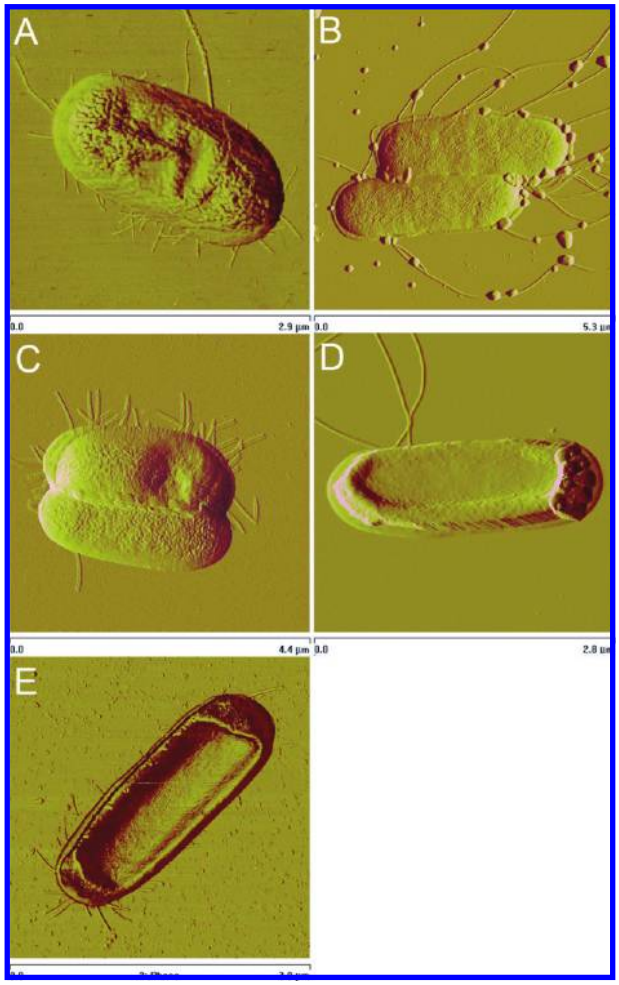


Figure 2. AFM images of (A) *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC, (B) H647, (C) *motA*3::*cat* H683-pTP2fliC, (D) Δ*asd*::*kan*^{R} H71-pF1, and (E) *E. coli* H681-pBBScfa.

Table 1. Surface Antigens of *S.* Typhimurium Strains Used in This Work[a]

	Δ*asd*::*kan*^{R} H71-pHC	Δ*asd*::*kan*^{R} H71-pF1	H647	*motA*3::*cat* H683-pTP2fliC
CFA/I	Y	N	N	N
flagella	Y	Y	Y	Y
flagella motion paralyzed	N	N	N	Y
F1	N	Y	N	N
LPS	Y	Y	Y	Y

[a] Y = yes and N = no.

Typhimurium strains expressed flagella, but the flagella motion of the *motA*3::*cat* H683-pTP2fliC strain was paralyzed. Only one *S.* Typhimurium strain (Δ*asd*::*kan*^{R} H71-pHC) and the *E. coli* strain expressed CFA/I fimbriae. LPS is a native antigen for all *S.* Typhimurium strains, and no attempt was made to modify the LPS expression for the strains studied here. Only one strain (Δ*asd*::*kan*^{R} H71-pF1) expressed the F1 antigen. Strain H647 was used to test the immobilization efficiency of the antibodies raised against flagellin and LPS.

Evaluation of Antibody Efficacy in Bacterial Immobilization. In those experiments in which no micropattern was used (Figure 1A), the antibody solution was deposited by a micropipet on the silicon substrate as small droplets so that the antibody-modified areas were separated from the unmodified areas by a sharp border. Because of the specificity of the antibody−antigen interaction, the bacterial cells, if immobilized, would be found only inside the antibody-modified areas. Therefore, a sharp separation of the bacteria-covered areas from those not covered was expected, provided that the antibodies covalently coupled to the substrate bound to the bacterial surface antigen efficiently. An optical image focused around the edge of the antibody-coated areas would demonstrate the immobilization efficacy of the antibody−antigen interaction. This is shown in Figure 3A,B, where a sharp separation of the bacteria-covered area is observed for both *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC and *E. coli* H681-pBBScfa when anti-CFA/I was used for substrate functionalization. The cell coverage within the antibody-modified area approached a dense monolayer. The cell density of *E. coli* H681-pBBScfa was slightly lower than that of *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC, most likely because of the smaller number of CFA/I fimbriae of *E. coli* relative to *S.* Typhimurium (compare parts A and E of Figure 2). The areas outside the antibody-modified regions (right-hand sides of the panels in Figure 3) serve as a negative control to evaluate the immobilization efficacy. Usually no cells or only sparsely attached cells were observed in these control areas. We conducted additional control experiments on similar substrates using no antibody (Figure 3J) and using an irrelevant antibody (anticytochrome *c*) (Figure 3K). These further confirmed that a specific antibody is essential for the immobilization of the living bacterial cells tested in our experiments. Since the CFA/I fimbriae have a terminal lectin motif, it is possible that the lectin−carbohydrate binding contributes to the immobilization of living bacterial cells.[29] However, the lack of immobilization indicated by Figure 3K suggests that this binding does not play a significant role in bacterial immobilization for the irrelevant antibody, anticytochrome *c*, used in the experiments.

The efficiency of immobilization using antiflagellin and anti-LPS was tested on *S.* Typhimurium H647, which expresses flagella but lacks CFA/I fimbriae. As expected, this strain could not be immobilized on substrate modified with anti-CFA/I antibody. However, *S.* Typhimurium H647 could be immobilized on substrates modified with antiflagellin (Figure 3D), although with a relatively low cell density as compared with CFA/I fimbriae (Figure 3A,B). The coverage density of H647 cells could not be enhanced by increasing the time the substrate was immersed in the bacterial culture. This is in spite of the antiflagellin showing a reasonably strong affinity for the purified *S.* Typhimurium flagella, as seen in Figure 4. We speculate that the low cell coverage density in Figure 3D is a result of the high-speed rotary motion of the flagella, which can be as high as 10 000 rpm at 35 °C,[30,31] hindering the ab−ag interactions. To verify this hypothesis, experiments were conducted in which a flagella-paralyzed *S.* Typhimurium strain, *motA3*::*cat* H683-pTP2fliC, was immobilized

(29) Jansson, L.; Tobias, J.; Lebens, M.; Svennerholm, A. M.; Teneberg, S. *Infect. Immun.* **2006**, *74*, 3488–3497.
(30) Magariyama, Y.; Sugiyama, S.; Kudo, S. *FEMS Microbiol. Lett.* **2001**, *199*, 125–129.
(31) Lu, Z. J.; Murray, K. S.; Vancleave, V.; Lavallie, E. R.; Stahl, M. L.; Mccoy, J. M. *Bio/Technology* **1995**, *13*, 366–372.

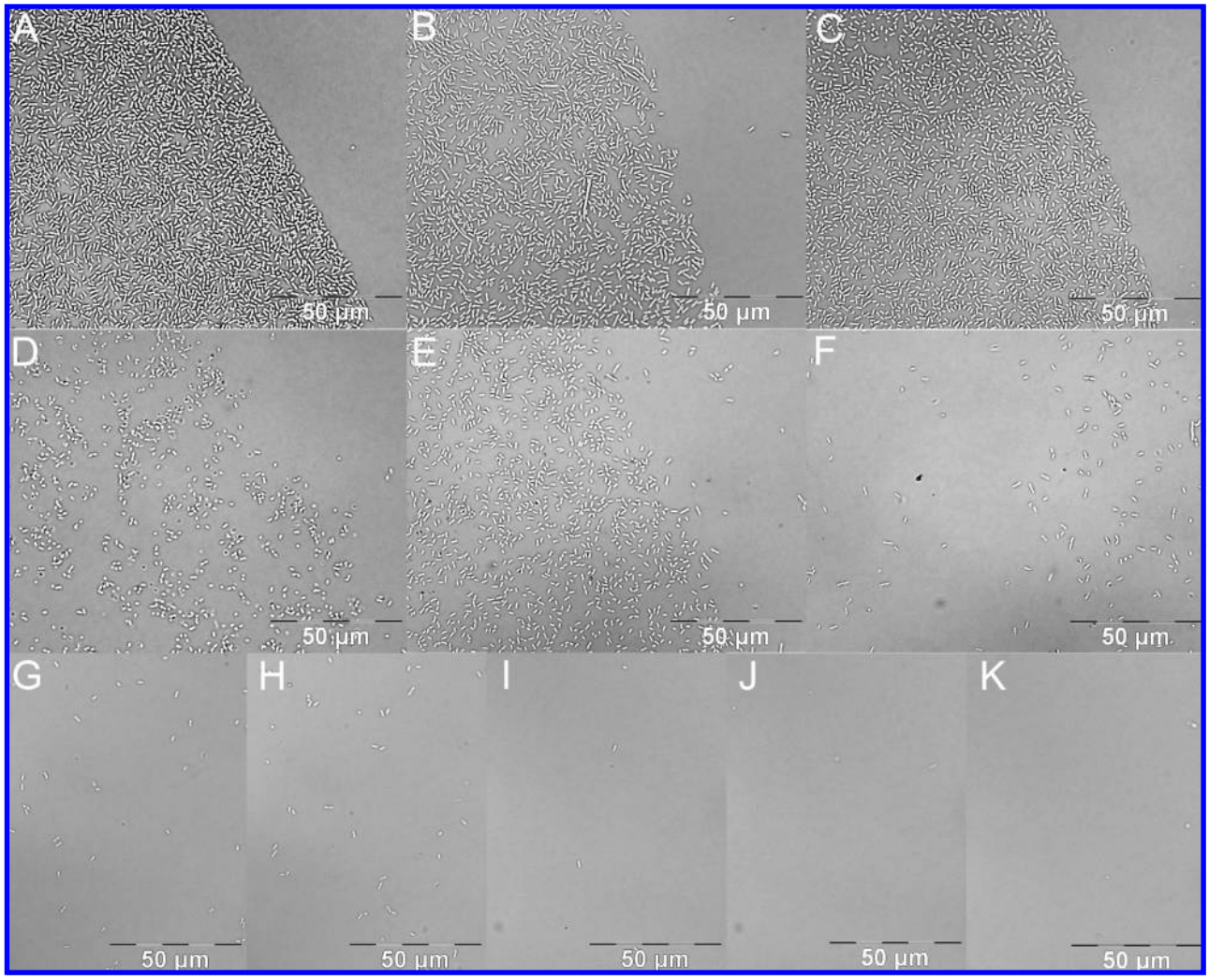


Figure 3. Immobilization of living bacteria on antibody-modified substrates: (A) *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC on substrate modified with anti-CFA/I; (B) *E. coli* H681-pBBScfa on substrate modified with anti-CFA/I; (C) *S.* Typhimurium *motA*3::*cat* H683-pTP2fliC (with flagella motion paralyzed) on substrate modified with antiflagellin; (D) *S.* Typhimurium H647 (with active flagella) on substrate modified with antiflagellin; (E) *S.* Typhimurium H647 on substrate modified with anti-O4-antigen (sc52224); (F) *S.* Typhimurium H647 on substrate modified with anti-O4-antigen (sc52223); (G) *S.* Typhimurium H647 on substrate modified with anti-O-antigen (sc52221); (H) *S.* Typhimurium H647 on substrate modified with anticore antigen (sc52219); (I) *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pF1 on substrate modified with anti-F1; (J) *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC cells on silicon surface without antibody (negative control); and (K) *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC cells on silicon surface modified with unrelated antibody, anticytochrome *c* (negative control).

on substrates modified with antiflagellin. Figure 3C shows the coverage density of the flagella-paralyzed cells, which was significantly enhanced compared with that of the H647 strain with functioning flagella (Figure 3D). In Figure 3D we notice that most of the H647 cells appear as dots (standing-up position) rather than rods (lying-down position), while, for an identical incubation time period of ~30 min, almost all the cells in Figure 3C appear as rods. This implies that the immobilized cells with functioning flagella prefer a standing-up orientation, presumably in an effort to free themselves from the substrate. This observation is consistent with our previous observation that Δ*asd*::*kan*^{R} H71-pHC cells immobilized by anti-CFA/I fimbriae express a large amount of flagella compared with the same in a planktonic state.[10]

The antibodies against *S.* Typhimurium LPS showed mixed results, in that only one of the four antibodies tested demonstrated efficient immobilization of H647. LPS is an important amphiphilic molecule which often extends out from the bacterial outer membrane. It is composed of three covalently linked domains: lipid A, core antigen (oligosaccharide) and O-antigen (polysaccharide).[32] The lipid A is embedded in the outer membrane phospholipid bilayer and hence it is expected that it would be difficult for the antibody for living bacteria to recognize it. Our observations support this claim in that the antibody against lipid A failed to provide any immobilization of *S.* Typhimurium (data not shown). The saccharides, including both the core antigen and O-antigen, protrude from the phospholipid bilayer of the outer membrane and, therefore, can serve as potential targets for immunoimmobilization. However, in our experiments no successful immobilization was observed for the antibody against the core antigen (Figure 3H), implying that the core antigen is shielded by the O-antigen so that the antibody against the core antigen fails to interact directly with the core antigen.

(32) Raetz, C. R. H. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; Vol. 2, pp 1035−1063.

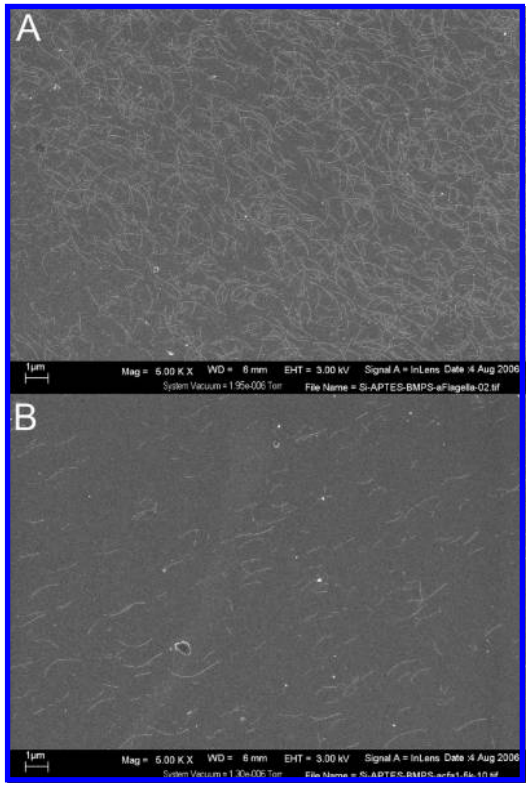
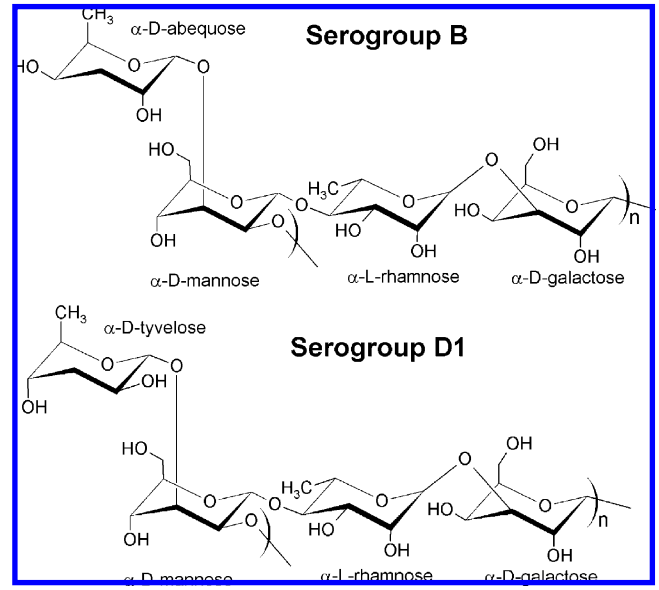


Figure 4. Immobilization of purified *S.* Typhimurium flagella on a silicon surface: (A) flagella on the antiflagellin-modified silicon surface and (B) flagella on the anti-CFA/I-modified silicon surface as a negative control.

At least 46 types of O-antigen have been identified for *Salmonella enterica* under various serogroups including B, D and E.[33] These O-antigens are long chains of polysaccharides with a total length of up to 40 repeating units, typically with three to six sugars in each repeating unit.[32] The sugar composition and the alteration of linkages among the sugars determine the serogroup that a specific strain belongs to.[34] We tested three commercially available antibodies targeting *S.* Typhimurium O-antigen: sc52221, sc52223, and sc52224. The last two antibodies (sc52223 and sc52224) are also labeled as specific to the O4-antigen. Only one of these three antibodies (sc52224) showed successful bacterial immobilization (Figure 3E). *Salmonella* serogroup B, including O-antigens 4, 5, and 12, shares a common tetrasaccharide repeating unit (with different sizes) given by α-D-mannose-1 → 4-α-L-rhamnose-1 → 3-α-D-galactose trisaccharide, to which an abequose is α1,3 linked, as shown in Scheme 1.[35,36] The authors are not aware of any report on the O-antigen structure of H647; however, we infer that H647 does not belong to serogroup B but that its O-antigen shares a large structural similarity with O4-antigen. Only one of the two monoclonal O4-specific antibodies showed positive results in immobilization experiments; if H647 belonged to serogroup B, both anti-O-antigens should have

Scheme 1. Schematics of the O-Antigen Structures of *S. entrica* under Serogroups B and D1[a]



[a] Note that α-D-abequose and α-D-tyvelose differ only in the 3-D orientation of their OH groups at the 2 and 4 positions.

immobilized the bacteria. Serogroup D1 has a repeating unit of α-D-mannose-1 → 4-α-L-rhamnose-1 → 3-α-D-galactose trisaccharide to which a tyvelose is α1,3 linked (Scheme 1).[36] Considering that such a structure is very similar to serogroup B in that tyvelose differs from abequose only in the 3-D orientation of their OH groups at the 2 and 4 positions, it is likely that H647 belongs to serogroup D1. This needs to be further confirmed by the ultimate identification of the O-antigen structure of H647 using other methods. For now, however, our results demonstrate that the antibodies targeting the O-antigen can be used for bacterial immobilization, provided that an appropriate antibody specific to the polysaccharide sequence of the O-antigen can be selected.

We also tested a polyclonal antibody raised against the proteinaceous capsular antigen (F1-antigen) to immobilize living bacteria. F1-antigen, originally discovered for *Yersinia pestis*, can form a dense amorphous capsule that covers the bacterium.[37,38] The *S.* Typhimurium strain Δ*asd*::*kan*^{R} H71-pF1 was constructed to express F1-antigen as a model bacterium. Because the F1-antigen capsule is temperature-sensitive,[39] the Δ*asd*::*kan*^{R} H71-pF1 was grown at 37 °C to ensure the expression of F1 antigen, as confirmed by immunofluorescence and Western blot analysis.[23] It was expected that such a capsular proteinaceous antigen would have a relatively strong interaction with the corresponding antibody. Surprisingly, however, Δ*asd*::*kan*^{R} H71-pF1 cells could not be immobilized on the substrate premodified with anti-F1 antigen (Figure 3I). Most likely this is because the F1 capsule, unlike the CFA/I fimbriae, is not tightly bound to the bacterial cell wall and therefore can easily slough off from the surface of the bacterium, leading to the failure of bacterial immobilization.

(33) Popoff, M. Y.; Minor, L. L. *Antigenic Formulas of the Salmonella Serovars*, 7th ed.; Institut Pasteur: Paris, France, 1997.
(34) Selander, R. K.; Li, J.; Nelson, K. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; Vol. 2, pp 2691−2720.
(35) Hellerquist, C. G.; Lindberg, B.; Svensson, S.; Holme, T.; Lindberg, A. A. *Carbohydr. Res.* **1968**, *8*, 43–55.
(36) Curd, H.; Liu, D.; Reeves, P. R. *J. Bacteriol.* **1998**, *180*, 1002–1007.
(37) Friedlander, A. M.; Welkos, S. L.; Worsham, P. L.; Andrews, G. P.; Heath, D. G.; Anderson, G. W.; Pitt, M. L. M.; Estep, J.; Davis, K. *Clin. Infect. Dis.* **1995**, *21*, S178–S181.
(38) Titball, R. W.; Williamson, E. D. *Vaccine* **2001**, *19*, 4175–4184.
(39) Karlyshev, A. V.; Galyov, E. E.; Smirnov, O. Y.; Guzayev, A. P.; Abramov, V. M.; Zavyalov, V. P. *FEBS Lett.* **1992**, *297*, 77–80.

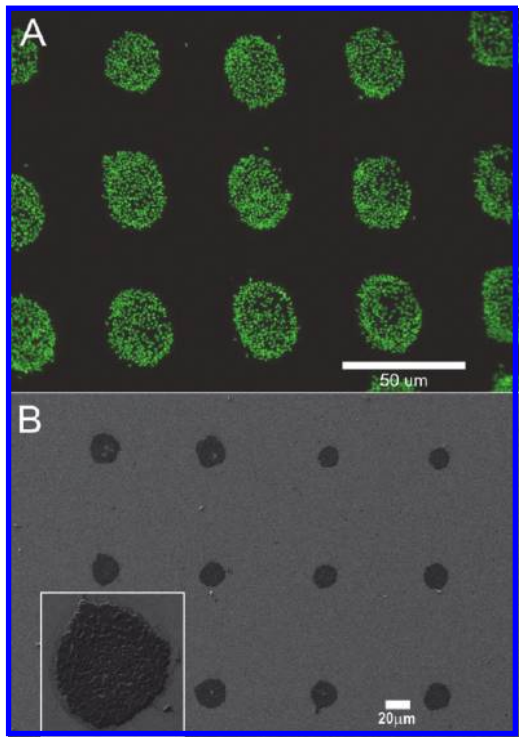


Figure 5. Microarrays fabricated with living *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC cells on a silicon chip: (A) Epifluorescence image of a matrix with 50 μm pitch and (B) FESEM image of a matrix with 100 μm pitch. The inset in part B shows an enlargement of one of the spots in part B.

Microarray of Living Bacterial Cells. In our previous work, the patterns of living bacteria were created by etching a passivated silicon chip using a focused ion beam (FIB).[10] This approach requires expensive FIB instrumentation and surface passivation using poly(ethylene glycol) PEG molecules. Taking into account the fact that in all the experiments *S.* Typhimurium showed minimal attachment to silicon substrates without the presence of antibody (Figure 3J), it is not necessary to passivate the substrate to prevent the nonspecific attachment of bacterial cells to these surfaces. For this reason microplotters offer a practical alternative for immobilizing live cells on flat surfaces. This approach was used to fabricate microarrays with a pitch of ~50 μm of living Δ*asd*::*kan*^{R} H71-pHC cells by means of a microplotter (Figure 5). The spot diameter of the protein pattern is a function of such factors as the tip size, the viscosity of the protein solution used as the "ink," the hydrophobicity of the substrate surface, and the moving speed of the glass tip of the microplotter.[26] In these experiments, glass tips with an inner diameter of 5 μm produced the smallest spot size, which was ~25 μm on APTES/BMPS-modified silicon (Figure 5B).

In principle, smaller spot sizes can be obtained by other techniques when necessary. The size of a bacterial colony is determined solely by the size of the antibody-patterned spot; hence, it is possible to fabricate a microarray of bacterial cells in which each spot has a single living cell by reducing the spot size of the antibody pattern down below ~1 μm in diameter through such techniques as microcontact printing[13] and DPN.[40] We have employed the DPN approach to prepare submicrometer antibody patterns on a gold surface with the aim of immobilizing a small number of organisms at each spot. A microarray of living *S.* Typhimuium H71-pHC cells immobilized on submicrometer-sized (~0.8 μm diameter) antibody patterns created by DPN is shown in Figure 6. It is notable that in some spots more than one organism is immobilized even though the size of the spot is smaller than an individual bacterium, indicating the efficacy of the ab−ag binding technique.

(40) Piner, R. D.; Zhu, J.; Xu, F.; Hong, S. H.; Mirkin, C. A. *Science* **1999**, *283*, 661–663.

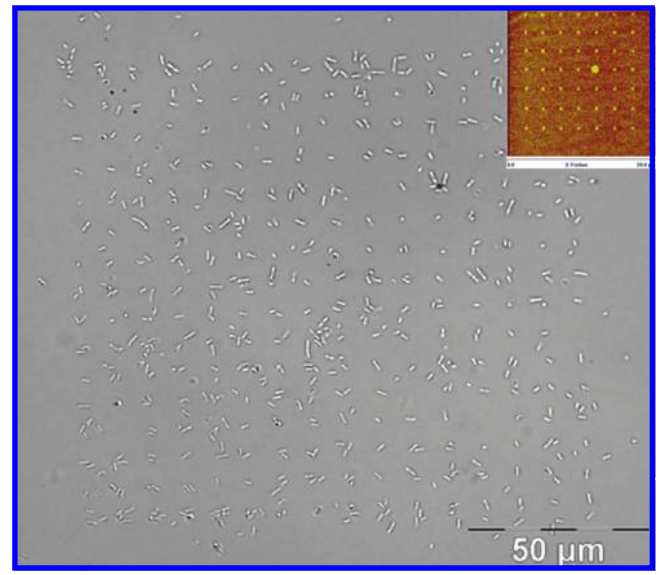


Figure 6. Reflection image of a microarray of single *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC cells prepared using DPN. Inset: an example of the MHA pattern on gold substrates before the covalent linking of antibody (lateral force image; scan size, 20 μm).

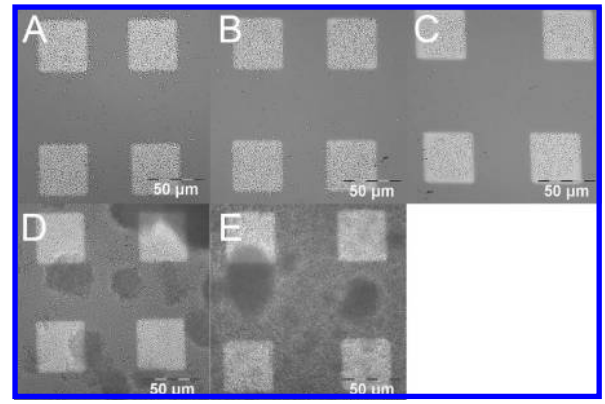


Figure 7. Determination of the stability of bacterial patterns: Silicon chips with anti-CFA/I patterns were incubated with *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC culture for (A) 1 day, (B) 3 days, (C) 10 days, (D) 17 days, and (E) 24 days.

Stability of Living Bacterial Patterns. The immobilized bacteria retained their cellular functions including division and continued to divide until a dense monolayer of bacteria filled the patterned area of the surface.[10] After that, the excess daughter cells were released into the medium and became part of the planktonic community during an initial period of about 2 weeks. Then a biofilm started to form on the substrate. This is shown in Figure 7, where optical images of patterned bacteria on silicon chips are displayed at various stages of their incubation. These silicon chips were prepared such that the patterned area was modified with anti-CFA/I fimbriae and the rest of the area was

passivated using PEG molecules to prevent nonspecific attachment.[10] A total of 15 identical silicon chips were incubated with *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC to form the initial bacterial patterns, and then all of the chips were rinsed with PBS and transferred to nonflow cell-free growth medium at room temperature with no extra cells other than what was immobilized on the chips at the time of transfer. The source of all the bacteria associated with the subsequent biofilm formation can be traced to this monolayer of bacteria. After a predetermined incubation period, one silicon chip was taken out and rinsed with PBS to remove the unattached and loosely attached cells, then imaged under the optical microscope to determine the bacterial content on the substrate surface and its viability. The purpose of the rinsing was to remove the loose bacteria from the medium and from the surface and observe only what remained on the surface. Rinsing was conducted by simply running ~3 mL of PBS buffer gently three times from a ~30° inclined chip surface held with a pair of tweezers (PBS was poured onto the end of the tweezers and allowed to run down over the chip surface), immersing the rinsed chip immediately in a nutrient-free PBS bath and observing the content of the surface under the bright field and epifluorescence microscopes. The epifluorescence images suggested that in all cases the great majority of the cells were alive. Furthermore, the bacterial patterns were maintained (no attachment outside the patterned areas) for about 2 weeks (Figure 7A−C), after which a bacterial biofilm started to form on the substrate. The biofilm remained attached to the silicon chips even after a gentle rinsing cycle with PBS (Figure 7D,E). It is notable that the biofilm grew not only inside the antibody-modified patterns but also on the PEG-covered areas in spite of the passivation by PEG molecules.

CONCLUSIONS

Our experiments demonstrate that in order to achieve efficient immobilization of living bacteria through immunobinding, an appropriate ab−ag pair must be identified. Fimbriae or pili, if present on the bacterial surface, are an excellent target antigen, as demonstrated in this and our previous work.[10] This is simply because fimbriae/pili protrude in large numbers outside the cell and, unlike flagella, do not rotate independently of the bacterium. Flagella, expressed by many bacterial species, can serve as an alternative target antigen, in case the bacterial species expresses no fimbriae/pili. However, the rapid rotation of a flagellum (~10 000 rmp) interferes with the ab−ag interaction and impedes the efficient immobilization of the bacterium. For this reason, a significant enhancement of immobilization can be achieved (compare parts C and D of Figure 3) if the flagella motion of the cells is paralyzed. For bacterial species lacking both fimbriae/pili and flagella, LPS can serve as a potential target antigen (particularly O-antigen) for immobilization, provided that a corresponding specific antibody is available. It should be emphasized that the structure of LPS varies considerably from species to species and sometimes even from strain to strain; it is important that the antibody recognizes the O-antigen. Antibodies raised against lipid A or core antigen are ineffective for bacterial immobilization. Similarly, capsular proteinaceous antigens, such as F1-antigen, and extracellular polysaccharides (K-antigen) are not recommended for immobilizing living bacteria because these molecules detach easily from bacterial surfaces and fail to hold the cells against the substrate surface.

A polyclonal antibody generated using whole bacterial cells as the immunogen is not recommended for immobilization purposes because an antibody obtained in this way recognizes both surface and intracellular antigens, while only surface-bound antigens contribute to bacterial immobilization. Similarly, antibodies targeting intracellular antigens should be avoided, which explains why poor immobilization efficiency was reported in previous studies.[13,21] The purity of antibodies is another critical factor that must be taken into account. Affinity-purified antibodies usually give higher immobilization efficiency than unpurified antiserum. The surface density of antibody molecules on the substrate is significantly reduced when antiserum is used because of the competition between the antibodies and other serum proteins for the linking sites on the substrate surface.[10]

In summary, antibodies targeting fimbriae/pili provide the most efficient immobilization and antiflagellin can offer similar immobilization efficacy provided that the flagella motion is paralyzed. Antibodies targeting the O-antigen of the bacterial LPS are an ideal option for bacteria lacking fimbriae/pili and flagella. Capsular antigens, such as F1, and their corresponding antibodies should be avoided in immunoimmobilization because these antigens can easily detach from bacterial surfaces.

ACKNOWLEDGMENT

This work is funded by NASA-EPSCOR under Grant NCC5-579, by U.S. Public Service Grant AI-41123, by the National Science Foundation under Grant CBET-0709358, and by the Office of Naval Research under Grant N000014-03-1-0692. It is in part supported by National Institutes of Health Grant P20 RR020185 and R21 AI080960-01A1, an equipment grant from the M.J. Murdock Charitable Trust, and the Montana State University Agricultural Experimental Station. The help of the ICAL staff, particularly of Ms. Linda Loetterle and Ms. Laura Kellerman, in handling bacteria and AFM analysis is greatly appreciated.

Received for review April 24, 2009. Accepted July 29, 2009.

AC9014484