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Micropatterns of Spores Displaying Heterologous Proteins

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Microbial spores, a robust and dormant state formed upon the deprivation of essential nutrients,[1] can persist in the soil for many years until they encounter a germination signal. The cell viability of microbial spores can be kept up almost permanently by shuttling between vegetative cells and spores. Spore germination is triggered by a variety of small molecules in rich media, including amino acids, sugars, purines, and simple salts.[2] The robustness of spores is, in part, attributable to the presence of the coat proteins that form the outermost layers enclosing spores.[3] Several genes encoding the spore coat proteins have been identified in *Bacillus* species.[4] Spore surface display technique has been developed especially for *Bacillus subtilis* for the possible application as a vaccine carrier.[5] In this paper we report the first example of micropatterns of recombinant *Bacillus thuringiensis* spores (BT spores), the surface of which displays heterologous proteins. We also demonstrate the successful germination of the recombinant BT spores patterned on the surface.

The generation of spatially well-defined, two-dimensional microstructures of cells has a great deal of potential in the development of high-throughput cellular analysis systems,[6] ultrasensitive cell-based biosensors,[7] and platforms for rare event detection,[8] as well as in the fundamental studies of cell biology on man-made surfaces.[9] In the areas of sensors/detectors, spore-based micropatterns would have advantages over the conventional cell-based micropatterns, including high stability and ease of manipulation. The micropatterns of cells have been generated mainly by employing adhesion receptor ligands such as RGD peptides[10] and fibronectin.[11] It has been, therefore, difficult to extend this strategy to other cell types or spores because suitable receptor ligands, which can be used for the pattern generation through biospecific interactions, have not been identified. We were particularly interested in micropatterning spores, because the patterned spores can be germinated to vegetative cells by controllable external stimuli while providing advantages of long-term storage and outstanding stability. With the aim of developing a versatile method for generating micropatterns of any spore-forming cells, we herein demonstrate that cell-wall engineering,[12] more specifically spore surface display technique, can be utilized to pattern BT spores on glass slides, and the patterned BT spores germinate into vegetative cells on the micropatterns. As a proof-of-principle, we constructed BT spores that displayed enhanced green fluorescent protein (EGFP) (EGFP-BT spores) on the spore surface.

Figure 1a shows the schematic representation of the micropattern generation of BT spores (See Supporting Information for a detailed description of experimental procedures). Glass surfaces patterned with rhodamine (TRITC)-conjugated streptavidin and poly(ethylene glycol) (PEG) were fabricated by four simple steps: generation of interchain anhydrides on the surface, microcontact printing (μCP) of biotin, passivation of the surface with PEG, and spatially controlled deposition of TRITC-conjugated streptavidin. Briefly, self-assembled monolayers (SAMs) of 6-hex-1-enyltrichlorosilane were formed on glass slides, and the vinyl groups on the SAMs were oxidized. The interchain anhydride groups were then generated by following the reported procedure.[13] After the formation of interchain anhydrides, μCP was performed to generate the micropatterns of biotin and TRITC-conjugated streptavidin.[14] The EGFP-BT spores were reacted with anti-rabbit GFP antibody and then with biotin-protein A.[15] The EGFP-BT spore/anti-rabbit GFP antibody/biotin-protein A complex in phosphate-buffered saline (PBS) solution was incubated on the streptavidin-patterned glass slide at room temperature for 1 h in the presence of Tween 20 and bovine serum albumin. After the substrate was washed with distilled water, the spore micropatterns on the glass slide were characterized by confocal microscopy.[16]

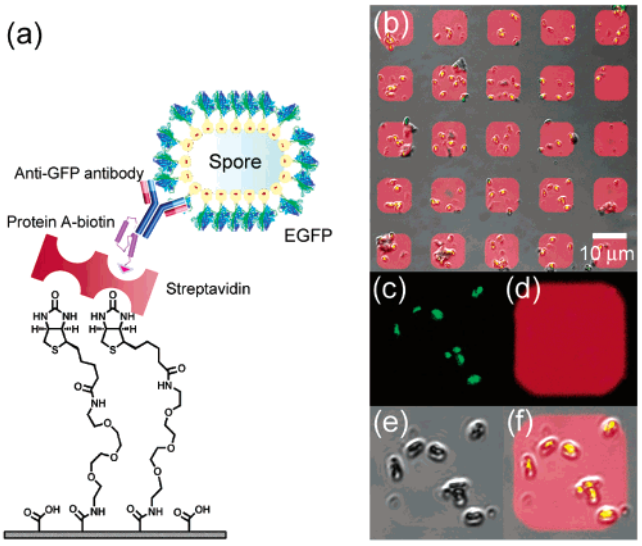

Figure 1. (a) Schematic description of the procedure for generating micropatterns of spores of *Bacillus thuringiensis*. (b−f) Fluorescence and optical micrographs of spore patterns.

Figures 1b−f show the fluorescence and optical micrographs of the EGFP-BT spore-patterned surfaces. The μCP of biotin and subsequent deposition of TRITC-conjugated streptavidin reliably generated the micropatterns of streptavidin. Poly(ethylene glycol) (PEG) effectively minimized the nonspecific adsorption of streptavidin onto the glass slides, which was confirmed by a high contrast of the red color (Figures 1b,d). EGFP-BT spores were observed only in the areas presenting streptavidin under fluorescence and optical microscopy (Figures 1c,e,f).[17] BT spores are ellipsoidal,

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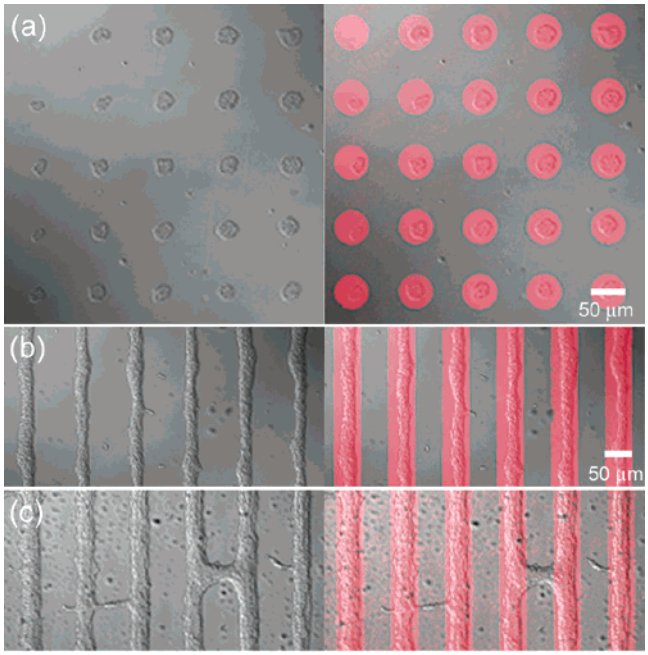

Figure 2. Fluorescence and optical micrographs of vegetative cells after (a,b) 10 h and (c) 15 h of incubation.

having an average size of approximately 1−2 μm: most of the deposited spores were ellipsoidal presumably because of the maximized interactions between biotin and streptavidin at that orientation.

In the presence of nutrients such as sugars and amino acids, spores germinate into vegetative cells, which can be characterized by spore swelling, either absorption or rupture of the spore coat, release of spore components, loss of heat/chemical resistance, and increased metabolic activity. To investigate the viability of the deposited spores and to generate the micropatterns of vegetative cells, we placed the micropatterned spores on the Luria-Bertani (LB) agar plate with the upside-down configuration and incubated them at 37 °C for 10 or 15 h. The BT spores maintained their viability under the micropatterning conditions and were successfully germinated into vegetative cells (Figure 2). The spatially defined microstructures of vegetative cells (live cells) were observed after 10 h of incubation (Figures 2a,b), and some crossovers of cells were observed after 15 h of incubation (Figure 2c). Once germinated, the vegetative cells did not display EGFP on their surface anymore. Nonetheless, it is noteworthy that the nonadherent vegetative cells were confined mostly in the biotin-patterned areas under the germination conditions, although the germinated cells, in principle, could cross over the patterned lines to the PEG-patterned areas. These results suggest that the strategy presented herein should be useful in generating micropatterns of any microbial cells by spatially addressing their spores onto the substrate and making them germinate.[18]

Microbial spores displaying heterologous proteins or other biologically active molecules would be an important tool in many (nano)biotechnological areas, such as target-directed vaccines, protein chips, high-throughput screening of peptide and antibody libraries, and biosensors. Combined with the microarray technique, spores presenting various biomolecules can be micropatterned in many desirable configurations. The demonstrated strategy can be extended to any spore-forming microbial cells, including bacteria, yeasts, and filamentous fungi, and therefore open up the possibility of micropatterning a wide range of microbial cells, leading to various applications taking advantage of both prokaryotic and eukaryotic characteristics.

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Supporting Information Available: Detailed experimental procedure for construction of plasmids, spore purification, flow cytometry analytical methods, surface modification, and pattern generation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Protein A is a highly stable surface receptor produced by *Staphylococcus aureus* and is capable of binding the Fc portion of immunoglobulins, especially IgGs, from a large number of species. See: Sjöquist, J.; Meloun, B.; Hjelm, H. *Eur. J. Biochem.* **1972**, *29*, 572−578.
(16) Fluorescence micrographs were obtained using an LMS510 laser scanning confocal microscope (Carl Zeiss, Germany). For imaging spore-displayed EGFPs, the substrate was excited by a 488 nm argon laser and the images were filtered by a long-pass 505 nm filter. Red fluorescence from rhodamine was observed by the excitation with a 543 nm He−Ne laser followed by filtering with a long-pass 575 nm filter.
(17) Before the pattern generation, spores were analyzed by flow cytometer to confirm that green fluorescence was observed only with EGFP-displayed BT spores. The number of EGFPs was estimated to be about 700 molecules per spore.
(18) Although the viability of spores is maintained almost permanently, we did not investigate the long-term stability of the system consisting of linkers and spores in this study. One possible method to eliminate potential stability problems of the linkers would be to coat the patterned substrate with a thin film that provides the protection of the system and is permeable to stimuli, such as stimulus of germination.

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