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RESEARCH PAPER

Quantitative Studies of Carbohydrate-protein Interaction using Functionalized Bacterial Spores in Solution and on Chips

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Abstract Carbohydrate-protein interaction is one of the most important molecular events deemed critical for numerous biological processes. Therefore, understanding this interaction is essential. In this study, we used bacterial spore display techniques to present multiple copies of streptavidin on the surface of spores to explore carbohydrate-protein interaction in solution and on chips. By applying bacterial spores displaying streptavidin, we developed a new method which allows sensitive, versatile, and passive detection of carbohydrate-protein interactions with a 10-fold increase in sensitivity. The linear relationship of interactions between carbohydrates and labeled concanavalin A (con A) in solution and on functionalized bacterial spore chips has also been confirmed. To the best of our knowledge, this is the first example of development and characterization of binding behavior in carbohydrate-protein interactions using bacterial spore-displayed streptavidin. We believe this strategy may enable new high-throughput screening of carbohydrate interactions as well as establish a basis for monitoring inhibitors of carbohydrate-binding proteins when developing new drugs.

Keywords: bacterial spore display, carbohydrate-protein interaction, concanavalin A, immobilization, lectin

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1. Introduction

Carbohydrate-protein interaction is one of the most important molecular events. Understanding the functions and roles involved in these interactions in numerous biological processes, including cell to cell communication, cell differentiation, cell adhesion and immune response is critical [1,2]. Evaluation of the specific carbohydrate interactions and their properties with their unlimited structural diversity is essential for understanding fundamental biological mechanisms in living cells. This knowledge can provide information necessary for developing novel therapeutics and improved diagnostic assays [2,3]. A variety of methods are used to study the interacton of carbohydrates with proteins including immobilization of protein on a gold surface using electrochemical methods [3,4], carbohyrate microarrays [5], and capillary electrophoresis [6].

However, a key limitation of a model using substrates with functional ligands is that it does not allow multivalent interactions, resulting in low binding affinity to target proteins. Thus, there is interest in alternate methods with increased complexity and multivalency. For example, multiple carbohydrate ligands have been assembled on linear polymers [7], two-dimensional metal surfaces [8], and liposomes [9] to enhance carbohydrate-protein interactions. However, there is still a lack of multivalency in these interactions; therefore, the need for a new efficient analytical

method to detect carbohydrate-protein interactions remains.

Microbial cell-surface display techniques could potentially improve the multivalent interactions between ligands and receptors by displaying heterologous peptides or proteins of interest on the surface of microorganisms [10-12]. Frequently, microbial cell surface display is exploited in the study of biomolecular recognition, and the technology has been used to probe protein-protein interactions [10], to develop biosensors [13], to use the whole-cell biocatalysts [14], and to identify peptide libraries for use in diagnostic purposes [15].

Recently, we reported on the advances of the microbial surface display, more specifically, spore display which allows proteins to be displayed on the spore surface by fusing them with an anchoring motif [13]. It is conceivable that streptavidin can be displayed on the spore surface in biosensing applications through a streptavidin-biotin interaction which is one of the strongest interactions with high affinity [16,17]. The bacterial spore is highly resistant to destructive environmental conditions such as ultraviolet and ionizing radiation, toxic chemicals, and heat, enabling it to remain dormant for an extended period of time [16,18]. These characteristics are advantages for using polyvalent bacterial spores to study biomolecular recognition.

We hypothesize that whole spores displaying proteins may be useful to study carbohydrate-protein interactions, as demonstrated in previous reports [17]. Using fluorescence microscopy and microarray techniques we developed a general method to monitor carbohydrate-protein interactions in *Bacillus thuringiensis* spores expressing streptavidin on the surface. The surface protein, InhA in *B. thuringiensis* was used as a fusion partner to express streptavidin.

2. Materials and Methods

2.1. Chemicals

The α-D-mannose-sp-biotin (sugar-O$(CH_2)_3$NHCO$(CH_2)_5$-NH-biotin) was purchased from GlycoTech (Gaithersburg, MD, USA). Concanavalin A (con A), Texas Red® conjugate, and biotin-fluorescein were purchased from Molecular Probes (Carlsbad, CA, USA). The aldehyde-coated glass slides were purchased from CEL & Associates (Los Angeles, CA, USA).

2.2. Construction of the InhA-streptavidin expression vector

The *inhA* gene containing its native promoter was amplified by polymerase chain reaction (PCR) from the chromosome of *B. thuringiensis* (BT) subspecies, *kurstaki* HD-1 (ATCC 33679), using the following two primers:

Table 1. Plasmids used in this study

Plasmid	Relevant genotypes	Reference or source
pS	cat	[19]
pSD1	*cat*, *inhA*	[19]
pSD-SA	*cat*, *inhA*, core streptavidin	This study

5'-ACGCGTCGACATGTAATTCCTCCCTAATTAT-3'
5'-CTAGCTAGCACGATATAAACGAAC-3'

The amplified fragments were digested with restriction enzymes *Sal*I and *Nhe*I (underlined sites) and cloned into *Bacillus* plasmid pS to make pSD1 [19]. The PCR-amplified core streptavidin gene from the chromosome of *Streptomyces avidinii* (ATCC 27419) using the following two primers:

5'-ATAAGAATGCGGCCGCATGGACCCCTCCAAGGAC-3'
5'-CCGCTCGAGCTACTGCTGAACGGCGTC-3'

was then cloned into *Not*I and *Xho*I (underlined sites) in pSD1 to make pSD-SA (Table 1). PCR experiments were performed with a PCR Thermal MP TP 3000 (Takara Shuzo, Tokyo, Japan) using a high fidelity PCR System (Roche, Basel Schweiz, Germany). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA). The plasmid was transformed into *BT* 4Q7 by electroporation (Bio-Rad, Hercules, CA, USA). Other general molecular biological experiments were carried out following standard procedures [20]. The sequences of cloned genes were analyzed with an automatic DNA sequencer (ABI Prism model 377, Perkin Elmer, Waltham, MA, USA).

2.3. Purification of bacterial spores

The recombinant *B. thuringiensis* harboring pSD-SA was cultivated in chemically defined sporulation media (CDSM) [21] at 37°C and 250 rpm for 48 ~ 60 h until sporulation. Spores containing vegetative cells were harvested from 50 mL of the culture broth by centrifugation (5000 rpm) and were resuspended in 200 μL of 20% (w/v) urografin (Sigma). This suspension was gently layered over 1 mL of 50% (w/v) urografin in a microcentrifuge tube and then centrifuged for 15 min at 4°C and 13,000 rpm. The collected fraction containing only free spores displaying streptavidin was used to study carbohydrate-protein interactions.

2.4. Fluorescence spectroscopy in carbohydrate-protein interactions

The purified spores displaying streptavidin were washed in deionized water and resuspended in phosphate-buffered saline (PBS, pH 7.4) containing 3% (w/v) skim milk for blocking. After washing three times with PBS, the spores were incubated with biotinylated α-mannose and 50 μg/mL of the Texas Red®-conjugated con A for 1 h at room

temperature. After washing again with the same buffer three times, the final samples were analyzed using a SpectraMax® M2 multi-well plate reader (Molecular Device, Sunnyvale, CA, USA).

2.5. Flow cytometric analysis

The purified spores were incubated overnight with a solution of biotinylated α-mannose in PBS containing 1% (w/v) bovine serum albumin at room temperature. The samples were then rinsed twice with the same buffer. Subsequently, the complexes of spores were stained with a solution of Texas Red®-conjugated con A, and the final reaction samples characterized using a flow cytometer (FACSCalibur™; BD Bioscience, San Jose, CA, USA) and Cell Quest Pro™ software (BD Bioscience). All samples were analyzed for relative fluorescence intensity with a FL2 fluorescence detector coupled with a 585/542 nm filter.

2.6. Microarray experiments

Bacterial spores were immobilizedon aldehyde-coated glass slides (CEL & Associates) by amine-aldehyde covalent interactions. The spores displaying streptavidin were resuspended in PBS and spotted on the glass surfaces in 270 mm intervals using a micro spotter (MicroGrid; BioRobotics, Cambridge, UK). The slides were washed with PBS for 30 min to remove unbound spores before analyzing the carbohydrate-protein interactions. The immobilized glass slides were kept under humidified conditions (above 60% humidity) for 1 h at room temperature after spotting, and dried with a stream of nitrogen gas. To minimize any background signal, the non-reacted aldehyde groups were reduced to non-reactive primary alcohols by treatment with sodium borohydride ($NaBH_4$). Slides were read using a microscanner (GenePix® 4000B, Axon, Concord, ON, Canada).

3. Results and Discussion

In general, carbohydrate-protein interactions are identified as having very low affinity between each other in biological systems [16]. However, this low affinity can be increased by presentation of multiple ligands to individual receptors. The polyvalent interaction between multiple ligands and their receptors can be much stronger than corresponding monovalent interactions. For this and other reasons, many researchers have used polyvalent microbial surface displays in the development of sensing devices [10,12,22].

Fig. 1 depicts the schematic representation of multivalent carbohydrate-protein interactions on the spore surface using bacterial spore display techniques. As expected, cell

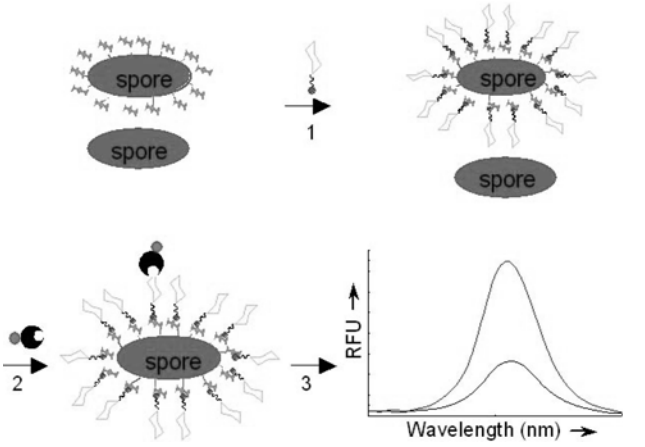

Fig. 1. Schematic representation of multivalent carbohydrate-protein interactions on the surface of bacterial spores. Precursor 1 is biotinylated mannose. Precursor 2 is labeled con A conjugated with Texas Red®.

expression in the absence of streptavidin is quite similar to the mean fluorescence of unlabeled cells, indicating that the control cells did not react with labeled con A (Fig. 2A). In contrast to the control experiment, the fluorescence values of cells displaying streptavidin incubated with 50 μg/mL of labeled con A were significantly shifted compared to those of the control experiment (Fig. 2B). This effect is due to the multivalent interaction of streptavidin displayed on the spore surface to corresponding target molecules. We calculated the molecules of displayed streptavidin bound by biotin-fluorescein interaction. One streptavidin can bind four biotin molecules. Fluorescence intensity of free streptavidin molecules bound with biotin-fluorescein was compared with that of spore-displayed streptavidin. As calculated, approximately 700 molecules of streptavidin were displayed per spore. Thus, the precursor of two was more effectively incorporated into the spore surface, indicating that the target protein, streptavidin, could be successfully displayed on the surface of bacterial spores. The multivalent interaction was further confirmed by the use of fluorescence microscopy. As predicted, a poor mean fluorescence value was observed in the absence of streptavidin (Fig. 3A, thin line). However, the fluorescence value of the displayed cells observed was approximately increased 10-fold in mean fluorescence over the background signal, as shown in Fig. 3A (solid line). This is similar to the result described previously.

To determine the specificity of carbohydrate-protein interactions, 1.4×10^8 colony forming units of streptavidin-displayed spores was incubated with 100 mL of biotinylated mannose (50 μg/mL) and washed to remove unbound reagents. The spores tethering biotinylated mannoses were incubated with a series of dilutions of labeled con A ranging from 0 to 300 μg/mL and measured by fluorescence

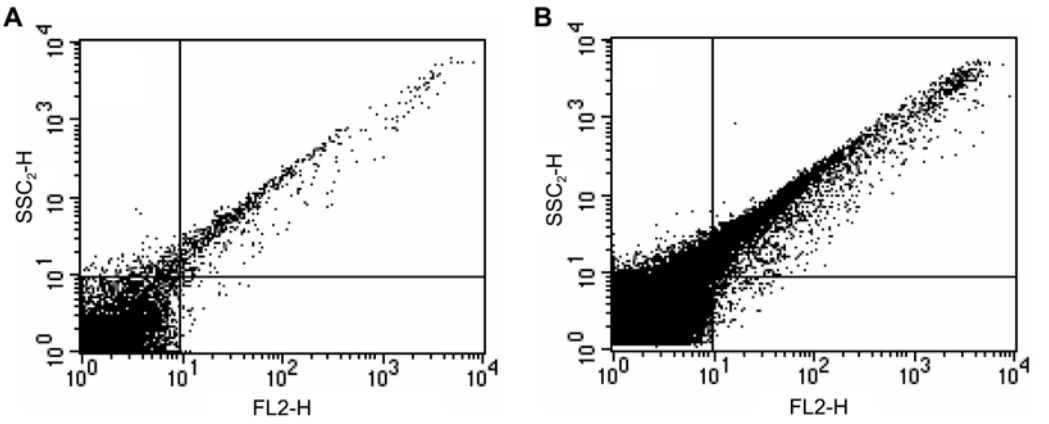

Fig. 2. Characterization of carbohydrate-protein interactions in solution by flow cytometry. The spores were sequentially incubated with precursor 1 and 2 for 1 h at room temperature and unbound labeling reagents removed by washing with buffer. Labeled spores were diluted to a final optical density (OD_{595} = 0.7) so that spores of 1.4×10^8 CFU/mL represent approximately 700 molecules of streptavidin displayed per spore surface. Measurements were made by flow cytometry; (A) unmodified spores, (B) spores displaying streptavidin.

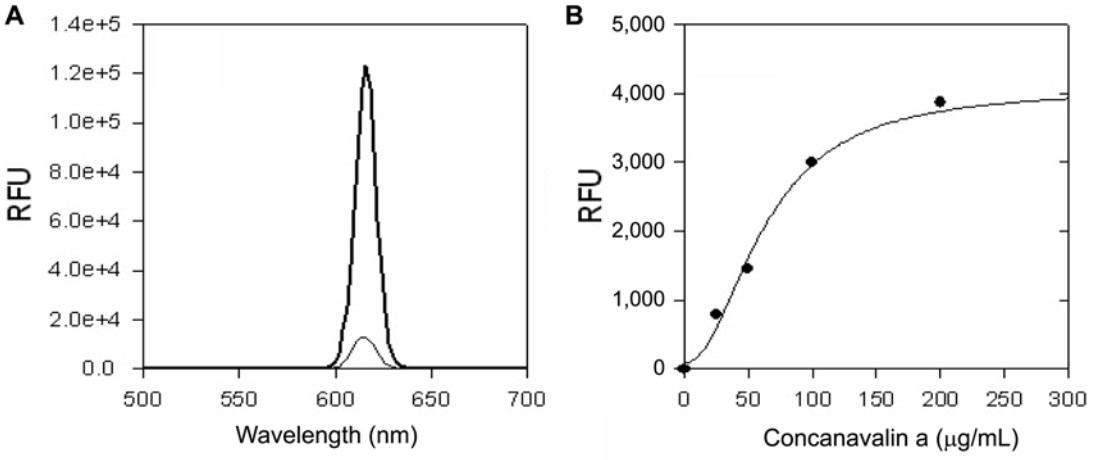

Fig. 3. Characterization of carbohydrate-protein interactions in solution by fluorescence spectroscopy. (A) Spores displaying streptavidin with labeled con A (solid line) and unmodified spores with labeled con A (thin line), (B) fluorescence intensities obtained by varying concentrations of labeled con A.

spectroscopy. Fig. 3B shows the fluorescence levels as a result of varying the labeled con A concentration. The fluorescence intensity was saturated with approximately 200 μg/mL of labeled con A and there is no increase in fluorescence at this concentration. Below the saturation concentration, a linear concentration relationship between carbohydrate and labeled con A was observed. This result suggests that streptavidins displayed on the spore surface retained binding affinity for their target carbohydrate molecules through multivalent interactions.

There are a number of methods for fabricating carbohydrate microarrays on functionalized solid substrates through surface chemistry techniques [7-9]. There is interest in presenting carbohydrates on the solid surface in a microarray format to provide an efficient way to simultaneously monitor multiple binding events between immobilized carbohydrates and their target proteins. With the goal of developing a versatile method for monitoring carbohydrate-protein interaction, we applied bacterial spores displaying streptavidin on functionalized glass slides to confirm our hypothesis.

For proof-of-concept, we used aldehyde-coated glass slides for direct binding of the spores expressing streptavidin with free amino groups. The spores expressing streptavidin were immobilized at 100 μm in diameter using a microarrayer. Following incubation with biotinylated carbohydrates (50 μg/mL), the spore chip tethered mannoses were incubated with the solution of labeled con A (50 μg/mL), washed with buffer, and dried at room temperature before measurement. Interestingly, carbohydrate-protein interactions were only observed on the spots of spore-immobilized chips which indicate that only cells displaying

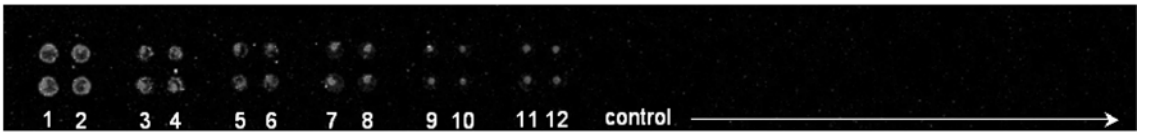

Fig. 4. Characterization of carbohydrate-protein interactions on a spore microarray. Bare slides were immobilized with the spores expressing streptavidin and reacted with biotinylated mannose (50 μg/mL). After washing, slides were incubated with labeled con A (50 μg/mL). For immobilization of spores, streptavidin-displayed spores were spotted at different concentrations (column 1-4, OD_{595} = 0.7; column 5-8, OD_{595} = 0.35; column 9-12, OD_{595} = 0.125) and incubated with labeled proteins for 5 min. Negative controls are representative of *BT* spores without surface-displayed protein at different concentrations. The change of fluorescence intensity was observed using an Axon scanner. Spot sizes are 100 μm in diameter and 270 μm in spacing.

streptavidin were recognized by labeled con A through streptavidin-biotin interactions. No fluorescence was detected in the negative control *BT* spores without displayed protein (Fig. 4). Also of interest, fluorescence intensity positively correlated with the concentration of cells added to the reaction mixture on the microarray. As shown in Fig. 4, carbohydrate-protein interactions could be easily observed even at a low spore concentration (OD_{595} = 0.125). It is reasonable that nonspecific binding of immobilized spores in the microarray were much lower, while the specificity of protein interactions was much higher. We suggest that streptavidin displayed on the surface of spores can participate in carbohydrate-lectin interactions. We were able to minimize nonspecific interactions of target proteins and reduced the signal-to-noise ratio of the spore microarray on the functionalized glass surface.

In this study, new strategies for displaying proteins on spore surfaces through bacterial surface display techniques, and the binding behavior of carbohydrate-protein interactions in solution and on a spore array were evaluated. We constructed the fusion expression system to display multiple copies of streptavidin as a model protein, resulting in enhanced sensitivity of carbohydrate-protein interactions. Although streptavidin and mannose were used as examples for displaying on the spore surface and for studying a binding behavior to con A, respectively, other proteins or mono-saccharides could be effectively used to monitor multiple binding interactions.

4. Conclusion

In conclusion, we developed a general method with high sensitivity and signal-to-noise ratio to study carbohydrate-protein interactions in solution and on a chip. This approach has several advantages over conventional methods including ease of manipulation and production to display any proteins or peptides at a lower cost and with outstanding higher stability (*e.g.* long-term storage and heat stability). It is also possible that other coat spore proteins can be used as an anchoring motif for surface expression by fusing them to target proteins. As described earlier, combined with the microchip technique, bacterial spores displaying active biomolecules could be an important tool in a variety of diverse biotechnological applications.

To the best of our knowledge, this is the first example of development and characterization of binding behavior in carbohydrate-protein interactions using bacterial spore display techniques. We believe that this strategy may enable new high-throughput screening and monitoring of inhibitors for interactions with carbohydrate binding proteins while developing new drugs.

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