Development of a bioreactor system using *Bacillus subtilis* spores

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Report:

To develop a system that utilizes *Bacillus subtilis* spores that display useful proteins on their surfaces as bioreactor particles called sporobeads, I first localized spore coat proteins in the spore layers and identified proteins located in the outermost layer of the spore coat. Next, I measured fluorescence intensities of the spores with green fluorescent protein (GFP) fused with these spore coat proteins to assess their abundance and stability on the spore surface. Four proteins were found to be able to display exogenous protein at both 4 ºC and room temperature (20-25 ºC), under wet and dry conditions for at least 3 weeks. Furthermore, disruption of a gene essential for germination, *cwlD*, did not affect the stability of proteins displayed on the spore surface. These results indicate that these 4 proteins are promising candidates for anchor proteins in this sporobead system.

Research aims

Displaying exogenous proteins or peptides on the surface of small particles is useful in many fields, including medical science, pharmaceutical science, bioremediation, the fermentation industry, and macromolecular material production. Efforts to develop such display systems on the surface of bacterial, mammalian, and insect cells have been reported. This research aims to develop a display system using *B. subtilis* spores. *B. subtilis* spores have many advantageous features for their utilization for this purpose. 1) *B. subtilis* is safe for humans, as *B. subtilis* subsp. natto is known to be used for natto production. 2) Easy and low-cost incubation and knowledge of the natto industry can be applied. 3) Since *B. subtilis* spores are highly resistant to harsh environments, they can be used under a wide range of conditions and are amenable to long storage or transportation. In addition, it is known that fixed enzymes are more stable than those free in solution. 4) To display proteins normally expressed in a cell on a cell surface, the protein must clear several hurdles including secretion efficiency, attachment to the membrane, and refolding after secretion. *B. subtilis* spores do not face these problems, because spore coat proteins are expressed in the mother cell and assembled on the forespore (Fig. 1). Therefore, this system is useful for a wide variety of purposes and in many reaction conditions. This research aims to identify the best anchoring protein to establish a protein displaying system with *B. subtilis* spores.

Methods

It is necessary to identify proteins exposed on the spore surface from among 70+ spore coat proteins, to establish a protein display system using *B. subtilis* spores (Fig. 1). To date, localization of each protein within the spore coat layer has only been accomplished using immuneelectron microscopy, and the results continue to be poorly understood. Therefore, we developed a method to identify the position of each spore coat protein within the spore coat layers using fluorescence microscopy combined with image analysis and statistical processing [1]. Using this technique, we discovered the previously unidentified outermost spore layer [2, 3]. We analyzed the location of several spore coat proteins and chose several proteins found on the spore surface as candidate anchoring proteins on which to display enzymes. Fluorescence intensities of spores expressing GFP-surface protein fusions were measured to assess the abundance and stability of displayed proteins. One potential flaw in this system is that if the substrate or products of a bioreactor reaction induce spore germination, the enzyme-displaying particles will be lost. To eliminate this possibility, we disrupted a gene essential for spore germination, *cwlD*, and assessed the abundance and stability of displayed protein on these mutant spores to identify the best anchor protein.

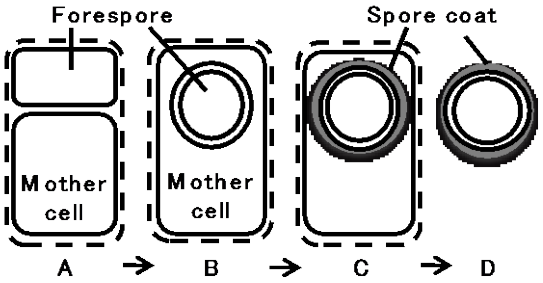

Fig. 1 Endospore formation in *Bacillus subtilis*. Sporulation of *B. subtilis* is initiated in response to nutrient limitations. The first morphological change in sporulation is the appearance of an asymmetrically positioned septum that divides the cell into a larger mother cell and a smaller forespore (A). Next, the mother cell membrane migrates around the forespore membrane and pinch off the forespore within the mother cell (B). Over 70 proteins are expressed in the mother cell and assemble on the forespore to form the spore coat (C). Matured spore is released upon lysis of the mother cell (D).

Results

Localization analysis of spore coat proteins in the spore layers identified 6 proteins at the outermost layer of the spore. We then assessed the abundance and stability of GFP anchored to these proteins on spores. Four proteins, CgeA, CotW, CotX and CotZ, were found to display GFP stably and abundantly at 4 ºC as well as room temperature, in wet and dry conditions. Fig. 2 shows the data for dry conditions. Although each anchor protein exhibited a different initial fluorescence intensity, all proteins retained at least half of their initial fluorescence after 23 days of storage at 4 ºC or room temperature (Fig. 2). These results indicate that CgeA, CotW, CotX, and CotZ can display exogenous protein on the surface of spores for at least a few weeks. In addition, since there was no significant difference in effects of the temperature (i.e., 4 ºC and room temperature) on the ability to display proteins, this system can retain enzymes stably on spores, without low temperature storage.

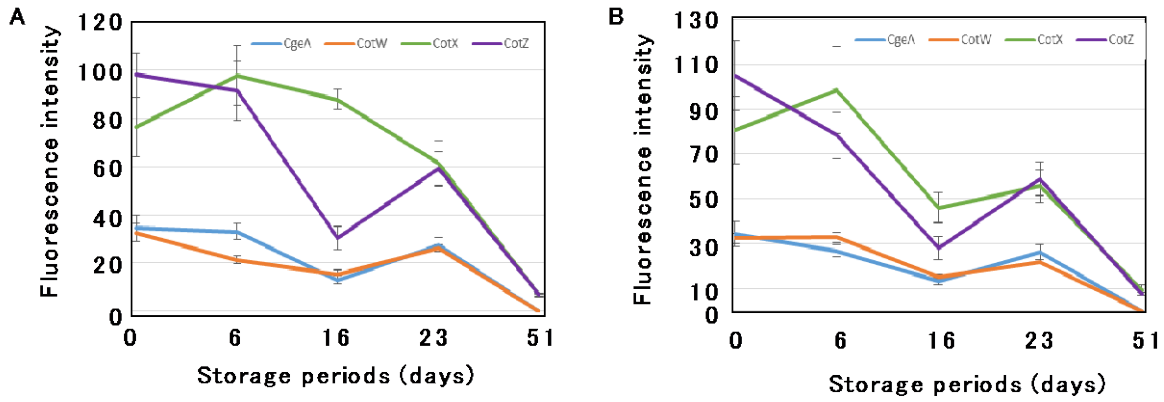

Fig. 2 Stability of GFP displayed on the spore surface during long term storage.
GFP was displayed on the spore by fusing to the indicated spore surface proteins and purified spores were dried and stored at room temperature (A) or 4 °C (B). Fluorescence intensities were measured at indicated time points. The mean and standard deviation are shown.

This spore display system can be used in bioreactors by fusing enzymes required for a reaction to these anchoring proteins. However, if the substrate or reaction product induces spore germination, the enzyme particles would be lost. Thus, we tested whether GFP is stably displayed on spores lacking an essential gene for spore germination. GFP fused to four proteins was retained at equivalent levels to that on wild type spores and on spores lacking an essential germination gene.

Taken together, the above results indicate that four proteins identified in this study, CgeA, CotW, CotX, and CotZ, can display exogenous proteins for several weeks at room temperature, and spores lacking a germination gene were also stable.

Conclusion

This research identified four spore coat proteins that are suitable for long term display of exogenous proteins on the surface of spores. Thus, these proteins are promising candidate anchoring proteins for use of spores in bioreactors.

References

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