Developing an Expression System with High Heterologous Protein Accumulation Capacity Using *Bacillus subtilis* Mother Cell

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Research objective

Basic and applied research depend heavily on the expression and purification of heterologous proteins. Heterologous expression systems must be simple to use and have a high yield. Moreover, expression systems, including purification methods, must match the structures and properties of the different proteins. Therefore, several expression systems capable of producing heterologous proteins have been developed.

Bacillus subtilis is a spore-forming bacterium. They form intracellular spores upon nutrient depletion. Spores are dormant cells resistant to heat, radiation, and chemicals. They can diffuse into aqueous environments. The outer crust and inner coat proteins of spores are responsible for their resilience. In contrast, polysaccharides in the outer layer of the spore crust are responsible for diffusion into the aqueous environment. During sporulation, the cells divide unequally into larger “mother” and smaller “forespore” cells. The mother cells encapsulating the forespores help them mature into spores. Although the mother cell and the forespore have identical chromosomal DNA, their RNA polymerases have different σ factors. The mother cell RNA polymerases have $\sigma^E \rightarrow \sigma^K$ factors, whereas the forespore RNA polymerases have $\sigma^F \rightarrow \sigma^G$ factors. CotY is highly expressed in the crust; σ^K is involved in *cotY* transcription. Polysaccharide synthesis in spores requires enzymes encoded by the *spsA-L* operon. The crust and polysaccharide layers are linked by a CgeA anchor protein (1). Once spores mature within the mother cell, their cell membranes and cell walls are degraded by lytic enzymes (such as CwlB, CwlC, and CwH), resulting in the release of mature spores from the mother cell. The mother cells of *cwlBCH* mutant *B. subtilis* have a thick cell wall, and spores within them are less likely to be released (2).

Here, by exploiting *B. subtilis* sporulation mechanisms, we aimed to develop a new *B. subtilis* heterologous expression system in which the proteins accumulate in mother cells, facilitating their recovery.

Methods

The mother cells are less susceptible to degradation by intracellular proteases because they specialize in the biosynthesis of spore-related proteins. Gene expression in the mother cell is almost exclusively limited to σ^E- and σ^K-regulated genes. Therefore, it is likely that mother cells express fewer proteins than vegetative cells. This facilitates the purification of heterologous proteins from mother cells. Here, we employ the following strategies to design a *B. subtilis* heterologous expression system, which causes the target protein to highly accumulate in the mother cells: 1) we synchronized the expression of the heterologous proteins with

sporulation, which coincides with the maximum growth phase of *B. subtilis*; 2) we expressed the protein under the mother cell-specific P_{cotY} promoter; 3) we used pKM-1, a high copy number plasmid vector; 4) we disrupted the *spsA-L* operon to divert the host resources—including transcriptional and translational machinery dedicated to polysaccharide synthesis and the substrates and energy dedicated to protein synthesis—to the heterologous protein production; and 5) we disrupted *cwlBCH* expression to thicken the cell wall of mother cells, which will cause the target protein to accumulate within the mother cells. Thick cell walls inhibit the leakage of proteins from mother cells across the cell wall barrier into the extracellular culture medium. Additionally, thick cell walls prevent the re-entry of secreted proteases from the culture medium into the mother cells, protecting heterologous proteins from proteolysis. Furthermore, it is easier to harvest thick-walled mother cells by centrifugal precipitation because they encapsulate spores. Thus, high-molecular-weight proteins cannot pass through the thick cell wall and are recovered as a precipitate comprising mother cells that encapsulate the proteins and spores. We found that repeated washing using agitated resuspension in phosphate-buffered saline and centrifugation helped to effectively remove the extracellularly secreted proteases. Subsequently, heterologous proteins within the mother cells can be isolated by dissolving the cell wall. Incubation of the washed mother cell precipitate with a lysozyme-containing buffer released the enclosed heterologous protein, which could then be eluted. Centrifugation separates the mother cell proteins (as supernatant) from the spores and cell wall debris (as precipitates). Here, we demonstrated the efficacy of this new heterologous protein accumulation and recovery method (Figure 1).

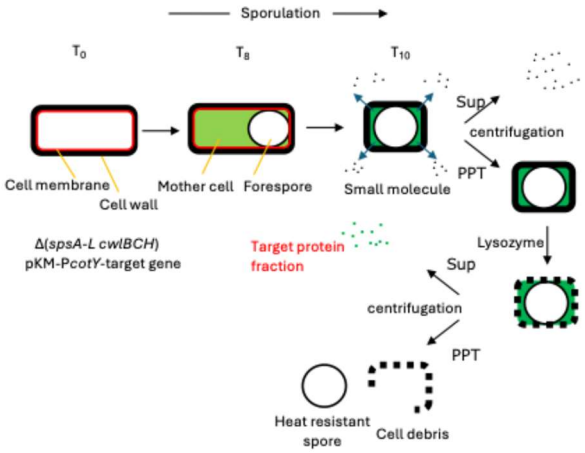

Figure 1 Schematic diagram of the method for accumulation and recovery of heterologous proteins in *Bacillus subtilis* mother cells.

Results

First, we constructed a multicopy plasmid (pKM-1-P_{cotY}) with the *cotY* promoter, and *gfp* and *lacZ* fused downstream of P_{cotY}. We examined sporulation-specific heterologous protein expression under the *cotY* promoter. The expression of heterologous proteins in these plasmid-bearing strains was observed 6 h after the onset of sporulation (T_6). The polysaccharide layer was located in the outermost spore and had a high-volume fraction (1). The spore polysaccharide synthesis enzymes are encoded by the *spsA-L* operon. We increased the expression of the heterologous protein gene by introducing a *spsA-L* operon mutation into a heterologous protein-expressing strain. Next, we introduced the *ΔcwlBCH* mutation into the heterologous protein-expressing strain, resulting in delayed lysis of the cell wall of mother cells, thus prolonging the retention of the heterologous protein in the mother cell. When cultured, the *ΔcwlBCH* strains form mature spores within the cell wall-enclosed mother cells after T_{10}.

Next, we cultured the double mutant *Δ* (*spsA-L cwlBCH*) strain to produce spore-containing mother cells. The spore-containing mother cells obtained were dense and could be easily isolated as precipitates by centrifugation. Subsequently, the spore-containing mother cell precipitate was resuspended in phosphate-buffered saline by vortexing, and re-pelleted by centrifugation. This washing step was repeated to increase the purity of the spore-containing mother cell fraction. Eventually, the cell walls of the mother cells were dissolved by incubating the repelleted mother cells with lysozyme buffer under constant stirring. This step releases the heterologous protein accumulated in the *Δ* (*spsA-L cwlBCH*) strain into the buffer solution. The eterologous protein was subsequently isolated from the supernatant obtained by centrifuging the lysed spore-containing mother cells (and the precipitate-containing spores). The ratio of each protein to total protein in the supernatant of the *Δ* (*cwlBCH spsA-L*) strain with pKM-1-P_{cotY}-*lacZ* and pKM-1-P_{cotY}-*gfp*, respectively, was >35% (Figure 2). Evidently, the use of the *Δ* (*cwlBCH spsA-L*) strain as a host can provide a highly purified heterogeneous protein fraction.

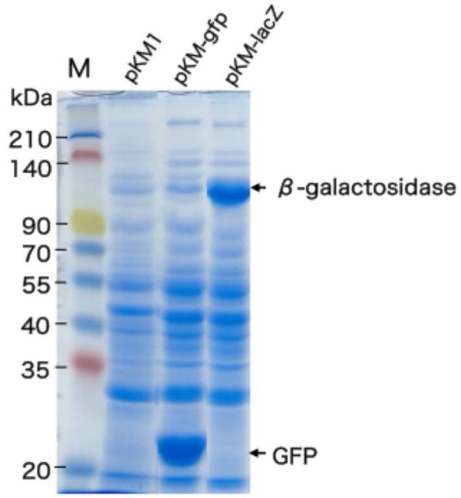

Figure 2 β-galactosidase and GFP expressed, accumulated and recovered in mother cells of Δ(*spsA-L cwlBCH*) strain

Conclusion

We constructed a novel protein expression system for efficient heterologous protein production using a multicopy vector containing the mother cell-specific promoter (P_{cotY}) and a double-mutant strain lacking *spsA-L* operon and *cwlBCH* expression. This new heterologous expression system increases protein yield by helping to accumulate heterologous proteins in mother cells, from which they can be easily recovered. The observed >35% increase in relative yield of heterologous proteins when using simple protein extraction methods such as centrifugation and lysozyme treatment indicates that this new heterologous expression system is an effective heterologous protein accumulation and recovery system.

References

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