

Toward Recombinant Cellulolytic Bacteria: Stabilizing Displayed Cellulases on Vegetative *Bacillus subtilis* Cells

Grace L. Huang,¹ Jason Gosschalk^{1*} (jaygosschalk@ucla.edu), Ye Seong Kim,¹ Xiyan Sun,² and Robert Clubb¹

¹UCLA-DOE Institute of Genomics and Proteomics, University of California, Los Angeles, 611 Charles Young Drive East, Los Angeles, CA 90095, USA. ²Chinese Academy of Sciences, Kunming Institute of Botany, Yunnan Kunming, China.

Project Goals: We are studying how eubacteria display proteins and polymers on their surface, toward the goal of creating surface engineered microorganisms that can degrade lignocellulose. To gain broad insight, our surface engineering efforts are concentrated on *Bacillus subtilis*, a model Gram-positive microorganism that is used industrially. For practical applications, surface-displayed cellulases need to be bound to the peptidoglycan at a high density and complexity, while remaining highly stable. In this study, we have systematically explored several well-established protease-deficient *B. subtilis* strains' ability to display cellulases and assessed the impact of surface-display on bacterial growth, cellular stress and morphology. The results of this work can be used to guide the construction of cellulase-displaying microbes that convert plant biomass into second generation biofuels, chemicals, and materials.

Lignocellulosic biomass is a promising feedstock from which to sustainably produce useful biocommodities, but its recalcitrance to hydrolysis limits its commercial utility. One attractive strategy to overcome this problem is to use consolidated bioprocessing (CBP) microbes that directly convert biomass into chemicals and biofuels. Several industrially useful microbes possess desirable consolidated bioprocessing characteristics, yet they lack the ability to degrade biomass. Engineering these microbes' surfaces to display cellulases and cellulosome-like structures could endow them with potent cellulolytic activity, enabling them to be used in CBP. To gain general insight into factors controlling protein display, we engineered the surface of the model Gram-positive bacterium *B. subtilis*. We constructed a *B. subtilis* protein display reporter system in which the *Clostridium thermocellum* Cel8A endoglucanase is fused to the LysM cell wall binding module. The effects of LysM positioning, extracellular proteases, and solution conditions on the copy-number and stability of the reporter protein were determined. We demonstrated that heterologous surface enzyme activity is rapidly lost, even when *B. subtilis* is genetically modified to eliminate all of its extracellular proteases (AprE, Epr, Bpr, Vpr, NprE, NprB, Mpr, WprA, HtrA and HtrB). This problem presumably occurs because the membrane's proton motive force (pmf) dissipates when nutrients are scarce, leading to autolysis and the concomitant release of cytoplasmic proteases that degrade the heterologous surface proteins. We overcame cellular autolysis using a two-step procedure in which the pmf is maintained by glucose or glycerol additives. This procedure enables the production of cellulase-coated *B. subtilis* cells that are stable for more than two days, as substantiated by whole-cell enzyme activity measurements and cell fractionation experiments in tandem with immunoblotting. We observed that cell morphology is affected by protein display in certain strains and that the secretory stress response is activated as a result of both Cel8A-LysM display and deficiency of the HtrA and HtrB proteases; we have made strides toward determining the ideal *B. subtilis* strain and growth conditions for CBP. The ability to produce stable enzyme-coated vegetative *B. subtilis* is a step toward their practical use in biotechnological applications, and lays the foundation for their further optimization.

This work was supported by the U.S. Department of Energy Office of Science, Office of Biological and Environmental Research program under Award Number DE-FC02-02ER63421. GL Huang was supported by a Ruth L. Kirschstein National Research Service Award GM007185.