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Engineering Thermophilic Microorganisms to Selectively Bind Strategic Metals in Low Temperature Geothermal Brines

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ABSTRACT

Geothermal brines can contain comparatively high concentrations of rare earth elements (REEs), high-value metals, and/or critical metals that offer the opportunity to add value to geothermal energy production. However, existing technologies are either insufficiently selective or too expensive to economically extract these elements. Here, we test whether engineered thermophilic cell surface proteins can unite the affinity, selectivity, and robustness needed to act as a low-cost, selective, and reusable metal adsorbent. Using purified proteins in small-scale batch binding experiments, we demonstrate that engineered thermostable protein variants can bind Zn^{2+} or Gd^{3+} more tightly and more selectively than their native counterparts. While this system awaits more extensive testing, the results presented here suggest that using engineered thermophilic microorganisms to display engineered metal-binding proteins is an extremely promising approach for strategic metal binding in the low-temperature geothermal arena.

1. INTRODUCTION

A variety of methods have been explored to recover high-value metals and rare earth elements (REEs) from geothermal fluids, including chemical sorption, biosorption, chemical precipitation and coagulation, electrochemical removal, and membrane separation (Bourcier, 2005). However, these technologies remain economically unviable. The major impediment to making these technologies feasible is their lack of binding selectivity (Bourcier, 2005; Lo, 2014); without specificity, the remainder of the extraction process becomes elaborate and costly.

Metal binding to bacteria has been well established experimentally (Gadd, 2009). On a dry weight basis, the metal binding capacities of bacteria compare favorably to the binding capacities of commercial ion exchangers (10^{-5} to 10^{-3} mol metal/g (dry wt) bacteria) (e.g., Vijayaraghavan, 2008 and references therein). Additionally, several mesophilic bacteria have been engineered to display metal-binding motifs on their cell surface. These engineered strains bind metals with increased capacity (Valls, 2000; Kuroda, 2003; Nishitani, 2010) and in a few cases, selectivity (Pazirandeh, 1995; Park, 2016). However, none of these bacterial sorbents yet have both the selectivity and the robustness to elevated temperature and salinity necessary for cost-effective strategic mineral binding under low-temperature geothermal conditions.

Our overall goal is to develop engineered thermophilic microorganisms as a new biosorbent technology that specifically binds two strategic metals, Zn^{2+} and Gd^{3+}, from geothermal fluids. The key innovation to this new technology is the high-density display of highly-selective binding sites for Zn^{2+} or Gd^{3+} on the surface of a robust thermophilic microorganism. Here we describe progress towards that goal. Specifically, we created and isolated engineered cell surface proteins, surface layers (S-layers), designed to selectively adsorb strategic metals. Using small batch scale reactions, we then demonstrated that engineered S-layers bind strategic metals more tightly and more selectively than their native counterparts.

2. RESULTS AND DISCUSSION

2.1 Creation of Thermostable Protein Nanosheets Containing Metal-Binding Domains

To create a robust biosorbent, we required a robust, thermophilic bacterium that can survive geothermal conditions, a means for high-density display of proteins on the cell exterior, and suitable metal binding domains (**Figure 1**). We chose *Geobacillus stearothermophilus* as our bacterial host because is a well-studied, salt-tolerant, thermophilic bacterium that can be genetically modified**.** Because it is native to *Geobacillus stearothermophilus* and it can accommodate significant insertions on its C-terminus (Moll, 2002), we chose the protein SbsB as our S-layer scaffold. To identify suitable Zn^{2+}- and Gd^{3+}- binding domains, we first analyzed the composition of low-temperature geothermal brines to determine the binding and selectivity targets. To bind 90% of the Zn^{2+} and Gd^{3+} present in geothermal brines with 90% selectivity, we determined the following requirements for metal-binding domains: i) K_d for Zn^{2+} and Gd^{3+} < 30 µM and 10 nM, respectively, ii) K_d for Zn^{2+}/Co^{2+} >10 or K_d for Gd^{3+}/Ca^{2+} >10^5, iii) minimal non-specific metal binding residues such as cysteine or histidine. A thorough literature search identified two Gd^{3+}-binding domains (Daughtry, 2012) and three Zn^{2+}-binding domains (Rich, 2012) that fit these criteria. Importantly, Jiao and co-workers have shown the Gd^{3+} binding domains can bind REEs when displayed on the surface of mesophilic bacteria (Park et al., 2016).

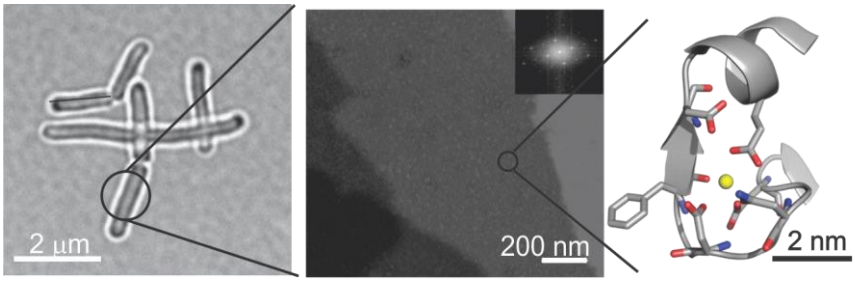

Figure 1: Overview of our approach. The thermophilic, salt-tolerant microorganism *G. stearothermophilus* (left) presents S-layer proteins at high density on its surface (middle), which in turn display selective metal-binding domains (right) to the extracellular solution.

While the overall goal of our work is to express engineered S-layers on the surface of *G. stearothermophilus*, as a proof-of-principle, we first expressed and purified these S-layers from the mesophile *Escherichia coli*. We generated two different DNA constructs to express S-layers displaying Gd^{3+} and a Zn^{2+} binding domains in *E. coli*. We then constructed stretches of DNA via chemical DNA synthesis and polymerase chain reaction, and used the Gibson assembly method to link these DNA segments into the pET28a vector for expression in *E. coli*. The final vectors were verified by DNA sequencing.

Using literature protocols (e.g. Moll et al., 2002), we were unable to purify significant amounts of SbsB variants from *E. coli*. As a result, we developed a new expression and purification methodology for SbsB in *E. coli*, based on a published purification for another S-layer (Norville et al., 2011). Our new approach expresses the SbsB variants at 18ºC and boasts several improvements over the previous approach. Most significantly, we can now purify the protein to >90% purity (**Figure 2A**) with yields of ~ 5 mg/L, which is ~5x-fold higher than our previous yield of ~1 mg/L. Additionally, the proteins can be purified under non-denaturing conditions, which greatly simplifies the purification. We were also able to assemble three of these S-layer proteins into S-layer nanosheets by addition of Ca^{2+} (**Figure 2B**). These engineered nanosheets are indistinguishable from native nanosheets (**Figure 2C**). Thus, this work generated adequate quantities of S-layer nanosheets to test their Zn^{2+}- and Gd^{3+}-binding capabilities.

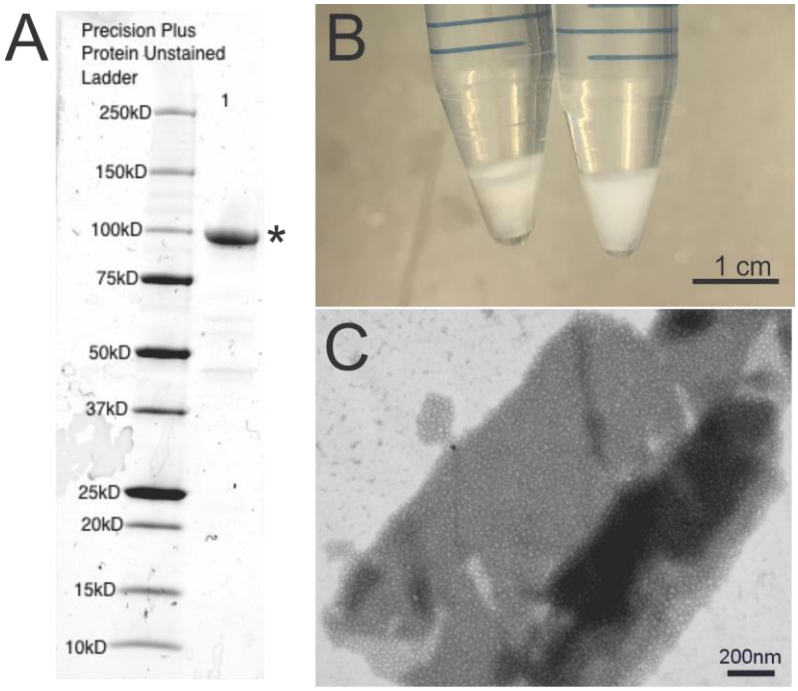

Figure 2: Purified S-layers displaying metal-binding domains make nanosheets. A) SDS-PAGE of purified SbsB nanosheets, showing the protein composition of nanosheets is >90% pure SbsB. SbsB is indicated by the *. B) Image of purified engineered SbsB nanosheets (white precipitate) that form in solution upon Ca^{2+} addition. C) Scanning transmission electron micrograph of SbsB nanosheets engineered to display a lanthanide-binding domain. The engineered protein forms sheets that are indistinguishable from the native sheets.

2.2 Engineered S-Layer Proteins Bind Zn^{2+} Metals with Higher Affinity and Selectivity than Native S-layer Proteins

Next, we compared Zn^{2+} binding to the engineered S-layer nanosheets versus the native SbsB S-layer nanosheets. To do so, we incubated varying concentrations of S-layer nanosheets with a fixed concentration of Zn^{2+} (1 µM) for 20 min at 40°C, pelleted the nanosheets and their bound metal by centrifugation, and measured the unbound Zn^{2+} present in the supernatant by inductively coupled plasma mass spectrometry (ICP-MS). At concentrations greater than 0.05 nM, both S-layer nanosheets bearing a Zn^{2+}-binding domain and the native S-layers bind significant amounts of Zn^{2+} (**Figure 3A**). However, by 0.2 µM, the engineered variant has bound all the Zn^{2+} initially present in the solution while ~25% of the Zn^{2+} was still unbound by the native S-layer. These data demonstrate that engineered S-layer proteins bind Zn^{2+} more tightly than native S-layers.

We also set out to test if this same Zn^{2+}-binding variant could bind Zn^{2+} with higher selectivity than the native S-layers in the presence of an analogue geothermal brine. We incubated both the native and engineered S-layers with 1 µM Zn^{2+} in the presence of ~3 M NaCl, ~288 mM KCl, and ~114 mM $CaCl_2$ for 20 minutes, removed the metals bound to the protein nanosheets by centrifugation, and measured the Zn^{2+} present in the supernatant by ICP-MS. Because of heavy metal contamination in the salts used to make the analogue brine, the actual concentration of Zn^{2+} was markedly higher (~1.5 µM) than what was added in the form of $ZnCl_2$ (1 µM). Above 0.05 µM S-layer, the Zn^{2+}-binding variant bound significantly more Zn^{2+} than the native SbsB S-layer (**Figure 3B**). For example, at ~0.2 µM S-layer, the engineered variant bound 0.59 ± 0.08 µM Zn^{2+} while the native protein sheets bound only 0.02 ± 0.06 µM Zn^{2+}. These observations indicate that the engineered S-layer variant can bind Zn^{2+} more selectively than the native S-layer in the presence of the high cation concentrations found in a geothermal brine.

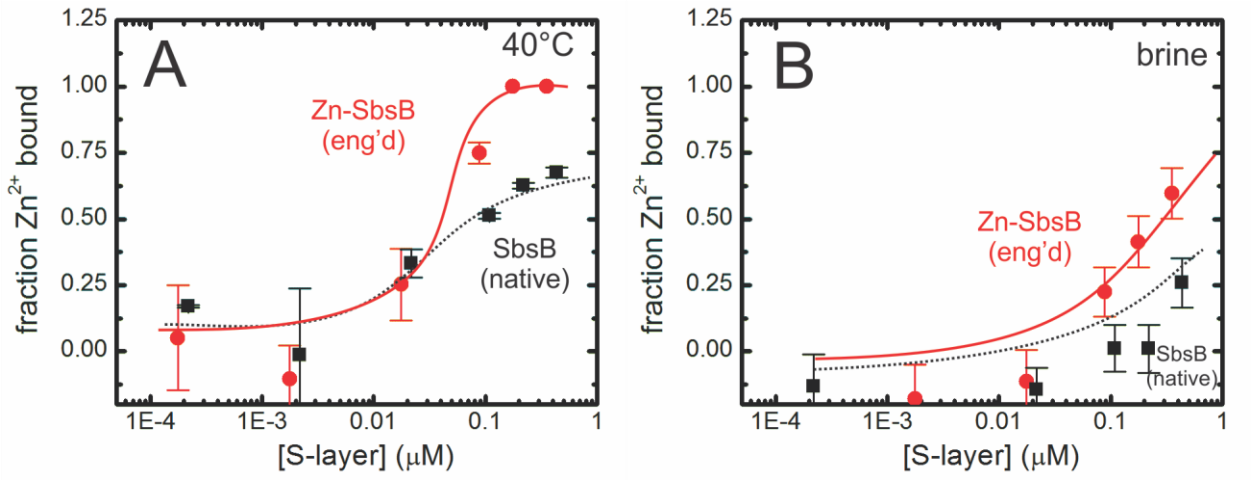

Figure 3: Engineered S-layer nanosheets bind Zn^{2+} with higher affinity and selectivity than native S-layers nanosheets. A, B)Fraction Zn^{2+} bound as a function of S-layer protein added for native SbsB S-layers (black squares) and SbsB S-layers displaying a Zn^{2+}-binding domain (red circles) at A) 40 °C and B) in the presence of an analogue geothermal brine. In the concentration range of 0.1- 1 µM, the engineered S-layers bind more Zn^{2+} than wild-type S-layers, indicating they have a higher binding affinity and selectivity for Zn^{2+}. Lines are provided to guide the eye.

2.3 Engineered S-Layer Proteins Bind Gd^{3+} Metals with Higher Affinity than Native S-layer Proteins

To test if our S-layer platform could also recover REEs, we also compared the Gd^{3+}-binding abilities of the native SbsB S-layer to S-layers engineered to display a Gd^{3+}-binding domain. As before, we incubated varying concentrations of S-layer nanosheets with a fixed concentration of Gd^{3+} (1440 nM) for 20 min at 40°C, pelleted the nanosheets and their bound metal by centrifugation, and measured the unbound Gd^{3+} present. At the lowest S-layer concentration tested, all the variants bound ~10% of the Gd^{3+} added (**Figure 4**). However, while additional native SbsB nanosheets only marginally increased the Gd^{3+}-binding, increasing the concentration dGd-SbsB S-layer nanosheets dramatically increased Gd^{3+} binding, binding 80% of the Gd^{3+} at 250 nM S-layer (**Figure 4**). These data demonstrate that engineered S-layer proteins can also bind the REE Gd^{3+} more tightly than native S-layers.

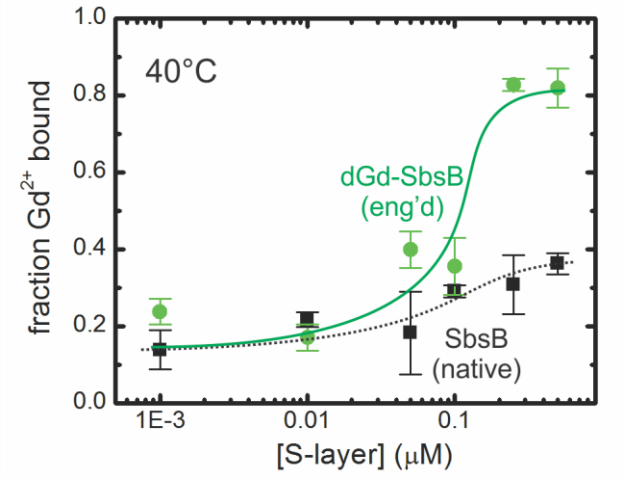

Figure 4: Engineered S-layers bind Gd^{3+} with higher affinity than native S-layers. Fraction Gd^{3+} bound as a function of S-layer protein added for wild-type SbsB S-layers (black squares) and SbsB S-layers displaying the dGd binding domain (green circles) at 40°C. Lines are provided to guide the eye.

2.4 Conclusions

In summary, we have created and demonstrated engineered S-layer proteins that bind Zn^{2+} more tightly and more selectively than their native counterparts. We have also demonstrated a S-layer variant that binds Gd^{3+} more tightly than the native SbsB S-layer. This is the first critical test of a new strategy to use engineered thermophilic microorganisms as selective, robust biosorbants for strategic metal recovery as part of low-temperature geothermal energy production.

In future work, we will test binding of our engineered proteins at higher temperatures (60°C, 80°C, 90°C) and Gd^{3+}-binding in the presence of metal cation concentrations akin to geothermal brines. The results of these experiments will be critical for identifying the most promising engineered S-layer candidates. With this information in hand, we will introduce the DNA coding for the engineered S-layer proteins into *G. stearothermophilus*, and then proceed to test these engineered microorganisms in flow-through reactors. Taken together, these experiments will push the development of engineered thermophilic microorganisms as low-cost, selective, and reusable approach for binding strategic metals in the geothermal arena and beyond.

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