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Bacillus subtilis Spore Display of Laccase for Evolution under Extreme Conditions of High Concentrations of Organic Solvent

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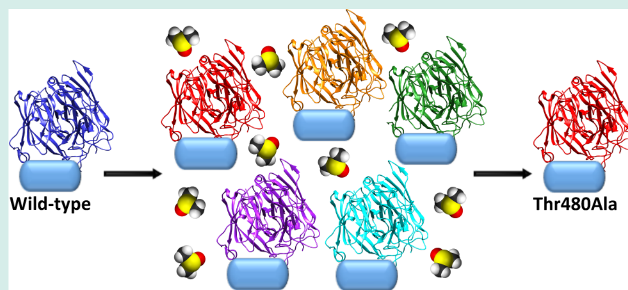
Supporting Information

ABSTRACT: Protein libraries were displayed on the spore coat of *Bacillus subtilis*, and this method was demonstrated as a tool for directed evolution under extreme conditions. *Escherichia coli*, yeast, and phage display suffer from protein folding, and viability issues. On the other hand, spores avoid folding concerns by the natural sporulation process, and they remain viable under harsh chemical and physical environments. The naturally occurring *B. subtilis* spore coat protein, CotA, was evolved for improved activity under conditions of high organic solvent concentrations. CotA is a laccase, which is a copper-containing oxidase enzyme. A CotA library was expressed on the spore coat, and ~3000 clones were screened at 60% dimethyl sulfoxide (DMSO). A Thr480Ala variant (Thr480Ala-CotA) was identified that was 2.38-fold more active than the wild-type CotA. In addition, Thr480Ala-CotA was more active with different concentrations of DMSO ranging from 0 to 70%. The mutant was also found to be more active compared with the wild-type CotA in different concentrations of methanol, ethanol, and acetonitrile.



KEYWORDS: *protein display, directed evolution, spore, laccase, organic solvent stability*

Enzymes from mesophilic organisms have the advantage of catalyzing specific chemical reactions under mild conditions in aqueous solutions; however, many chemical transformations require organic cosolvents for solubilization of hydrophobic substrates.[1] As a result, enzymes may not remain catalytically active beause of instability. Therefore, an efficient protein engineering strategy is necessary. Protein display strategies, which include Gram-negative bacteria, yeast, and phage have proven to be effective directed evolution tools for protein optimization.[2] However, these display methods may not be compatible to screen for proteins under harsh conditions, such as organic solvents. Organic solvents damage the cell membranes, which results in lysis and death;[3] hence, the cells cannot be cultured to retrieve the gene that codes for the improved protein. In short, the phenotype/genotype connection is severed.

Another concern is protein folding. For example, proteins displayed on the outer membrane of *Escherichia coli* are expressed in the reducing environment of the cytoplasm,[4] and then they travel across the inner and outer membranes into an oxidizing environment. As a consequence, correct disulfide formation is hindered. The issues associated with protein display on the surface of *E. coli* also exist for phage and yeast.

Protein display on the *Bacillus subtilis* spore coat may alleviate the viability and protein folding problems.[2d] Spores can withstand chemical and environmental extremes, and they remain viable.[5] Therefore, the phenotype/genotype connection remains intact. Next, proteins that are displayed on the spore coat do not travel across membranes, which is due to the natural sporulation process.[6] Furthermore, ATP-dependent chaperone proteins are also present during sporulation.[7] Spore display offers additional advantages. For example, immobilized proteins are generally more stable. In addition, immobilization requires that the protein is expressed, purified, and attached to an inert surface. The spore coat is inert; hence, the immobilization process is done in a single step during sporulation. Next, spores can be easily removed from a reaction by filtration. Furthermore, recombinant *B. subtilis* spores are easy to construct using genetics tools and genomic data. Finally, *B. subtilis* is naturally competent with transformation efficiencies that are on the order of 10^5−10^6/μg of plasmid DNA.[8]

Proteins displayed on the spore coat surface have been used for several biotechnological applications.[2d] For example, antigens were displayed and used for vaccine development.[9] Next, β-galactosidase (116 kDa/monomer) was displayed, and it was used for a transglycosylation reaction in biphasic systems.[10] In addition, streptavidin, which is a tetrameric protein (15 kDa/monomer), was displayed in its active form.[11] Finally, we were the first group to report that spores are

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effective as a tool for directed evolution of a naturally occurring spore coat protein.[12] The target protein was CotA, which is a laccase. In this study, a library of CotA genes was expressed on the spore coat, and it was evolved for improved substrate specificity.[12]

Laccases are copper-containing oxidoreductases that are found in plants, insects, fungi, and bacteria. They catalyze one-electron oxidation of a broad range of organic and inorganic substrates at the mononuclear T1 site with the concomitant four-electron reduction of dioxygen to water at the trinuclear T2/T3 site.[13] Laccases have applications in the textile industry, paper bleaching, chemical synthesis, biofuel cells, and bioremediation.[14] The physiological roles of laccase are also varied and include lignin degradation and formation, pigment formation, and detoxification.[14c]

In this report, we take advantage of the inert properties of the spore to demonstrate that this system can be used to screen under harsh conditions without compromising the viability. A CotA library was constructed and displayed on the *B. subtilis* spore coat. CotA was evolved for improved activity in high concentrations of organic solvent. Wild-type CotA was used to validate the organic activity screen. First, wild-type CotA and its natural promoter (wt-CotA) were integrated into the nonessential *amyE* gene in the *B. subtilis* genome via double crossover recombination. The *B. subtilis* strain had the endogenous *cotA* gene knocked out. Next, the *B. subtilis* cells were sporulated in 96-deep-well plates. CotA activity with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 60% dimethyl sulfoxide (DMSO) was recorded. The average of the coefficient of variance was 15.9% for three 96-well microtiter plates (data not shown).

Libraries of CotA were created by error-prone PCR, integrated into the genome, and screened. Positive clones identified in the screen had a V_{max}/OD_{580} 1.5-fold greater than wt-CotA. The most active variant was isolated from ∼3000 clones in one round of evolution. The sequence revealed a single base mutation, which resulted in a threonine (ACA)-to-alanine (GCA) amino acid substitution at position 480 (Thr480Ala-CotA, Figure 1).

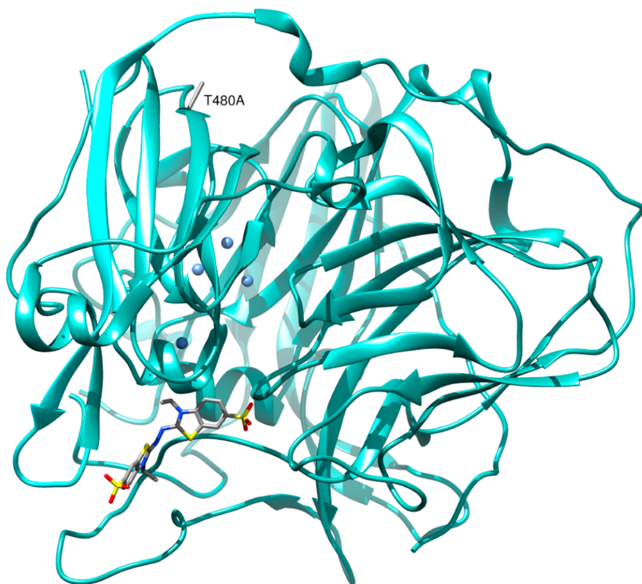

Figure 1. Ribbon diagram of Thr480Ala-CotA bound with ABTS.

The activity of Thr480Ala-CotA in DMSO was verified with purified large-scale expressions. All measurements were done in triplicate with three separate sporulations. The activity ($V_{max.T480A\text{-}CotA}/V_{max.wt\text{-}CotA}$) for Thr480Ala-CotA was 2.38-fold greater than the wt-CotA at the screening condition (DMSO 60%) (Figure 2). Furthermore, the V_{max} of Thr480Ala-CotA was improved at different concentrations of DMSO and buffer alone (Figure 2A). Next, the variant was measured at different concentrations of methanol, ethanol, and acetonitrile. In general, the V_{max} for Thr480Ala-CotA was 2−3-fold greater than the wt-CotA in buffer alone and all organic solvents (Figure 2). In short, the mutant was more active in both aprotic and protic polar solvents. Protic polar solvents appear qualitatively to have a greater effect on the activity. Similar results were found for laccase libraries secreted from yeast.[15] The substrate 4-hydroxy-3,5-dimethoxybenzaldehyde azine (SGZ) was also evaluated to demonstrate that the improvement in activity is non-substrate-dependent. The activity of the mutant SGZ in different concentrations of DMSO ranged from 3.4- to 9.5-fold greater than wt-CotA (Supporting Information (SI) Figure S1). This shows that the increased activity is not dependent on the substrate.

The V_{max} (μM/min/$OD_{580\ nm}$ of spores) and K_M (μM) for wt-CotA and Thr480Ala-CotA were measured at 60% DMSO, which was the screening condition (Figure 3). The $(V_{max}/K_M)_{Thr480Ala\text{-}CotA}/(V_{max}/K_M)_{wt\text{-}CotA}$ was 1.7-fold. The $(V_{max}/K_M)_{Thr480Ala\text{-}CotA}/(V_{max}/K_M)_{wt\text{-}CotA}$ in buffer alone was determined to be 1.9 (Figure 3).

The structure of wt-CotA revealed a H-bond with the T480 side chain with an adjacent antiparallel strand (SI Figure S2).[16] Furthermore, the alanine substitution has low β-sheet propensity.[17] This combined effect may result in destabilizing the local secondary structure, which could be transmitted to the loop above the mutation. This loop is at the C-terminus of a β-sheet, and the N-terminus loop forms part of the substrate-binding pocket. As a result, T480A-CotA may have an altered binding site, and the K_M would be increased (SI Figure S2). Next, the V_{max} for Thr480Ala-CotA is 2.9 and 3.6-fold greater than wt-CotA for 60% DMSO and buffer, respectively. The ABTS orientation may be altered for efficient electron transfer to the T1-site. The rate-limiting step is electron transfer from the substrate to the T1 site.[18] These conclusions are based upon the purified CotA structure.[16,19] Hence, it is difficult to interpret changes in the structure because Thr480Ala-CotA is embedded in the spore coat, and the structure may be different from the soluble enzyme.

The spore viability was determined in different concentration of DMSO. The percent viability was 100%, 115% ± 29, and 102% ± 27 for 0, 60, and 90% DMSO, respectively (SI Figure S3). Organic solvent does not affect viability.

This report demonstrates for the first time that spore display was a suitable engineering tool to evolve proteins under harsh conditions; namely, improved activity under high concentrations of organic solvent. Organic solvent does not compromise the spore viability. Hence, spore display may be a general tool to screen protein libraries with extreme properties.

■ EXPERIMENTAL PROCEDURES

Chemicals used were analytical grade or higher. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), citric acid, disodium phosphate, copper(II) chloride, dimethyl sulfoxide, ethanol, methanol, and acetonitrile were procured from Sigma-

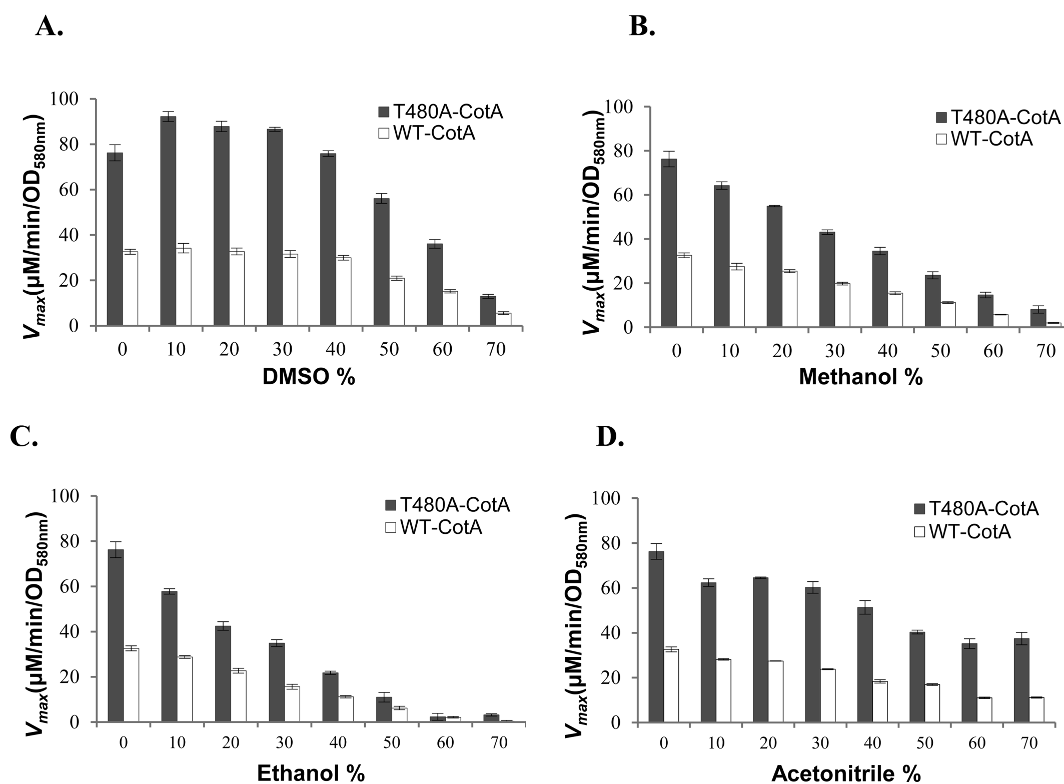

Figure 2. V_{max} (μM/min/$OD_{580\ nm}$) of wt-CotA (white bars) and Thr480Ala-CotA (shaded bars) in 0–70% organic solvent: (A) DMSO, (B) methanol, (C) ethanol, and (D) acetonitrile.

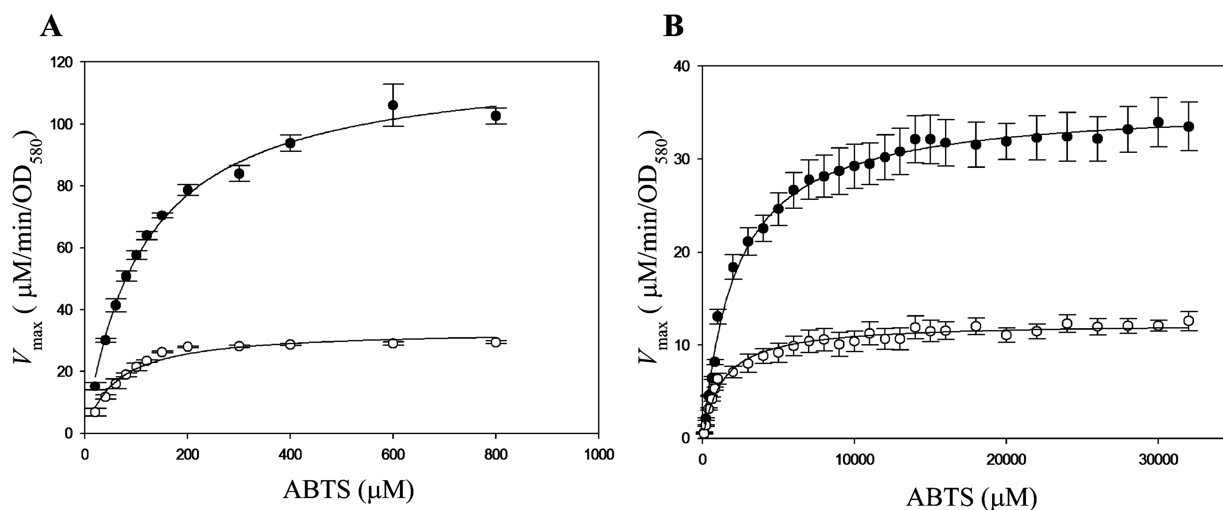

Figure 3. V_{max} (μM/min/$OD_{580\ nm}$ of spores) and K_M (μM) for wt-CotA (open circle) and Thr480Ala-CotA (closed circle) in 60% DMSO and citrate/phosphate buffer (pH 4). (A) 60% DMSO: wt-CotA (V_{max} = 12.4 ± 0.2; K_M = 1313.2 ± 104.3); T480A-CotA (V_{max} = 35.8 ± 0.3; K_M = 2186.0 ± 100.7). (B) Citrate/phosphate buffer: wt-CotA (V_{max} = 33.3 ± 1.1; K_M = 58.8 ± 7.2); T480A-CotA (V_{max} = 120.6 ± 2.5; K_M = 112.3 ± 6.9).

Aldrich (St. Louis, MO). Ampicillin, spectinomycin, and chloramphenicol were purchased from Fisher Scientific (Pittsburgh, PA). DNA purifications kits were purchased from Qiagen (Valencia, CA). Primers were procured from Fisher Scientific (Pittsburgh, PA). Enzymes were purchased from Invitrogen (Carlsbad, CA) and Agilent Technologies (La Jolla, CA).

Library Creation. The plasmid pDG1730CotA, which contains the CotA gene and its natural promoter, was previously created.[12] A Genemorf II Random Mutagenesis kit (Agilent Technologies, La Jolla, USA) was used to create the library. A typical 50 μL Genemorf II reaction contained of pDG1730CotA (3000 μg,), dNTP mix (40 nmol, 10 nm each), primers BacsubF (5′-GCGCGCAAGCTTGTGTC-CATGGCGTT-3′; 125 ng) and Psg1729R (5′-GCGCGGATCCTTATTTATGGGGATCA-3′; 125 ng), Mutazyme II DNA polymerase (2.5 U/μL), and 5 μL of buffer. The program consisted of 1 cycle at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 56.3 °C for 30 s, 72 °C for 4 min, 1 cycle at 72 °C for 10 min; 1 cycle at 4 °C until use.

The PCR product was digested with *Hind*III and *Bam*HI and was cloned into the same sites in the plasmid pDG1730. The resulting plasmids were transformed into *E. coli* DH5α cells and plated on LB plates containing ampicillin (100 μg/mL). The transformants were scraped from the plates, and a frozen cell stock was prepared. In addition, the plasmids were minipreped from scraped cells (Qiagen, Valencia, USA). The library was linearized with the restriction enzyme *Xho*I and integrated into the *amyE* locus into *B. subtilis* strain 1S101 (Bacillus Genetic Stock Center, Columbus, OH, USA) by double-crossover recombination. As a control, CotA and its natural promoter was also integrated into the *amyE* locus (wt-CotA). Genomic DNA was isolated and sequenced from 10 random clones to estimate the error rate (Rutgers-NJMS-Molecular Resource Facility, Newark, NJ, USA). There were 1.6 nucleotide substitutions per gene, which corresponded to 0.9 amino acid substitutes per gene (SI Figure S4).

Organic Stability Assay. Sporulation was induced by media exhaustion.[20] A scrape was resuspended from the frozen stock into LB media (5 mL) and incubated in 37 °C at 250 rpm for 30 min. The culture was diluted and plated on Schaeffer plates with spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL). Single colonies were picked into 96 deep-well plates containing 1 mL of 2SG media, which contained spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL). The plates were covered with breathable sealing film for culture plates and incubated at 37 °C for 48 h at 250 rpm. Next, the plates were centrifuged (3000*g*), and the media was discarded. The pellets were resuspended in Tris buffer (200 μL, 50 mM, pH 7.2) and lysozyme (50 μg/mL). The plates were incubated at 37 °C for 1 h, then they were centrifuged (3000*g*) for 20 min, and the supernatant was discarded. The spores were resuspended in 200 μL of sterile water, which contained 0.25 mM $CuCl_2$, and incubated at room temperature for 60 min. The plates were centrifuged (3000*g*) for 20 min, and the supernatants were discarded. The pellets were resuspended in aqueous DMSO (60%) and $CuCl_2$ (0.25 mM) and incubated at 25 °C for 4 h. A 200 μL reaction contained the spore solution (80 μL), citrate/phosphate buffer (100 μL, pH 4), and $CuCl_2$ (0.25 mM). The OD_{580} was ~0.2 to minimize turbidity. The reaction was initiated by the addition of ABTS (20 μL, 1.0 mM), and the plates were stirred before the measurement. After 10 min, the plates were centrifuged at 2400*g*, and the supernatant (80 μL) was transferred into a 96-well plate. A plate reader (SpectraMax M2, Sunnyvale, USA) was used to record the end point (420 nm; $\varepsilon = 36\,000$ M^{-1} cm^{-1}). A single column was reserved for wt-CotA as a control. Positive clones were selected that had 1.5 times the mean value of wt-CotA.

Organic Stability Rescreen. The positive clones were rescreened to verify improved activity. Wt-CotA and the positives were sporulated on a 50 mL scale culture. First, the strains were streaked from frozen stocks onto LB agar plates and incubated in 37 °C overnight. Next, a single colony was picked and streaked on a LB agar plate with spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL) and incubated overnight. Then a fresh single colony was picked and streaked on a Schaffer agar plate with spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL) and incubated overnight. The cells were scraped off the plate and used to inoculate 2SG media (50 mL) with spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL). The spores were produced by media exhaustion. The culture was centrifuged at 9000*g* for 30 min, and the supernatant was discarded. The spores were purified with lysozyme treatment with salt and detergent washes.[20]

The purified spores were incubated in 60% DMSO/H_2O with 0.25 mM $CuCl_2$ (incubation solution) for 4 h at 25 °C. Next, 450 μL of the incubation solution was added to 450 μL citrate/phosphate buffer (pH 4). The reaction was initiated by adding 100 μL of ABTS (1 mM) in citrate/phosphate buffer (pH 4). The values of V_{max}/OD_{580} of the mutants were compared with wt-CotA. Positive clones were identified that had a V_{max}/OD_{580} 1.5-fold greater than wt-CotA. The genomic DNA was isolated, and the primers BacsubF and Psg1729R were used to PCR amplify the CotA gene. The PCR product was sequenced (Rutgers-NJMS-Molecular Resource Facility, Newark, NJ, USA) to determine the mutation.

Kinetic Analysis. The kinetic parameters were determined with three different sporulations in triplicate. The reaction mixute was similar to the rescreening conditions. The final OD_{580} of spores was around 0.1−0.2. The initial rates were acquired from the linear portion of the reaction curve. The ABTS concentrations were varied from 0.1 to 30 mM. The kinetic parameters were determined by curve-fitting (SigmaPlot 12.0, Systat Software Inc., San Jose, CA, USA).

Cell Viability. Viability measurements were performed with three different sporulations in triplicate. The spores were diluted in sterilized H_2O, and the spore concentration was measured at OD_{580}. The spores were added to a final concentration of 0, 60, and 90% DMSO and incubated 24 h at 37 °C. Next, the same amount of spores was plated on LB agar plates with spectinomycin (100 μg/mL) and chloramphenicol (5 μg/mL). The plates were incubated at 37 °C for 16 h, and the colonies were counted.

■ ASSOCIATED CONTENT

Supporting Information

Spore viability in organic solvent. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Author Contributions

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Notes

The authors declare no competing financial interest.

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