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Engineering CotA Laccase for Acidic pH Stability Using *Bacillus subtilis* Spore Display

Silu Sheng[1], Han Jia[1], Sidney Topiol[2], and Edgardo T. Farinas[1]*

[1]*New Jersey Institute of Technology, Department of Chemistry and Environmental Science, University Heights, Newark, NJ 07102, USA*
[2]*Center for Healthcare Innovation, Stevens Institute of Technology, Hoboken, NJ 07030, USA*

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**Corresponding author*
Phone: +1-973-642-7363;
Fax: +1- 973-596-3371;
E-mail: edgardo.t.farinas@njit.edu

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Bacillus subtilis spores can be used for protein display to engineer protein properties. This method overcomes viability and protein-folding concerns associated with traditional protein display methods. Spores remain viable under extreme conditions and the genotype/phenotype connection remains intact. In addition, the natural sporulation process eliminates protein-folding concerns that are coupled to the target protein traveling through cell membranes. Furthermore, ATP-dependent chaperones are present to assist in protein folding. CotA was optimized as a whole-cell biocatalyst immobilized in an inert matrix of the spore. In general, proteins that are immobilized have advantages in biocatalysis. For example, the protein can be easily removed from the reaction and it is more stable. The aim is to improve the pH stability using spore display. The maximum activity of CotA is between pH 4 and 5 for the substrate ABTS (ABTS = diammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate). However, the activity dramatically decreases at pH 4. The activity is not significantly altered at pH 5. A library of approximately 3,000 clones was screened. A E498G variant was identified to have a half-life of inactivation ($t_{1/2}$) at pH 4 that was 24.8 times greater compared with wt-CotA. In a previous investigation, a CotA library was screened for organic solvent resistance and a T480A mutant was found. Consequently, T480A/E498G-CotA was constructed and the $t_{1/2}$ was 62.1 times greater than wt-CotA. Finally, E498G-CotA and T480A/E498G-CotA yielded 3.7- and 5.3-fold more product than did wt-CotA after recycling the biocatalyst seven times over 42 h.

Keywords: Protein display, directed evolution, spore, laccase, protein stability

Introduction

Laccases are in the multicopper oxidase family and they are found in plants, fungi, and microorganisms. The physiological functions are diverse, which include lignin synthesis [1], lignin degradation [2], pathogenesis [3], and morphogenesis [4]. Laccases catalyze one-electron oxidation of a wide variety of organic and inorganic substrates [1, 5]. These enzymes contain a mononuclear type-1 (T1) or blue copper center and a trinuclear copper cluster composed of a type-2 (T2) and type-3 (T3) centers. One-electron substrate oxidation occurs at the T1 site, which is coupled to four-electron reduction of dioxygen to water at the T2/T3 cluster. Laccases have applications in green chemistry, bioremediation, and biofuel cells [3]. These enzymes only require a substrate and dioxygen. As a result, expensive cofactors or additional proteins are not needed for function.

Immobilized enzymes on a solid support have several advantages. Protein stability towards organic solvents and temperature is increased. In addition, the economics is also improved because it is easy to separate the immobilized enzyme from the reaction solution, and the biocatalyst can be used again. Enzymes can be immobilized through entrapment on a porous solid matrix, encapsulation, adsorption based on ionic or van der Waals interactions, and covalent binding [6]. Typically, immobilized proteins are expressed, purified, and attached to an inert matrix. *Bacillus subtilis* spores can also be used for protein

immobilization [7]. Proteins displayed or immobilized on the spore coat do not require purification because of the natural sporulation process. Hence, expression and purification are not required. In addition, spores are inert and can withstand chemical and physical extremes. Finally, spores can also be used as a tool for protein engineering and optimization through directed evolution.

"Traditional" protein display technologies, such *Escherichia coli*, yeast, or phage, have proven to be efficient strategies, and these methods have accelerated protein discovery [8–12]. However, limitations exist that are due to cell viability and protein folding issues. As mentioned above, spores remain viable under harsh conditions. As a result, proteins can be evolved under harsh screening conditions and the gene coding for the improved protein remains intact. On the other hand, *E. coli* and yeast, which display the protein, may not remain viable under extreme conditions and the genotype/phenotype connection is lost. For example, cell lysis may occur under harsh conditions, and the link is disconnected between the improved protein and its gene. In addition, protein folding problems associated with the target protein crossing cell membranes are eliminated [13, 14], which is also due to the natural sporulation process [15]. Furthermore, ATP-dependent chaperones are present during the sporulation process to assist in protein folding. Finally, *B. subtilis* spore display has been used for a range of biotechnological applications, such as vaccine development [16–19] and whole-cell biocatalysts [20].

We recently showed that spores could be utilized for directed evolution. The laccase CotA, which is a *B. subtilis* spore coat protein, was the target [21]. A library of the *cotA* gene was expressed on the spore coat, and it was evolved for improved substrate specificity [22]. Spores are able to remain viable under extreme conditions, and CotA was evolved for improved activity in high concentrations of organic solvents [23]. *E. coli* or yeast protein display methods are not compatible with screening with high concentrations of organic solvent because the cell would lyse and the connection between the gene and the improved protein is lost.

The physiological role of CotA in *B. subtilis* has not been determined. Spores have a characteristic brown color. If *cotA* is knocked out of the genome, then the spores lose this color. The color protects the spores from UV light [24]. The crystal structures of diammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) (ABTS)-bound and substrate-free CotA have been solved, and they are nearly identical [25, 26].

Laccases have several biotechnological applications. They have been employed to degrade dyes and xenobiotics in wastewater treatment. In addition, the enzyme has been used as biosensors and biofuel cells. Furthermore, they are utilized in the paper industry for bleaching and delignification [6].

We demonstrated for the first time that spores could be used for directed evolution. The next goal was to optimize CotA as a whole-cell biocatalyst immobilized in an inert matrix of the spore. The aim was to improve pH stability using spore display. Acid-resistant enzymes have applications in degradation of polymeric or oligomeric carbon sources [27], biofuel production, textile industry, and fruit juice production [28]. The pH optimum for CotA is substrate specific and it has maximum activity between pH 4 and 5 for the substrate ABTS. Although the activity dramatically decreases at pH 4, it is not significantly altered at pH 5. CotA was engineered for improved pH stability at pH 4.

Materials and Methods

Library Creation

The plasmid pDG1730CotA was previously constructed [22], which contains the *cotA* gene under control of its natural promoter. Random mutagenesis libraries were created by error-prone PCR (ep-PCR) using GeneMorph II Random Mutagenesis (Agilent Technologies, USA). A typical 50 µl GeneMorph reaction contained pDG1730CotA (3,000 ng), primers BacsubF (125 ng; 5′-GCGCGCAAGCTTGTGTCCATGGCGTT-3′) and Psg1729 (125 ng; 5′GCGCGGATCCTTATTTATGGGGATCA-3′), dNTP mix (40 nmol, 10 nm each), and Mutazyme II DNA polymerase (2.5 U), and 5 µl buffer. The PCR program consisted of 30 cycles at 95°C for 30 sec, 56.3°C for 30 sec, and 72°C for 4 min.

Libraries were also made using Taq polymerase [29]. A typical 100 µl ep-PCR contained pDG1730CotA (100 ng), dGTP (20 nmol), dATP (20 nmol), dCTP (100 nmol), dTTP (100 nmol), primers BacsubF and Psg1729 (500 ng each), $MgCl_2$ (0.7 mM), Platinum Taq DNA Polymerase (5 U; Invitrogen, USA), and reaction buffer (10 µl). The PCR program consisted of 30 cycles at 95°C for 30 sec, 56.3°C for 30 sec, and 72°C for 4 min.

For each library, the PCR product was digested with HindIII and BamHI and was cloned into the same sites in the plasmid pDG1730. The resulting plasmids were transformed into *E. coli* DH5α and plated on LB plates containing ampicillin (100 µg/ml). The transformants were collected together and the plasmids were isolated. The library was integrated into the *amyE* locus into *B. subtilis* strain 1S101 (Ohio State University, *Bacillus* Genetic Stock Center, USA) by double cross-over recombination. This strain is a *cotA* knockout strain.

Spore Screening for Acid Stability

Expression of the library in 96-deep-well plates was performed as described previously [22]. The culture medium was discarded

from the CotA-expressed/displayed spores after centrifugation. The spores were resuspended in 200 µl of spore control solution (sterile water containing $CuCl_2$ (0.25 mM)) and incubated at room temperature for 30 min. Next, two assay plates were constructed for screening. One plate had 40 µl of spore control solution in each well as the control plate, and the other had 40 µl of spore control solution with 100 µl of citrate phosphate buffer (100 mM, pH 4) containing $CuCl_2$ (0.25 mM) as the assay plate. Both plates were at room temperature for 2 h. After incubation, 100 µl of citrate phosphate buffer (100 mM, pH 4) containing $CuCl_2$ (0.25 mM) was added into each well of the control plate. ABTS (1.0 mM, pH 4) was added into each well for both plates to initiate the reaction, and allowed to react for 20 min. The plates were centrifuged and the supernatants were transferred to another microtiter plate. The endpoint was measured at 420 nm (ε = 36,000 M^{-1} cm^{-1}). Next, the ratio of $Abs_{pH\ 4\ at\ 420\ nm}$: $Abs_{control\ at\ 420\ nm}$ was determined. Positive clones were selected that showed at least 1.5 the mean value of the wild-type ratio of $Abs_{pH\ 4\ at\ 420\ nm}$: $Abs_{control\ at\ 420\ nm}$. A rescreen was carried out to eliminate false positives. The assay was similar to that described above, but multiple colonies from a single clone were picked into a column of a 96-deep-well plate. A wild-type control column was also included.

Acid Inactivation

Large-scale sporulation was initiated by medium exhaustion, and the spores were purified using procedures described previously [30]. The half-life of acid inactivation ($t_{1/2}$) of spores was determined. Purified spores were incubated in aqueous $CuCl_2$ (0.25 mM) for 1 h at 37°C. Then, the spores were centrifuged and resuspended in citrate-phosphate buffer (100 mM, pH 4, 0.25 mM $CuCl_2$) and incubated at 37°C. The resulting solution had an OD_{580} between 0.1 and 0.3. A 200 µl reaction was initiated by adding ABTS (10 mM) into the spore solution. The measurements were performed every 3–10 min at 420 nm until the activity decreased to 20% of the initial activity. The data were recorded in triplicates.

Kinetic Measurements of CotA and Its Variants

All measurements were done in triplicates with at least three different batches of purified spores. The kinetic parameters of spores were determined at 37°C by using ABTS (1–8,000 µM) in citrate-phosphate buffer (100 mM, pH 4). The spores were first suspended in aqueous $CuCl_2$ (0.25 mM) solution for 60 min. Then, the reactions were initiated by adding 50 µl of spore solution into wells of each concentration of ABTS. The final OD_{580} of spores was between 0.2 and 0.3. The initial rates were acquired from the linear portion of the reaction curve. Kinetic parameters were obtained by curve fitting (SigmaPlot 12.0, Systat Software Inc., USA).

Product Yield of CotA and Its Variants

CotA recycling was investigated and the total product yield was determined over a 42-h period. The spores were resuspended in aqueous $CuCl_2$ (0.25 mM) solution for 60 min at 37°C. Next, the spores were centrifuged and the supernatant was discarded. The spores were resuspended in citrate-phosphate buffer (100 mM, pH 4) at 37°C with a final OD_{580} of 0.15. The spores were incubated for 2 h and then centrifuged, and the supernatant was discarded. The reaction was initiated by resuspending the spores with 1 ml of ABTS (20 mM) in citrate-phosphate buffer (100 mM, pH 4) at 37°C. The absorbance was taken at 420 nm after 15 min to determine the product yield. This cycle was repeated seven times over 42 h.

Results and Discussion

Most laccases have an optimum pH range from 2.0 to 4.0. However, the stability is compromised in this pH range [31]. Wt-CotA residual activity was determined after 2 h incubation in the pH range between 2 and 9. Wt-CotA is most active between pH 4 and 5 for the substrate ABTS. The activity at pH 5 was not significantly altered. However, the enzyme had a large loss of activity at pH 4. Hence, the goal was to improve $t_{1/2}$ at pH 4 for increased product yield (Fig. 1).

Error-prone PCR was used to construct a *cotA* library. The library was integrated by double cross-over recombination into the genome of a *B. subtilis* strain, which has the endogenous *cotA* gene knocked out, into the non-essential *amyE* locus. It should be noted that germination and sporulation are not effected in the *cotA* knockout strain. The library was under the control of the natural *cotA* promoter. Wt-CotA was also integrated into the *amyE* locus

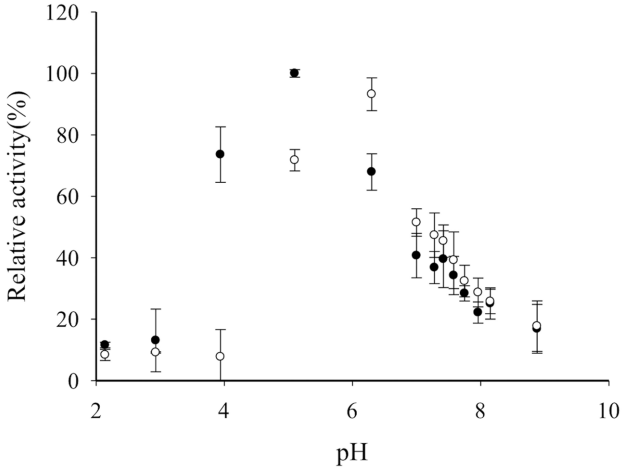

Fig. 1. Relative activity versus pH of wild-type CotA.
Spores were incubated in aqueous $CuCl_2$ (0.25 mM) at 25°C for 60 min. Next, the spore suspension (OD_{580} = 0.3) was added to citrate-phosphate buffer (100 mM, pH 2.0–9.0, 0.25 mM $CuCl_2$). Finally, the reaction was initiated with ABTS (1.0 mM). Closed circle: t = 0 min; Open circle: t = 120 min.

Table 1. Kinetic parameters.

Mutation	V_{max} [a]	K_M (μM)	V_{max}/K_M [b]	$t_{1/2}$ [c]
E29V	29.4 ± 1.4	67.2 ± 9.2	0.44	60.4 ± 3.7
L343S	14.7 ± 0.5	58.2 ± 6.4	0.25	33.4 ± 2.3
E498G	41.8 ± 3.7	857.6 ± 125.9	0.05	1,264.1 ± 78.0
E29V/L343S/E498G	18.5 ± 0.5	358.6. ± 29.4	0.05	302.6 ± 23.8
T480A	120.6 ± 2.5	112.3 ± 6.9	1.07	56.1 ± 1.1
T480A/E498G	45.5 ± 3.0	585.1 ± 76.4	0.08	3,165.6 ± 431.1
Wt-CotA	33.3 ± 1.1	58.8 ± 7.2	0.57	50.9 ± 1.8

Kinetic parameters and half-life of inactivation at pH 4 for WT-CotA and CotA variants. [a]μM/min/$OD_{580\ nm}$. [b](μM/min/$OD_{580\ nm}$)μM^{-1}. [c]Half-life (minutes) at pH 4.

and it was used as a control.

Approximately 3,000 mutants were screened, and variants with a ratio of $Abs_{pH\ 4\ at\ 420\ nm}$: $Abs_{control\ at\ 420\ nm}$ 1.5 times that of the wild type were isolated in an endpoint assay. A mutant, Y19, was identified with a $t_{1/2}$ 5.9 times greater than wt-CotA (Table 1 and Fig. S1). The sequence of Y19 was determined from the isolated genomic DNA, and three amino acid substitutions (E29V (GAA to GTA), L343S (TTG to TCG), and E498G (GAA to GGA)) were found. Val^{29} and Ser^{343} are on the surface, whereas Gly^{498} is close to the T1 copper center (Fig. 2).

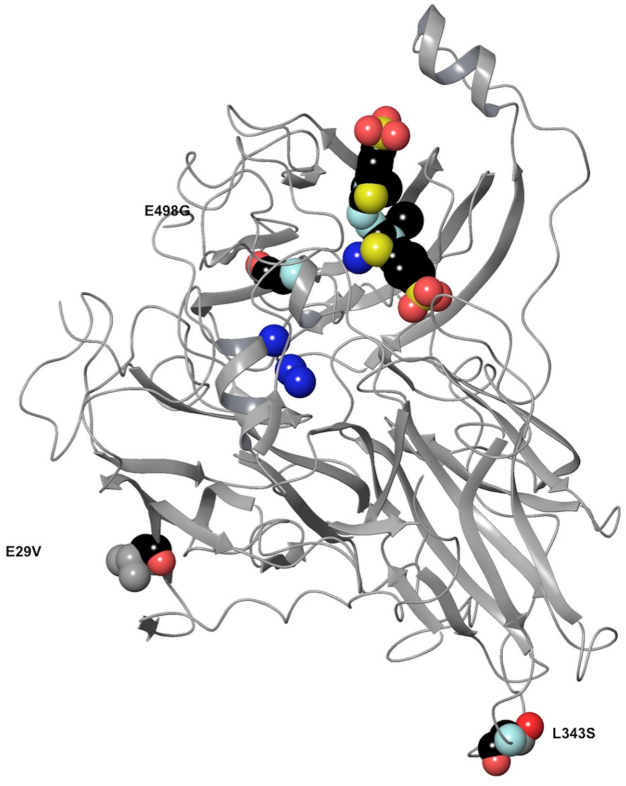

Fig. 2. Molecular model of ABTS-Y19.
Molecular model of the ABTS-Y19 complex was generated using Maestro software from Schrodinger (Schrodinger, Maestro ver. 9.2, Portland, 2011). Copper, ABTS, and the amino acids are rendered as CPK models.

The CotA variants that carry only one mutation were also constructed to determine the contribution the amino acid substitution had on pH stability and catalytic efficiency (Table 1 and Figs. S2A–S2E). The $t_{1/2}$ of E498G variant was 1,213.2 min longer than wt-CotA, which was 24.8-times greater. The E498G substitution is the only amino acid required for the increased $t_{1/2}$ (vide infra). The V_{max}/K_M for E498G was 8.8% compared with wt-CotA (S2E). A lowering of V_{max}/K_M is not unusual because this property was not included in the pH stability screen (Table 1 and Fig. S2B). The change in V_{max}/K_M for the variant was mainly due to a 14.6-fold increase in K_M, which was partially compensated with a 1.3-fold increase in V_{max}.

In a previous investigation, Glu^{498} was mutated to aspartate, threonine, and leucine, and it was suggested that these amino acid substitutions near His^{497} resulted in minor changes in substrate binding and electron transfer to the T1 site [32, 33]. Substrate oxidation at the T1 site has been proposed to be the rate-limiting step in the reaction [34, 35]. The mutagenesis investigations also proposed that Glu^{498} was necessary for proton-assisted reductive cleavage of the O-O bond at the trinuclear copper center. The E498G variant does not have a side chain that can facilitate the cleavage. However, the amino acid substitution is also expected to alter substrate binding and electron transfer. Glycine is known to have greater conformational flexibility because the side chain is hydrogen. The mutation may have altered the position ABTS for efficient electron transfer and the V_{max} was increased. On the other hand, this was at a cost to substrate binding, which resulted in a higher K_M. In addition, the glycine substitution is adjacent to His^{497}, which coordinates to Cu^{2+} in the T1 site. Hence, the Cu^{2+}

coordination geometry may also be affected, such that metal binding has increased, and the protonation of the histidine ligands is hindered. Protonation of the histidine would result in release of Cu^{2+} from the T1 site and loss of enzymatic activity. The enzyme is imbedded in the spore coat, and the structure of the mutant has not been solved. Hence, it is difficult to extract conclusions based on the structure of ABTS bound to CotA [25]. The structure of the soluble CotA may not be identical to the variant that is embedded and restrained in the spore coat.

In a previous study, wt-CotA was evolved for increased activity in high concentrations of organic solvents [23]. An assay was performed at 60% dimethyl sulfoxide, and a variant was identified to be 2.4-fold more active after screening 3,000 clones. The clone was found to have a threonine to alanine amino substitution at position 480 (T480A-CotA). T480A-CotA was also more active with different concentrations of DMSO ranging from 0 to 70%. The mutant was also found to be more active compared with the wt-CotA in different concentrations of methanol, ethanol, and acetonitrile [23]. The amino acid substitution in T480A-CotA was introduced into E498G-CotA to create T480A/E498G-CotA. The $t_{1/2}$ for T480A/E498G-CotA at pH 4 was found to be 3,165.6 ± 431.1 min, which was 62.1-fold greater than wt-CotA (Table 1 and Fig. S5). When compared with wt-CotA, the V_{max} for T480A/E498G-CotA was slightly increased by 1.4-fold; whereas the K_M increased 10-fold (Fig. S2F). The V_{max} in the double mutant was due to the T480A amino acid substitution, and the E498G mutation was mainly responsible for the increase in K_M (Table 1). The crystal structure has not been determined for T480A-CotA, E498G-CotA, and T480A/E498G-CotA. As a result, it is difficult to conclude the effect that the T480A mutation has towards improved pH stability of T480A/E498G-CotA. However, they were not more active in organic solvents (data not shown). In short, T480A/E498G-CotA and E498G-CotA have comparable kinetic properties, but T480A/E498G-CotA has a greater $t_{1/2}$ compared with wt-CotA.

An important feature of a biocatalyst is that it can be reused for several cycles. E498G and T480A/E498G have a larger K_M compared with wt-CotA, which results in a lower V_{max}/K_M (Table 1). However, the variants had a higher product yield compared with wt-CotA, which was due to the longer $t_{1/2}$ (Table 1). The spores were recycled seven times over 42 h (Table 2 and Fig. 3). Wt-CotA, E498G-CotA, and T480A/E498G-CotA had a total yield of 24.8, 92.9, and 131.3 mmol, respectively. E498G-CotA and T480A/E498G-CotA yielded 3.7- and 5.3-fold more product then did wt-CotA. This also demonstrates that the biocatalysts can be recycled. T480A/E498G was the most stable biocatalyst and retained 42% of its activity after the 42 h period.

The surface amino acid substitutions, E29V and L343S variants, did not significantly contribute to the pH stability of Y19 (Table 1). The E29V mutant had a slight decrease in V_{max}/K_M (0.77-fold) and a modest increase in $t_{1/2}$ (1.2-fold) compared with wt-CotA (Figs. S2C and S2E). Next, the L343S mutant had a decreased V_{max}/K_M and a decrease in $t_{1/2}$ compared with wt-CotA of 0.44- and 0.66-fold, respectively (Fig. S2D). It appears that the E29V and L343V amino acid substitutions have a deleterious effect. The spore coat proteins were extracted and the amount of wt-CotA and the mutants were comparable, as assessed by SDS-PAGE gels (Fig. S3). The band intensity in lanes 4–8

Table 2. Product yields.

Time (h)	Wt-CotA	E498G	T480A/E498G
0	11.1 ± 0.1	20.2 ± 0.3	19.8 ± 0.2
2	3.3 ± 0.5	19.6 ± 0.2	19.4 ± 0.2
4	2.8 ± 0.6	15.4 ± 0.9	18.9 ± 0.9
8	1.9 ± 0.7	12.4 ± 1.6	18.0 ± 1.6
18	1.6 ± 0.2	9.1 ± 0.2	15.9 ± 0.2
25	1.8 ± 0.4	7.6 ± 1.2	14.4 ± 1.2
30	1.3 ± 0.2	5.3 ± 0.9	13.4 ± 0.9
42	1.0 ± 0.3	3.3 ± 0.3	11.5 ± 0.3
Total	24.8	92.9	131.3

Product yield (mmole) of $ABTS^+$ over 42 h with wt-CotA, E498G, and T480A/E498G.

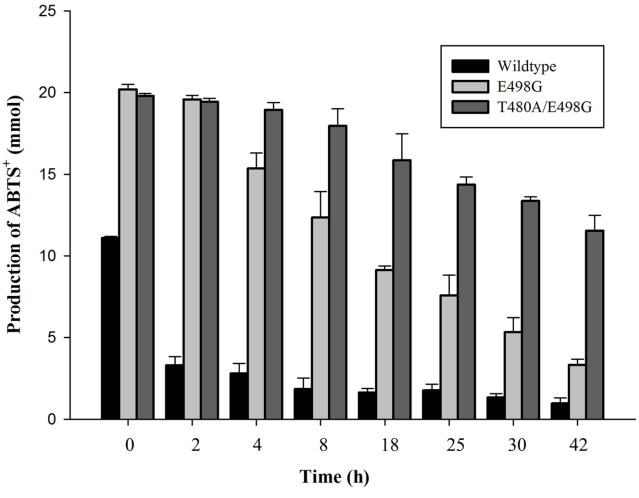

Fig. 3. $ABTS^+$ product yields for wt-CotA (black bars), E498G-CotA (light shaded bars), and T480A/E498G-CotA (dark shaded bars) determined for seven cycles over a 42 h period.

were quantified using ImageJ (https://imagej.nih.gov/ij/). The values for lanes 4–8 were 7,045, 6,798, 7,716, 7,171, and 7,012, respectively. The average of the intensities is 7,151 ± 344. The amounts of protein present in the spore coat were similar between the samples.

Lastly, the cell viabilities of wt-CotA and mutant were not affected with incubation at pH 4 (Fig. S4).

In conclusion, a library of CotA variants was expressed on the surface of *B. subtilis* spores, and an acid-stable mutant was identified. Next, a T480A/E498G-CotA variant was constructed, which combined the amino acid substitution from an organic solvent-tolerant and acid-stable variants. This mutant had a significantly increased $t_{1/2}$. The spores were able to be recycled, and the total amount of product formed after approximately 2 days was greater for the variants than for wt-CotA.

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