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LccA, an Archaeal Laccase Secreted as a Highly Stable Glycoprotein into the Extracellular Medium by *Haloferax volcanii*▽†

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Laccases couple the oxidation of phenolic compounds to the reduction of molecular oxygen and thus span a wide variety of applications. While laccases of eukaryotes and bacteria are well characterized, these enzymes have not been described in archaea. Here, we report the purification and characterization of a laccase (LccA) from the halophilic archaeon *Haloferax volcanii*. LccA was secreted at high levels into the culture supernatant of a recombinant *H. volcanii* strain, with peak activity (170 ± 10 mU · ml^{-1}) at stationary phase (72 to 80 h). LccA was purified 13-fold to an overall yield of 72% and a specific activity of 29.4 U · mg^{-1} with an absorbance spectrum typical of blue multicopper oxidases. The mature LccA was processed to expose an N-terminal Ala after the removal of 31 amino acid residues and was glycosylated to 6.9% carbohydrate content. Purified LccA oxidized a variety of organic substrates, including bilirubin, syringaldazine (SGZ), 2,2,-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and dimethoxyphenol (DMP), with DMP oxidation requiring the addition of $CuSO_4$. Optimal oxidation of ABTS and SGZ was at 45°C and pH 6 and pH 8.4, respectively. The apparent K_m values for SGZ, bilirubin, and ABTS were 35, 236, and 670 μM, with corresponding k_{cat} values of 22, 29, and 10 s^{-1}, respectively. The purified LccA was tolerant of high salt, mixed organosolvents, and high temperatures, with a half-life of inactivation at 50°C of 31.5 h.

Multicopper oxidases (MCOs) are a family of enzymes that include laccases (*p*-diphenol: dioxygen oxidoreductases; EC 1.10.3.2), ascorbate oxidases (EC 1.10.3.3), ferroxidases (EC 1.16.3.1), bilirubin oxidases (EC 1.3.3.5), and other enzyme subfamilies (27, 65). MCOs couple the oxidation of organic and/or inorganic substrates to the four-electron reduction of molecular oxygen to water. These enzymes often have four Cu atoms classified into type 1 (T1), type 2 (T2), and type 3 (T3) centers, in which a mononuclear T1 center on the surface of the enzyme provides long-range intramolecular one-electron transfer from electron-donating substrates to an internal trinuclear T2-T3 center formed by a T2 Cu coordinated with a T3 Cu pair. The T2-T3 cluster subsequently reduces dioxygen to water.

Enzymes of the laccase subfamily oxidize a broad range of compounds, including phenols, polyphenols, aromatic amines, and nonphenolic substrates, by one-electron transfer to molecular oxygen and thus have a wide variety of applications from biofuels to human health. The best-known application is the use of a laccase from the lacquer tree *Rhus vernicifera* in paint and adhesives for more than 6,000 years in East Asia (29). Laccases have also been used in the delignification of pulp, bleaching of textiles and carcinogenic dyes, detoxification of water and soils, removal of phenolics from wines, improving adhesive properties of lignocellulosic products, determination of bilirubin levels in serum, and transformation of antibiotics and steroids (60). In addition, laccases have demonstrated potential for use in biosensors, bioreactors, and biofuel cells (61).

Laccases, once thought to be restricted to eukaryotes (fungi, plants, and insects), appear to be widespread in bacteria (10). Laccase-like MCOs are now known to have numerous biological roles in bacteria, including sporulation, electron transport, pigmentation, metal (copper, iron, and manganese) homeostasis, oxidation of phenolate-siderophores, phenoxazinone synthesis, cell division, and morphogenesis (9). In contrast to the widespread occurrence of laccases in bacteria and eukaryotes, only a few MCOs have been identified in archaea, and this is based only on genome sequences (e.g., the hyperthermophilic crenarchaeote *Pyrobaculum aerophilum* and the halophilic euryarchaeotes *Haloferax volcanii* and *Halorubrum lacusprofundi*). Most archaea with sequenced genomes, however, are anaerobes. Since MCOs reduce molecular oxygen to water, this likely accounts for the limited number of MCOs among archaea.

Many archaea thrive under harsh environmental conditions, including high temperature, extreme pH, and/or low water activity. Thus, they have many biochemical and physiological properties that are ideal for industrial applications. Here, we report the identification of a highly thermostable and salt/solvent-tolerant laccase (LccA) from the halophilic archaeon *H. volcanii* that catalyzed the oxidation of a wide variety of phenolic compounds. LccA was readily secreted and purified from the culture broth as a blue multicopper oxidase that was glycosylated and processed by the removal of 31 amino acid residues from its N terminus.

MATERIALS AND METHODS

Materials. Biochemicals were from Sigma-Aldrich (St. Louis, MO). Other organic and inorganic analytical-grade chemicals were from Fisher Scientific

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TABLE 1. Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Description	Source or reference
Strains		
E. coli		
DH5α	λ$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Life Technologies
GM2163	F$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL 136 dam13*::Tn*9 xylA5 mtl-1 thi-1 mcrB1 hsdR2*	New England Biolabs
Rosetta (DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^r)	Novagen
H. volcanii		
DS70	Wild-type isolate DS2 cured of plasmid pHV2	68
H26	DS70 *pyrE*	1
SB01	H26-pJAM822	This study
US02	H26-pJAM824	This study
Plasmids		
pET24b	Km^r; *E. coli* expression vector	Novagen
pBAP5010	Ap^r; Nv^r; 11-kb *H. volcanii-E. coli* shuttle vector derived from pBAP5009; includes *Halobacterium cutirubrum* rRNA P2 promoter (P2$_{rrn}$); T7 transcription terminator and pHV2 *ori* for expression of cloned genes in *H. volcanii*	33
pJAM202	Ap^r Nv^r; pBAP5010 containing P2$_{rrn}$-*psmB-H6*	34
pJAM202c	Ap^r Nv^r; pJAM202 with P2$_{rrn}$-*psmB-H6* deleted	70
pJAM821	Km^r; pET24b with *lccA-strepII* for expression in *E. coli* Rosetta (DE3)	This study
pJAM823	Km^r; pET24b with *lccA* for expression in *E. coli* Rosetta (DE3)	This study
pJAM822	Ap^r Nv^r; pJAM202 with P2$_{rrn}$-*lccA-strepII* for expression in *H. volcanii*	This study
pJAM824	Ap^r Nv^r; pJAM202 with P2$_{rrn}$-*lccA* for expression in *H. volcanii*	This study
Primers		
HvoB0205 (*lccA*) up	5′-tgggcgt*CATATG*acagactggtctaggcggcgg-3′[a]	This study
HvoB0205 (*lccA-strepII*) down	5′-aa*AAGCTT*cacttctcgaactgcgggtgcgacca*AGTACT*ggcgacttcgtcgccgcttc-3′[b]	This study
HvoB0205 (*lccA*) down	5′-aa*AAGCTT*tcaggccacttcgtcgccgcttc-3′[c]	This study

[a] The NdeI site is in italics.
[b] The HindIII and ScaI sites are in italics.
[c] The HindIII site is in italics.

(Atlanta, GA). Restriction endonucleases, T4 DNA ligase, and Vent DNA polymerase were from New England Biolabs (Ipswich, MA). AccuPrime GC-rich DNA polymerase was from Invitrogen (Carlsbad, CA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Agarose for routine analysis of DNA was from Bio-Rad Laboratories (Hercules, CA). SeaKem GTG agarose, used for the separation and isolation of DNA fragments prior to ligation, was from FMC Bioproducts (Rockland, ME).

Strains, media, and plasmids. The strains, primers, and plasmids used are summarized in Table 1. *Escherichia coli* DH5α was used for routine recombinant DNA experiments, and *E. coli* GM2163 was used to generate plasmid DNA for transformation of *H. volcanii* (16). *E. coli* strains were grown at 37°C in Luria-Bertani medium. *H. volcanii* strains were grown at 42°C in complex medium (ATCC 974 and yeast-peptone-Casamino Acids) and lactate-minimal medium (LMM), as previously described (1, 16, 34). Ampicillin (0.1 mg · ml^{-1}), novobiocin (0.1 μg · ml^{-1}), and $CuSO_4$ (50 to 500 μM) were included as needed. Cultures were grown in liquid (150 to 200 rpm) and solid (1.5% [wt/vol] agar plates) media.

Genome analysis. NCBI Local BlastP with BioEdit sequence editor software v7.0.4.1 (25) was used to compare the theoretical *H. volcanii* DS2 proteome (http://archaea.ucsc.edu/, April 2007 version) to fungal and bacterial proteins with known laccase activity. The protein sequences included phenol oxidase A of the lignocellulolytic ascomycete *Stachybotrys chartarum* (42), spore coat protein A (CotA) from *Bacillus subtilis* (43), and polyphenoloxidase (PPO) from *Streptomyces lavendulae* (66). Phylogenetic and molecular evolutionary analyses of the primary protein sequences were conducted using MEGA v3.1 (39). Pairwise and multiple-sequence alignments were performed using Clustal W (67). Nonconserved regions in the N and C termini were excluded from the protein sequence alignment. Evolutionary distances were estimated from the protein sequences using the proportion (p-) distance substitution model. Consensus tree inference was by neighbor joining with a bootstrap phylogeny test (1,000 replicates; seed number for bootstrap, 64,238) and pairwise gap deletion. TatP v1.0 (4), TATFIND v1.4 (57), and EnsembleGly (8) servers were used for prediction of twin arginine translocation (TAT) and glycosylation motifs in the LccA protein.

DNA isolation, analysis, and strain construction. Genomic DNA was prepared from *H. volcanii* DS70 (a strain of DS2 cured of plasmid pHV2) (68) for PCR by transfer of isolated colonies to 30 μl deionized H_2O using toothpicks. The cells were boiled (5 min), and the resulting lysate was chilled on ice (10 min) and centrifuged (14,000 × *g*; 10 min at 4°C). The supernatant (5 to 10 μl) was used as a template for PCR with primer pairs specific for the *H. volcanii* laccase gene (*lccA*; HvoB0205), as listed in Table 1. A mixture of AccuPrime GC-rich and Vent DNA polymerase at a 9:1 ratio was used for PCR, with buffer and nucleotide concentrations according to the recommendations of Invitrogen. PCR was performed with an iCycler (Bio-Rad Laboratories), and the sizes of the products were analyzed by electrophoresis using 0.8% (wt/vol) agarose gels in TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5) with Hi-Lo DNA molecular weight markers as standards (Minnesota Molecular, Minneapolis, MN). The gels were photographed after being stained with ethidium bromide at 0.5 μg · ml^{-1} with a Mini visionary imaging system (Fotodyne, Hartland, WI). PCR-generated DNA fragments of appropriate size for the *lccA* gene (1.8 kb) were isolated from 0.8% (wt/vol) SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) gels in TAE buffer using the QIAquick gel extraction kit (Qiagen) and ligated into the NdeI-to-HindIII sites of pET24b to generate plasmids pJAM821 and pJAM823 for expression of *lccA* in recombinant *E. coli* Rosetta (DE3). In addition, the *lccA* gene was excised from these plasmids and ligated into the NdeI-to-BlpI sites of the *H. volcanii* shuttle vector (pJAM202) to position the *lccA* gene downstream of a constitutive rRNA promoter, P2. These expression plasmids (pJAM822 and pJAM824) were transformed into *H. volcanii* H26 to generate strains SB01 and US02 for high-level synthesis of LccA with and without a C-terminal StrepII tag (-WSHPQFEK), respectively. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The fidelity of cloned DNA sequences and the 100% identity of *lccA* genes from DS2

and DS70 strains were confirmed by Sanger DNA Sequencing (ICBR Genomics Facility, University of Florida).

Enzyme purification. *H. volcanii* SB01 and US02 cells were grown in media supplemented with 100 μM $CuSO_4$ (LMM or YPC; 25 ml per 125-ml Erlenmeyer flask) to stationary phase. Cells were harvested by centrifugation (15,000 × *g*; 20 min at 4°C), and the culture broth was filtered with Whatman 3MM chromatography paper. The protein was concentrated by gradual addition of 1 volume of cold absolute ethanol, incubation on ice (1 h), and centrifugation (7,000 × *g* at 4°C for 10 min). The resulting precipitate was suspended in 25 mM Tris-HCl buffer at pH 8.4 (T buffer) to 1/5 the original sample volume and recentrifuged to eliminate insoluble material. US02 precipitate (from an original culture of 360 ml) was concentrated to 3 ml using a Centricon PL-30 centrifugal filter according to the instructions of the supplier (Millipore, Billerica, MA) and separated into two 1.5-ml fractions (where F1 and F2 represent the more viscous lower and upper fractions, respectively) and diluted 6.7-fold with T buffer. For both SB01 and US02 samples, proteins were filtered (0.20 μm) immediately prior to application to an anion-exchange MonoQ HR 5/5 or 10/10 column (Pharmacia) equilibrated with T buffer, as indicated in Table 1. Elution was carried out with a linear NaCl gradient (0 to 1 M in 1 ml T buffer) with fractions containing peak activity at 625 to 675 mM NaCl. Additional steps were included for purification of LccA from SB01, including application of active fractions (0.25 ml per run) to a Superdex 200 HR 10/30 column (Pharmacia) equilibrated in T buffer with 150 mM NaCl, followed by a MonoQ HR 10/10 column equilibrated in T buffer and developed with a linear NaCl gradient (600 to 800 mM in 1 ml T buffer). The fractions were monitored for purity by assay with 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine [SGZ]) under standard assay conditions (see below for details) and staining with Coomassie blue R-250 after separation by reducing 12% SDS-PAGE according to the method of Laemmli. Prestained SDS-PAGE standards and Kaleidoscope Standards (Bio-Rad) were used as protein standards for PAGE. The native molecular mass was determined by applying samples to a calibrated Superose 200 HR 10/30 column as recommended by the supplier (Pharmacia). The molecular mass standards for calibration included cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (443 kDa) (Sigma). Samples were stored at 4°C.

Enzyme activity and protein estimation. Enzyme activity was determined using the following substrates: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Sigma; A1888), 2,6-dimethoxyphenol (DMP) (Sigma; D135550), 2-methoxyphenol (guaiacol; Sigma; G5502), SGZ (Sigma S7896), bilirubin (Sigma; B4126), and ferrous ammonium sulfate. The enzymes were assayed by change in absorbance using a BioTek Synergy HT multidetection microplate reader with a 96-well plate and the following extinction coefficients: ε_{420} = 36,000 (ABTS), ε_{468} = 49,000 (DMP), ε_{436} = 26,600 (guaiacol), ε_{526} = 65,000 (SGZ), ε_{440} = 56,300 (bilirubin), and ε_{315} = 2,200 (ferrous ammonium sulfate) in units of M^{-1} cm^{-1}. The standard assay conditions were 185 mM NaCl at 45°C with the following buffers and substrate concentrations: 100 mM sodium acetate at pH 6.0 for ABTS (5 mM), DMP (2 mM), and guaiacol (5 mM); 25 mM Tris buffer at pH 8.4 for SGZ (1 mM); and 200 mM Tris buffer at pH 8.4 for bilirubin (450 μM). Copper sulfate (1 mM) was included for the DMP oxidation assay. Oxidation of ferrous ammonium sulfate (1 mM) was monitored with and without salt (185 mM NaCl) in 100 mM MES (morpholineethanesulfonic acid) buffer at pH 5, as well as 25 mM Tris-HCl buffer at pH 7.0 and 8.4. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min under standard conditions. Kinetic studies were conducted using purified LccA at a final concentration of 0.61 $\mu g \cdot ml^{-1}$ with 5 to 160 μM SGZ, 50 to 1,250 mM ABTS, and 90 to 540 μM bilirubin. Kinetic parameters (K_m, V_{max}, and k_{cat}) were determined using the Michaelis-Menten equation. The protein concentration was determined based on the method of Bradford using a Bio-Rad protein assay with bovine serum albumin as the standard (Bio-Rad Laboratories). All assays and protein purifications were performed at least in triplicate.

For detection of laccase activity in gels, nonboiled samples were separated on 7.5% PAGE gels devoid of SDS and β-mercaptoethanol. The gels were immersed in T buffer containing 1 mM SGZ, and activity was rapidly visualized as a pink color. For detection of total protein, the gels were counterstained with Coomassie blue R-250 stain and destained according to the instructions of the supplier (Bio-Rad). Images of activity and protein staining were acquired using a VersaDoc 1000 imaging system (Bio-Rad).

N-terminal sequencing. Purified LccA was separated by reducing 12% SDS-PAGE and transferred by electroblotting to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) at 100 V for 100 min at 4°C. Proteins were stained in the membrane with 0.2% (wt/vol) Coomassie blue R-250 in 40% acetic acid for 30 s and rinsed with deionized water. The LccA protein band was excised and subjected to automated Edman degradation for N-terminal sequencing (18).

Glycoprotein analysis. LccA was separated by reducing SDS-PAGE and stained in gel for glycosylation using Pro-Q Emerald 300 (Invitrogen P21857) and total protein was separated using Sypro Ruby (Bio-Rad; 1703126) with a VersaDoc 4000 imaging system according to the instructions of the manufacturer (Bio-Rad Laboratories). The carbohydrate content of purified LccA was estimated by the colorimetric method of Dubois et al. (15) using glucose as a standard. Pure LccA was deglycosylated by the trifluoromethanesulfonic acid (TFMS) method (17) for 0 to 10 h on ice and analyzed by reducing SDS-PAGE. *Trametes versicolor* laccase (Sigma Fluka; 53739) and bovine carbonic anhydrase (Sigma-Aldrich; C7025) were included for glycoprotein analysis as positive and negative controls, respectively.

Mass spectrometry analysis. Purified LccA was provided for mass spectrometry (MS) analysis as an in-solution sample and as protein bands separated by reducing 7.5% SDS-PAGE. Gels were stained with Bio-Safe Coomassie (Bio-Rad), and LccA-protein bands were excised from the gel and destained with 100 mM NH_4HCO_3 (pH 7.5) in 50% (vol/vol) acetonitrile (4°C overnight). Protein samples were reduced, alkylated in gel or in solution, digested with trypsin (Promega), and desalted. Peptides were further separated by capillary reverse-phase high-performance liquid chromatography (HPLC) using a PepMap C_{18} column (15 cm by 75-μm inside diameter [i.d.]) and an Ultimate Capillary HPLC System (LC Packings, San Francisco, CA) with a linear gradient of 5 to 40% (vol/vol) acetonitrile for 25 min at 200 nl/min. Tandem MS (MS-MS) analysis was performed online using a hybrid quadrupole time-of-flight instrument (QSTAR XL hybrid LC/MS-MS) equipped with a nanoelectrospray source (Applied Biosystems, Foster City, CA) and operated with the Analyst QS v1.1 data acquisition software. Spectra from all experiments were converted to DTA files and merged to facilitate database searching using the Mascot search algorithm v2.1 (Matrix Science, Boston, MA) against the theoretical *H. volcanii* proteome (http://archaea.ucsc.edu/, April 2007 version). The search parameters included trypsin as the cleavage enzyme and carbamidomethylation as a fixed modification. Variable modifications included deamidation of asparagine and glutamine; oxidation of methionine; methyl-esterification of aspartate and glutamate; and N-acetylation and phosphorylation of serine, threonine, and tyrosine. The mass tolerance for all QSTAR analyses was 0.3 Da, and peptides with assigned probability-based Mascot ion scores of less than 30 were excluded.

RESULTS AND DISCUSSION

LccA is related to laccases of the multicopper oxidase family. Basic local alignment search tool (BLAST) analysis of the *H. volcanii* theoretical proteome revealed a 63,397-Da polypeptide of pI 4.34 (HVO_B0205, termed LccA) related to laccases of the MCO family (Fig. 1). The gene locus encoding LccA resided on pHV3 (440 kb), one of three large replicons with *cdc6*- and *orc1*-associated replication origins separate from the 2.848-Mb chromosome (48). Archaeal proteins that cluster with the MCO family are rare, and none have been characterized to date. LccA included the conserved cysteine and histidine residues required for coordination of the type 1, 2, and 3 copper centers and activities of blue copper laccases (Fig. 1). In addition to the conserved catalytic residues, the N terminus of LccA included a twin arginine motif (RRRFL) preceding a hydrophobic patch, suggesting it may be translocated in a folded state through the twin arginine translocation (Tat) system. LccA was also predicted to be cleaved between Ala31 and Ala32 to generate a mature protein of 60.1 kDa and to be glycosylated based on the presence of O- and N-linked glycosylation motifs, Thr-X_5-Pro-X-Pro and Asn-X-(Thr/Ser) (where X represents any amino acid) (Fig. 1).

Consensus tree inference revealed that LccA was closely related to laccases of the Gram-positive bacteria, including 38 to 40% identity with and 52 to 53% similarity to the thermostable CotA proteins of *B. subtilis* and *Bacillus licheniformis* (38, 43) (see Fig. S1 in the supplemental material). Among the

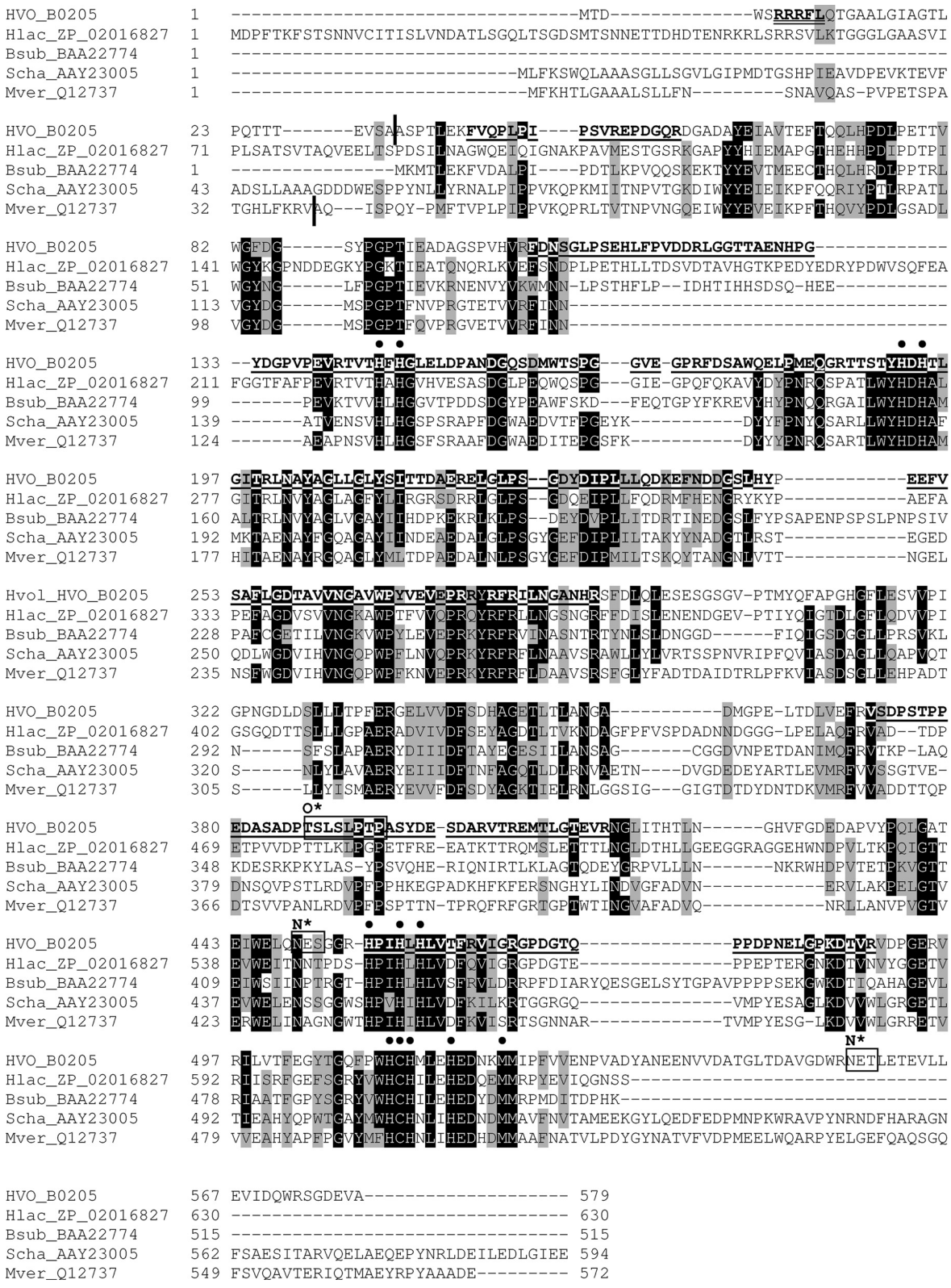

FIG. 1. Sequence alignment of *H. volcanii* LccA (Hvo_B0205) to multicopper oxidases. The vertical lines represent cleavage sites with experimental evidence based on the N-terminal sequence of the mature LccA (HVO_B0205) and *Myrothecium verrucaria* bilirubrin oxidase (Mver_Q12737) (37). TAT motifs, indicated by a double underline, are based on *in silico* analysis using the TatP 1.0 server. Note that TatP also predicts both cleavage sites described above. Residues that bind the four copper atoms to form the type 1, 2, and 3 copper sites of known multicopper oxidases are indicated by dots above the amino acid residues. Potential O- and N-linked glycosylation sites with EnsembleGly scores above 0.8 are indicated by O* and N*, respectively, above the residues, with the Thr-X_5-Pro-X-Pro and Asn-X-Thr motifs (where X represents

archaea, LccA was closely related (45% identity) only to Hlac_1049, encoded on the main chromosome of *Halorubrum lacusprofundi*. Other archaeal genomes (completed as of November 2009) did not encode LccA homologs, including the haloarchaea *Halobacterium* sp. NRC-1 (*Halobacterium salinarum* R1), *Haloquadratum walsbyi* DSM 16790, *Haloarcula marismortui* ATCC 43049, and *Natronomonas pharaonis* DSM 2160. Furthermore, LccA had less than 30% identity to PAE1888, an uncharacterized protein with enhanced transcript levels under denitrifying conditions in *P. aerophilum* (12). In contrast to LccA, PAE1888 clustered with bacterial MCOs involved in oxidative stress and/or metal homeostasis, such as McoA of *Aquifex aeolicus*, CueO of *E. coli*, and Mco of *Staphylococcus aureus* (21, 62, 63). Consistent with this, the methionine-rich helix proposed to be involved in metal sequestration that lies over the type 1 sites of the metal-oxidizing MCOs, such as *E. coli* CueO (53), is not conserved in LccA. This sparse distribution of LccA homologs among the archaea and the close relationship of LccA to laccases of the firmicutes (e.g., *B. subtilis* CotA) suggested the haloarchaeal gene was acquired by horizontal gene transfer from bacteria and that it encodes a laccase that oxidizes phenolic compounds.

Laccase activity secreted by recombinant *H. volcanii*. To characterize LccA, our initial efforts focused on modifying the *H. volcanii lccA* gene for laccase production in recombinant *E. coli* using the pET expression system with Rosetta (DE3) and plasmids pJAM821 and pJAM823 (Table 1). Although numerous *H. volcanii* proteins have been successfully produced using this type of bacterial system (34, 52), LccA protein and laccase activity were not detected by SDS-PAGE and SGZ oxidation assay of culture broth and cell lysates of these recombinant *E. coli* strains. "Salt-loving" haloarchaeal proteins, enzymes with elaborate metal clusters, glycoproteins, and proteins secreted by the TAT system are all notoriously difficult to produce in conventional expression systems, such as *E. coli* and *Saccharomyces cerevisiae* (6, 11, 41). Thus, a number of factors may be limiting the production of LccA in *E. coli*.

To overcome these limitations, *lccA* was engineered for high-level expression in the native organism *H. volcanii*, known for its halophilic properties and robust TAT system (57). The *lccA* gene was positioned downstream of a strong rRNA P2 promoter on a multicopy plasmid (pHV2 based) with and without coding sequence for a 1-Da C-terminal StrepII tag (-WSHPQFEK), and the resulting plasmids were transformed into *H. volcanii* H26 to generate strains SB01 and US02, respectively (Table 1). These engineered strains were grown in various media supplemented with $CuSO_4$ (50 to 500 μM) and compared to their parent (H26) for the production of laccase activity by monitoring the oxidation of SGZ. Using this approach, laccase activity associated with *H. volcanii* cells was negligible for all three strains examined, reaching at most 0.69 ± 0.12 mU · ml^{-1} after 80 to 120 h of growth (Fig. 2). In

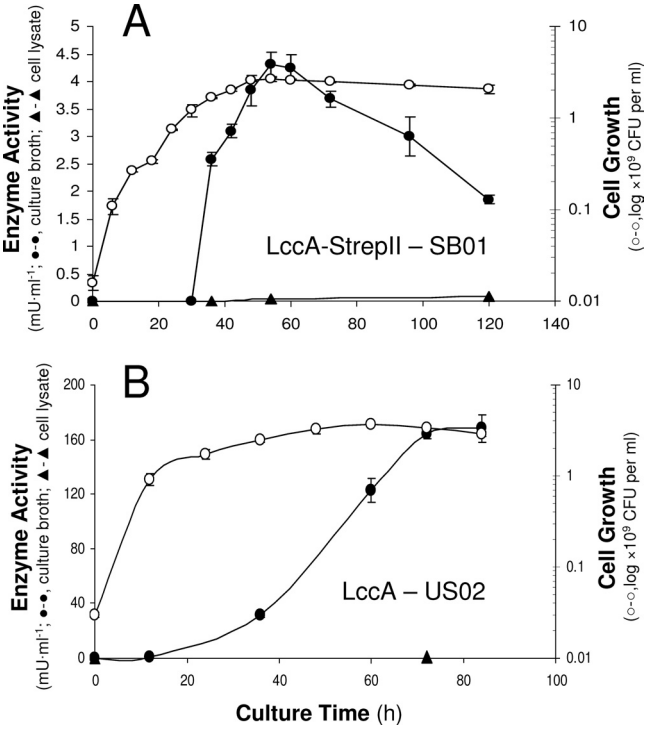

FIG. 2. Laccase activity of *H. volcanii* SB01 and US02 strains at various stages of growth. *H. volcanii* SB01 (A), US02 (B), and the parent, H26 (data not shown), were inoculated to a final optical density at 600 nm (OD_{600}) of 0.01 in medium supplemented with novobiocin and 100 μM copper sulfate. LMM and YPC were optimal for production of laccase activity for SB01 and US02, respectively. The cells were grown for up to 120 h at 42°C in an orbital shaker (150 to 200 rpm). Samples were withdrawn at intervals and used for estimation of growth (1 OD_{600} unit ≈ 1×10^9 CFU per ml) and assay of laccase activity. For analysis of cell lysates, the cells were harvested by centrifugation (10 min at 10,000 × *g* and 4°C), washed twice in T buffer supplemented with 2 M NaCl, resuspended in T buffer, and lysed by sonication (15 s on, 45 s off at 20% amplitude for 3 min). The culture broth (filtered at 0.4 μm) and cell lysate were assayed for laccase (SGZ-oxidizing) activity under standard conditions. Data are presented as means ± standard deviations.

contrast to these cellular fractions, significant levels of laccase activity were detected in the culture broth of *H. volcanii* SB01 and US02, with the highest level of activity detected for strain US02 in YPC medium with 100 μM $CuSO_4$ (Fig. 2). Under these conditions, the laccase activity of US02 reached a maximum of 170 ± 10 mU · ml^{-1} at stationary phase (72 to 80 h), whereas the level of laccase activity secreted by SB01 was 40-fold lower, reaching levels of only 4.3 ± 0.23 mU · ml^{-1} at 54 h. Laccase activity was not detected in the culture broth of US02 and SB01 until stationary phase (Fig. 2) and was not detected at significant levels throughout all phases of growth

any amino acid) boxed. Amino acid sequences in boldface and underlined represent peptides detected by MS-MS analysis of the laccase purified from *H. volcanii* US02 (details of MS-MS analysis are shown in the supplemental material). Black and grey shadings represent identical and functionally conserved amino acid residues, respectively. HVO_B0205, *H. volcanii* laccase LccA (this study); Hlac_ZP-02016827, *Halorubrum lacusprofundi* putative multicopper oxidase (GenBank accession no. 153896174); Bsub_BAA22774, *Bacillus subtilis* spore coat protein A (CotA) with laccase activity (28, 43); Scha_AAY23005, *Stachybotrys chartarum* phenol oxidase A (42); Mver_Q12737, *M. verrucaria* bilirubrin oxidase (37).

TABLE 2. Purification of LccA from the culture broth of *H. volcanii* cells[a]

Fraction	Total activity (U)	Protein (mg)	Yield (%)	Sp act (U · mg^{-1})	Purification (fold enrichment)
H. volcanii SB01					
60-h culture broth (130 ml)	740	6.7[a]	100	0.11	1
60-h culture broth (filtered)	770	6.4	100	0.12	1.1
Ethanol precipitate	1,010	6.3	140	0.16	1.5
MonoQ 5/5, pH 8.4	470	0.92	63	0.51	4.6
Superdex 200 HR 10/30	170	0.23	23	0.76	6.9
MonoQ 10/10, pH 8.4	95	0.07	13	1.3	12
H. volcanii US02					
72-h culture broth (360 ml)	57	25	100	1.4	1
Ethanol precipitate	55	13	94	4.3	2
Centricon PL-30 and MonoQ 10/10, pH 8.4	F1, 41.5; F2, 14.2	F1, 1.4; F2, 2.1	F1, 72; F2, 24	F1, 29.4; F2, 6.7	F1, 13; F2, 2.9

[a] *H. volcanii* SB01 and US02 were grown for 60 h in HvLMM and 72 h in YPC (42°C; 200 rpm), respectively. LccA protein was purified from the culture broth as indicated. Activity was monitored by oxidation of SGZ under standard assay conditions (see Materials and Methods). F1 and F2 represent the lower (viscous) and upper fractions generated after concentration by centrifugal filtration using a Centricon PL-30, respectively (see Materials and Methods for details).

for the parent strain H26 (data not shown). The reason for the lag in laccase productivity for US02 and SB01 remains to be determined; however, a similar time course has been observed for the secretion of the *Natrialba magadii* subtilisin-like Nep protease from recombinant *H. volcanii* (14). While the addition of a strong, relatively constitutive promoter upstream of *lccA* enhanced laccase productivity by over 170-fold, addition of a C-terminal StrepII coding sequence significantly decreased LccA productivity. Thus, enhanced expression of *lccA* coding sequence alone was optimum for secretion of laccase activity by *H. volcanii*.

Although the genetic systems of archaea are not as advanced as those of bacteria or yeast, the productivity of the engineered *H. volcanii* strain US02 was surprisingly high, with 170 mU · ml^{-1} laccase activity produced in the medium in less than 3 days. This level of productivity is comparable to that of yeast systems, such as *Yarrowia lipolytica* and *Pichia methanolica*, developed for secretion of the white-rot fungal laccase from *T. versicolor*. Typically, these yeast systems require 5 to 6 days of cultivation for production of 230 to 1,260 mU · ml^{-1} of laccase activity (24, 32). It should also be noted that the secretion of LccA from *H. volcanii* contrasts with the vast majority of systems developed for production of bacterial MCOs. In these systems, the bacterial MCO is not secreted and is instead purified from the soluble and/or insoluble fractions of recombinant *E. coli* cells (e.g., production of the *Thermus thermophilus* Tth laccase [45], *B. subtilis* and *B. licheniformis* CotA [38, 43], *Bacillus halodurans* Lbh1 [58], *S. aureus* Mco [63], *A. aeolicus* McoA [21], and *Bacteroides* RL5 from the rumen metagenome [3]).

LccA purified from the culture broth of *H. volcanii*. LccA was purified 13-fold from the culture broth of *H. volcanii* US02 to a specific activity of 29.4 U · mg^{-1} by ethanol precipitation, concentrated using a Centricon PL-30 centrifugal filter, and fractionated by MonoQ chromatography as outlined in Table 2. LccA-StrepII was similarly purified from SB01 with the addition of gel filtration (see Fig. S2 in the supplemental material) and MonoQ chromatography steps at the final stages of purification (Table 2). In contrast to US02-purified LccA, the specific activity of LccA-StrepII was over 20-fold lower at only 1.3 U · mg^{-1}. Thus, addition of a C-terminal StrepII tag not only reduced the productivity of the strain, but also reduced LccA enzyme activity. Incorporation of StrepTactin chromatography did not improve the overall activity or yield of LccA-StrepII from SB01 and thus was not further pursued (data not shown).

The LccA protein isolated from US02 (as described above) was analyzed for purity by reducing SDS-PAGE and MS. Purified LccA migrated as a single protein band of 75 to 80 kDa

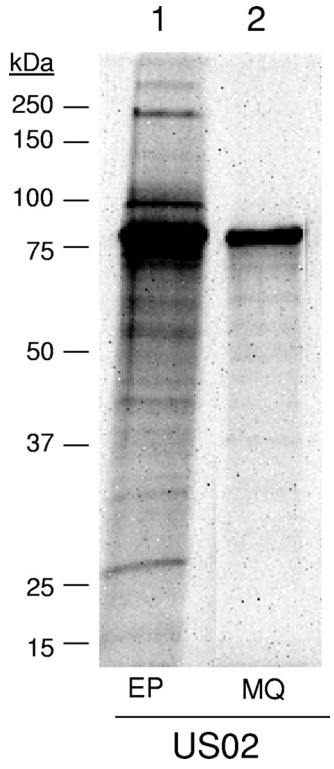

FIG. 3. LccA purified to electrophoretic homogeneity from the culture broth of *H. volcanii* US02. Shown is reducing 10% SDS-PAGE of LccA at various stages of purification, including ethanol precipitate (EP) (lane 1) and MonoQ 10/10, pH 8.4 (MQ) (lane 2), as indicated. Molecular mass standards are on the left. Proteins were stained with Coomassie brilliant blue R-250.

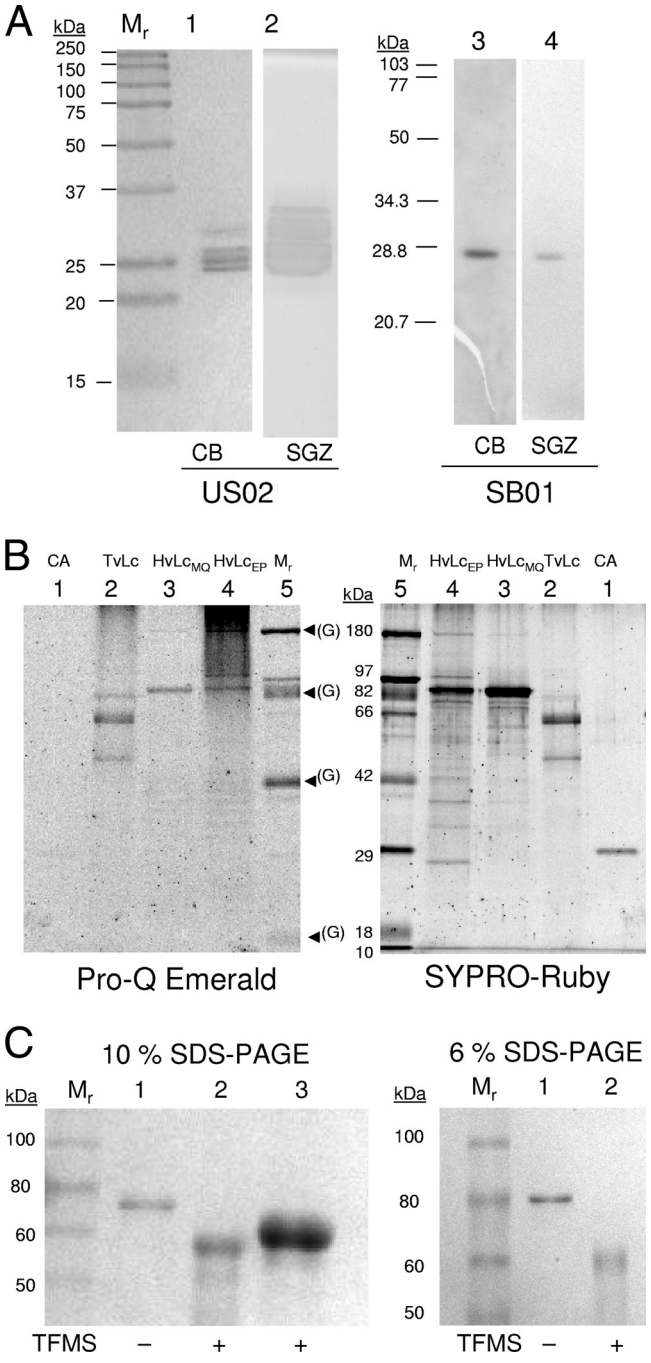

FIG. 4. LccA purified from US02 is a glycoprotein. (A) Native gels of LccA at its final stages of purification from US02 (Superdex 200 HR 10/30, lanes 1 and 2) and SB01 (MonoQ 10/10, pH 8.4, lanes 3 and 4). The gels were stained for total protein with Coomassie brilliant blue R-250 (CB) and for laccase activity by in-gel oxidation of SGZ (SGZ), as indicated. Prestained kaleidoscope and low-range protein standards (Bio-Rad) were included for US02 and SB01, respectively, as indicated at the left of each gel. (B) Pro-Q Emerald glycoprotein analysis of US02-purified LccA. Proteins were separated by reducing 10% SDS-PAGE. The gels were stained for glycosylation with Pro-Q Emerald (left) and for total protein with Sypro-Ruby (right), as indicated. The proteins included carbonic anhydrase (CA) (lanes 1); *T. versicolor* laccase (TvLc) (lanes 2); LccA MonoQ 10/10, pH 8.4, fractions ($HvLc_{MQ}$) (lanes 3); LccA ethanol precipitate ($HvLc_{EP}$) (lanes 4); and CandyCane molecular mass standards (Molecular Probes), including a mixture of glyosylated (G) and nonglycosylated proteins, as indicated (lanes 5). (C) Deglycosylation of LccA. LccA purified from US02 was treated with TFMS on ice for 0 h (lanes 1), 10 h (lanes 2), and 3 h (lane 3) and separated by reducing 6 and 10% SDS-PAGE, as indicated (see Materials and Methods for details).

(Fig. 3) that was specific for LccA based on MS analysis with 38 to 68% coverage of the LccA polypeptide sequence (Fig. 1; see Fig. S3 and Table S1 in the supplemental material). The 10- to 20-kDa difference in migration of LccA by SDS-PAGE compared to that calculated from the polypeptide sequence was consistent with its low pI and/or posttranslational modification by glycosylation.

Multiple isoforms of LccA. LccA (purified as described above) was separated by native PAGE and stained for SGZ-oxidizing activity and total protein. In contrast to SB01-purified LccA-StrepII, for which only a single protein band was detected, LccA purified from US02 migrated as at least four separate isoforms that were all active in SGZ oxidation (Fig. 4A). These results suggested that LccA was modified posttranslationally and that addition of a C-terminal StrepII tag abolished this modification. It should be noted that mobility in native gels depends on not only the hydrodynamic size, but also the charge of the protein. The LccA polypeptide sequence is predicted to be highly acidic, and this is likely to account for the rapid migration of all of the LccA isoforms compared to protein standards of similar molecular mass.

N-terminal cleavage and glycosylation of LccA. Many fungal laccases are modified posttranslationally, including removal of N-terminal residues, as well as the addition of high-mannose-type glycans to Asn residues (N glycosylation) (5, 22, 37, 41, 51, 69). Based on bioinformatics, LccA is predicted to be cleaved between Ala31 and Ala32 and to be N and/or O glycosylated (Fig. 1). To investigate these possibilities, the US02-purified LccA was N-terminally sequenced by Edman degradation and analyzed for glycosylation. Purified LccA was cleaved after Ala31 (Fig. 1) and glycosylated based on a carbohydrate content of 6.9% ± 0.06%, as determined by a phenol-sulfuric acid method (15), and staining using Pro-Q Emerald 300 (26) (Fig. 4B). The carbohydrate content of LccA was similar to the 6.9% previously reported for other fungal laccases (51). To further analyze and confirm LccA glycosylation, the purified protein was treated with TFMS, a strong acid known to remove glycans from polypeptide chains (17). A molecular mass shift of 10 to 20 kDa was observed by SDS-PAGE for the TFMS-treated LccA compared to the untreated control and resulted in its migration at ~60 kDa, consistent with the molecular mass calculated from the deduced amino acid sequence (Fig. 4C). TFMS treatment did not shift the migration of nonglycosylated control proteins (e.g., carbonic anhydrase), confirming that polypeptide chains remained intact under these conditions (data not shown). Together, these results provide evidence that LccA is secreted, cleaved, and glycosylated; however, the order of these events during its maturation remains to be determined.

Interestingly, the LccA-StrepII variant purified from SB01 was not associated with carbohydrate and did not stain with Pro-Q Emerald (data not shown). Furthermore, the N terminus of LccA-StrepII was not amendable to sequencing by Ed-

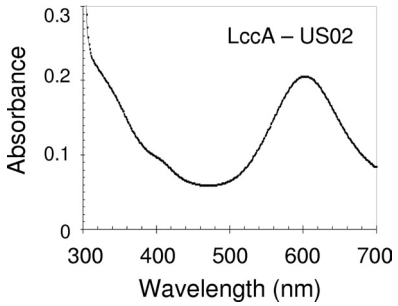

FIG. 5. UV-visible absorbance spectrum of LccA. The absorbance of LccA (1.5 mg · ml^{-1}) purified from *H. volcanii* US02 in T buffer containing 185 mM NaCl was analyzed from 300 to 700 nm.

man degradation, suggesting it may be blocked by N-terminal α amine group (N^{α}) acetylation. These results are consistent with the native PAGE analysis and suggest that addition of a StrepII tag to the C terminus of LccA not only reduces its activity, but also inhibits its N-terminal cleavage and glycosylation.

Spectroscopic and catalytic properties of LccA. LccA (purified from US02) was blue and exhibited an absorbance spectrum typical of blue multicopper oxidases, including a peak at 605 nm and a shoulder at 330 nm (Fig. 5). Many laccases isolated from fungi and bacteria are blue multicopper oxidases that coordinate four copper atoms into three types of copper-binding sites (27). All of these "blue" enzymes produce spectra with a maximum at 605 nm, corresponding to the T1, or blue, copper atom. The T2 copper site exhibits weakly visible absorbance, and the T3 copper site has two copper centers and is responsible for a shoulder at around 330 nm. The spectrum of LccA is consistent with its clustering with blue laccases, including the closely related and structurally characterized *B. subtilis* CotA (20). Thus, the conserved cysteine and 10 histidine residues of LccA are likely to coordinate these three types of copper sites.

LccA oxidized a variety of compounds. The four substrates most commonly used for determination of laccase activity were evaluated: ABTS, DMP, guaiacol, and SGZ. The oxidation of ferrous (Fe^{2+}) ammonium sulfate and bilirubin was also investigated. Although LccA did not oxidize Fe^{2+} or guaiacol in the presence or absence of copper supplementation, it did oxidize the remaining substrates, i.e., ABTS at 6.1 ± 0.39 U · mg^{-1}, DMP at 12.6 ± 1.1 U · mg^{-1}, SGZ at 29.4 ± 1.4 U · mg^{-1}, and bilirubin at 25.5 ± 1 U · mg^{-1} (the values reported are for US02-purified LccA under standard conditions [see Materials and Methods for details]). Note that the oxidation of DMP was highly stable for several hours but, unlike ABTS, SGZ, and bilirubin, required the addition of $CuSO_4$. LccA presented a higher specificity for SGZ than for ABTS, with k_{cat}/K_m values of 0.62 and 0.015 s^{-1} μM^{-1}, respectively (Table 3). While the specificity of LccA for SGZ was significantly lower than that of the DUF152 RL5 laccases, it is comparable to if not higher than that of *B. subtilis* CotA (Table 3). LccA specificity for bilirubin oxidation was also high, with a k_{cat}/K_m value of 0.12 s^{-1} μM^{-1} (k_{cat}, 29 s^{-1}; K_m, 236 μM).

Recently, laccases have been grouped based on their DMP oxidase activities (64). The first group oxidizes DMP without copper supplementation (e.g., the laccase of *Pyricularia oryzae* and PpoA of *Marinomonas mediterranea*), the second group readily oxidizes DMP after the addition of copper but is also rapidly inactivated (e.g., CueO of *E. coli*), and the third type shows a very low rate of DMP oxidation that lasts for hours, but only in the presence of added copper (e.g., CotA of *B. subtilis*). LccA oxidized DMP only in the presence of added $CuSO_4$; hence, it appears to be most similar to the last group of laccases.

To further evaluate the catalytic properties of LccA, the temperature, pH, and salt optima of the purified enzyme were

TABLE 3. Kinetic parameters of *H. volcanii* LccA compared to those of selected laccase-like enzymes

Enzyme, origin of encoding gene	Source of purified enzyme	ABTS oxidation			SGZ oxidation			Reference
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	
LccA, *H. volcanii*	Culture broth of *H. volcanii* SB01	700	10.0	0.014	67	19.4	0.30	This study
	Culture broth of *H. volcanii* US02	671	9.9	0.015	35	21.7	0.62	This study
CotA, *Bacillus subtilis*	Cell lysate of recombinant *E. coli*	106	16.8	0.16	26	3.7	0.14	43
CotA, *Bacillus licheniformis*	Cell lysate of recombinant *E. coli*	6.5	83	13	4.3	100	23	38
McoA, *Aquifex aeolicus*	Refolded from cell lysate of recombinant *E. coli*	128	2.1	0.016	38	0.48	0.013	21
LAC2, white-rot basidiomycete C30	Culture supernatant of C30	536	683	1.27	6.8	1,093	160	36
LAC1, white-rot basidiomycete C30	Culture supernatant of C30	56	10.7	5.22	1.8	30	17	36
RL5, metagenome library of bovine rumen (tentative *Bacteroides* origin)	Cell lysate of recombinant *E. coli*	26	18	0.69	0.43	660	1,534	3
BT4348, *Bacteroides thetaiotaomicron*	Cell lysate of recombinant *E. coli*	10	217	21.7	0.83	555	669	3
YifH, *E. coli*	Cell lysate of recombinant *E. coli*	23	24	1.05	1.1	362	329	3

determined using SGZ and/or ABTS as a substrate. LccA activity was optimum at 45 to 50°C (see Fig. S4 in the supplemental material), consistent with the optimal growth temperature of the organism (42 to 45°C) (54). Relative to this optimum, LccA was ~75% active at room temperature (21°C) and 40 to 50% active at 60°C. This temperature optimum of LccA is similar to a number of bacterial MCO optima, including 40 to 45°C for laccases of *Streptomyces* sp. (19, 47), 52°C for *Bacteroides thetaiotaomicron* BTH4389, and 44°C for *E. coli* YifH (3). It is also closely related to the temperature optimum for simultaneous saccharification and fermentation (SSF) processes, which include fungal cellulases and second-generation microbial biocatalysts (49). However, it is significantly lower than the optima of 75°C for *B. subtilis* CotA (43) and 70°C for *A. aeolicus* McoA (21).

Most extracellular and intracellular enzymes of haloarchaea, including *H. volcanii*, require salt for activity and stability. Consistent with this, the activity of LccA was optimal at ~200 mM salt, with 1.5-fold greater activity in KCl than in NaCl, and the enzyme displayed reduced activity after the removal of salt by dialysis (see Fig. S4 in the supplemental material). LccA was also active at relatively high concentrations of salt, with 65% activity at 1 M NaCl. The ability of LccA to rapidly oxidize phenolic substrates at molar concentrations of salt is not surprising, as the enzyme is secreted by a microbe isolated from the Dead Sea. Similar to LccA, the activity of Lbh1 from the halotolerant *B. halodurans* is stimulated by addition of NaCl (58).

While the oxidation of ABTS by LccA was optimal at low pH (pH 6.0), its optimum for SGZ oxidization was more alkaline (pH 8.4) (see Fig. S4 in the supplemental material). Most microbial laccases display optimal activity at low pH. Those with alkaline or near-neutral pH optima are not as common, e.g., *Acremonium murorum* (23), *B. halodurans* (58), *Coprinus cinereus* (69), *Streptomyces ipomoea* (46), and *Melanocarpus albomyces* (35). Unlike LccA, the few microbial laccases that are active at alkaline pH do not exhibit significant activity below pH 6.0. These unique catalytic properties of LccA underline the potential of *Archaea* as a source of high-value laccases.

LccA is stable at high temperature and high salt and with solvent. To assess the thermostability of LccA, the purified protein was preincubated at various temperatures from 37 to 70°C for up to 2.5 days. LccA was fully active after 1 h at 55°C and 5 h at 50°C, with a half-life of inactivation at 50°C of greater than 1 day (31.5 h) (see Fig. S5 in the supplemental material). The enzyme retained nearly all of its original activity at 37°C and 35 to 60% of its activity at 45 to 50°C after incubation for 2.5 days. These results revealed that LccA is highly thermostable, retaining most of its activity after prolonged incubation at moderately high temperatures. The multicopper oxidase of *A. aeolicus*, McoA, has remarkably stable activity, with the ability to oxidize metals after incubation for 5 h at 90°C (21). Likewise, the recently studied recombinant RL 5 laccase from the rumen metagenome has maximal activity at 60°C and is fully stable at this temperature for 3 to 4 h (3). CotA of *B. subtilis* is also thermostable, with a half-life of nearly 2 h at 80°C (43).

The purified LccA was also stable at a wide range of salt concentrations from 100 mM to at least 1.4 M NaCl, retaining nearly all of its original activity, if not higher activity (see Fig. S5 in the supplemental material). It is noteworthy that prolonged incubation of LccA in salt (for 24 h) did not alter this activity profile (data not shown). Recent analysis of *Streptomyces psammoticus*, a bacterium isolated from a mangrove swamp, revealed that it produces a laccase that is also salt tolerant, with full activity after incubation for 24 h in 0.8 M NaCl (47).

Commonly used water-miscible organosolvents (i.e., methanol, ethanol, dimethyl sulfoxide [DMSO], and dimethylformamide [DMF]) were examined for their compatibility with LccA stability and activity. Purified LccA was incubated in T buffer with 25% (vol/vol) solvent in the presence of salt (185 mM NaCl) for 1.5 to 24 h. The enzyme and solvent mixtures were diluted 4-fold and assayed for SGZ-oxidizing activity under standard conditions (see Fig. S5 in the supplemental material). LccA was relatively stable in all solvents examined, retaining nearly 75% of its activity after 24 h in methanol or ethanol and over 50% of its activity after incubation in DMSO or DMF. Prior studies (7, 40, 44, 55, 56) coupled with our current findings reveal that a wide variety of laccases, including LccA, are stable and/or can support catalysis and conversion of phenolic substrates in water-organic mixed solvents. Since most of the substrates and mediators of laccases are insoluble or poorly soluble in water and the degree of polymerization of phenoxyl radicals is higher in aqueous media (44), solvent stability is an important feature to consider in optimizing this group of enzymes. Many industrial applications would also benefit from the addition of water-miscible organic cosolvents to laccase-mediated reactions, particularly the conversion of insoluble substrates, such as lignin and its derivatives, to useful products (2, 13, 50).

Effects of inhibitors. A number of compounds were examined for their influences on LccA activity, including small ions (sodium azide or NaN_3), sulfhydryl group-containing redox reagents (L-cysteine and dithiothreitol [DTT]), denaturants (thiourea), and chelators (EDTA, 1,10-phenanthroline, and 2,2-dipyridyl) (Table 4). The most effective laccase inhibitors to date are small anions, especially N_3^-, which often bind to the trinuclear copper center and interfere with electron flow and substrate oxidation (31). Consistent with this, LccA was inhibited nearly 50% by the addition of 1 mM NaN_3. In contrast, some fungal laccases, such as that reported by Saito et al. (59), are relatively unaltered by NaN_3 (at 10 mM). Of the redox reagents examined, both L-cysteine and DTT were strong inhibitors of LccA activity and were effective at relatively low concentrations (i.e., 0.1 to 1 mM). As has been observed for other laccases, this inhibition is likely caused by reduction of the oxidized substrate by the sulfhydryl groups of the redox reagents and not by inhibition of the enzyme (30). The LccA protein was also susceptible to denaturation by thiourea, with 90% inhibition at 10 mM. Although the general chelator EDTA did not inhibit LccA activity at 50 mM, the transition metal chelators 2,2-dipyridyl and 1,10-phenanthroline were inhibitory at 10 mM, with 25% and 90% reductions in LccA activity, respectively. These results are consistent with the recalcitrant nature of the copper-containing laccases toward chelation by EDTA (59) yet reveal a likely role of metals in LccA activity, based on inhibition by phenanthroline.

TABLE 4. Effects of various compounds on activity of LccA

Compound[a]	Concn (mM)	Relative activity (%)[b]
Control		100.0 ± 0.9
L-Cysteine	0.1	83.0 ± 3.7
	1	UD[c]
Sodium azide	1	55.2 ± 2.7
DL-Dithiothreitol	0.01	90.5 ± 5.5
	0.1	UD
	1	UD
EDTA	0.1	100.6 ± 1.8
	1	106.3 ± 5.8
	5	123.6 ± 3.2
	50	123.7 ± 3.0
Thiourea	1	96.5 ± 3.9
	10	9.5 ± 1.3
1,10-Phenanthroline	1	85.3 ± 3.4
	10	10.9 ± 3.9
2,2-Dipyridyl	1	86.6 ± 3.3
	10	75.2 ± 2.3

[a] LccA purified from SB01 was incubated with the various compounds for 10 min at 45°C in T buffer with 185 mM NaCl and assayed for SGZ-oxidizing activity under standard conditions.
[b] The values represent the average of at least 3 replicates.
[c] UD, undetectable.

Conclusions. LccA is the first laccase of the multicopper oxidase superfamily to be characterized from the archaea, is one of only a few known archaeal glycoproteins, and may prove useful for a variety of industrial applications based on its ability to function from acidic to basic pH and to withstand high salt, high temperature, and mixed organosolvents. The LccA protein was secreted at high levels into the extracellular milieu of *H. volcanii* cells expressing multiple copies of the *lccA* gene and was readily purified from the culture broth in a highly active form. LccA was modified posttranslationally, including removal of 31 amino acid residues from its N terminus, addition of glycan residues (glycosylation), and apparent coordination of four copper atoms into three types of copper-binding sites (based on its UV-visible spectrum and the presence of conserved residues for copper binding).

In contrast to typical cells, which accumulate organic solutes and pump salt ions out, haloarchaea maintain homeostasis by accumulating high concentrations of intracellular cations or counterions (e.g., K^+). As a result, most haloarchaeal proteins, including LccA, possess a low pI and a highly acidic surface that serves as a hydration shell and prevents protein aggregation, thus enabling catalysis to occur at molar concentrations of salt. This unusual ability of haloarchaea to thrive in environments with low water activity (high salt, solvent, and/or desiccation) has made these organisms and their enzymes ideal candidates for biotechnology advances. Currently, we are examining the potential of LccA for the detoxification of lignin from lignocellulosics (e.g., steam-exploded sugarcane bagasse) for enhanced recovery of fermentable sugars for ethanol production.

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