Molecular and Biochemical Characterization of a Highly Stable
Bacterial Laccase That Occurs as a Structural Component of the
Bacillus subtilis Endospore Coat*

Lígia O. Martins‡§, Cláudio M. Soares‡, Manuela M. Pereira‡, Miguel Teixeira‡, Teresa Costa‡,
George H. Jones‡¶, and Adriano O. Henriques‡||

From the ‡Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2781-901 Oeiras, Portugal, §Universidade Lusófona de Humanidades e Tecnologias, Departamento de Engenharias e Tecnologias, Av. do Campo Grande, 376, 1749-024 Lisboa, Portugal, and ¶Department of Biology, Emory University, Atlanta, Georgia 30322

The Bacillus subtilis endospore coat protein CotA shows laccase activity. By using comparative modeling techniques, we were able to derive a model for CotA based on the known x-ray structures of zucchini ascorbate oxidase and Cuprinus cereneus laccase. This model of CotA contains all the structural features of a laccase, including the reactive surface-exposed copper center (T1) and two buried copper centers (T2 and T3). Single amino acid substitutions in the CotA T1 copper center (H497A, or M502L) did not prevent assembly of the mut-}

Bacterial endospores are cellular structures designed to resist to a wide range of physical-chemical extremes such as wet and dry heat, desiccation, radiation, UV light, and oxidizing agents, would promptly destroy vegetative cells. The remarkable level of resistance of the bacterial endospore is largely attributed to its unique structural features (1, 2). In Bacillus subtilis the dehydrated spore core, which contains a copy of the chromosome, is surrounded by a thick layer of a modified peptidoglycan called the cortex, which is essential for heat resistance. The cortex is protected from the action of lysozyme and harsh chemicals by a multi-layered protein coat, which also influences the spore response to germinants (1, 2). In B. subtilis the coat is structurally differentiated into a thin lamellar inner layer closely apposed to the cortex peptidoglycan and a thicker, striated and electron-dense outer layer (1, 2).

Received for publication, January 25, 2002, and in revised form, March 4, 2002
Published, JBC Papers in Press, March 7, 2002, DOI 10.1074/jbc.M200827200

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: SGZ, syringaldazine; ABTS, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); T1, type 1 copper; T2, type 2 copper; T3, type 3 copper; ZAO, zucchini ascorbate oxidase; LB, Luria-Bertani; DSM, Difco sporulation medium; IPTG, isopropyl-β-D-thiogalactopyranoside; EPR, electron paramagnetic resonance; bp, base pair(s).
Bacillus subtilis CotA Is a Highly Stable Laccase

Bacterial Strains and Culture Conditions

Overproduction and Purification of CotA

Structure of CotA Mutants by Comparative Modeling Techniques

Materials and Methods

Structure of CotA by Comparative Modeling Techniques

Engineered CotA Variants

Protein Expression and Purification

Materials and Methods

Construction of a B. subtilis Strain Bearing a Multicopy Allele of cotA

Purification of Spores and Extraction of Spore Coat Proteins

Structural and Functional Analysis of CotA

Results

Discussion

Acknowledgments

References

Supplementary Information

Online Resource

Figure Legends

Table of Contents

18850

vatization, textile dye or stain bleaching, and contaminated water or soil detoxification (16). These enzymes are widely distributed in plants and fungi, where they have been implicated in melanin formation, lignolysis, and detoxification (17). Several protein sequences with significant similarity to fungal laccases have been predicted in bacterial genomes (11), but other than in B. subtilis spores (12, 13), laccase activity was found in only three other bacterial species, the soil bacterium Azospirillum lipoferum (18) and the marine bacteria Marinomonas mediterranea and strain 2-40 (19–21). However, to date no bacterial laccase has been purified and characterized in detail.

Here, we have shown that CotA has all the molecular features typical of ascorbate oxidase and fungal laccase, namely an exposed reactive center, and confirmed that CotA oxidase activity is directly required for the formation of spore pigments. Furthermore we overproduced, purified, and characterized biochemically the recombinant enzyme and found spectroscopic and kinetic properties consistent with those reported for fungal laccases. We show that both the spore-associated enzyme or the purified protein are remarkably heat stable. CotA is naturally associated with the coat structure in an active form, and expression of cotA from a multicopy plasmid results in spores with greatly increased levels of CotA. Therefore, we suggest that the B. subtilis endospore coat structure can be used as a surface display system for biocatalyst applications involving the highly stable CotA laccase.

Materials and Methods

Structure of CotA by Comparative Modeling Techniques—The structures of ascorbate oxidase from zucchini (Ref. 22; FDB code 1AOZ) and laccase from Coprinus cinereus (Ref. 23; FDB code 1A65) were used to derive a structural model of CotA by comparative modeling techniques. The program Modeler version 4 (24) was used for this purpose. The ZAO and laccase structures were superimposed to generate a sequence alignment that reflected the equivalence of residues in the structure (that may differ from common sequence alignments). The CotA sequence was then aligned against the primary alignment. Because of the low sequence identity, this alignment had a considerable number of ambiguities which were taken into account in the final model (as “Results”).

The initial alignment of CotA was used to generate structural models that were then checked using two criteria, identification of zones displaying restraint violations using Modeler and checking several stereochemical and conformational criteria using PROCHECK (25). The alignment was changed to correct these problems, and a new cycle was started. Several of these cycles were performed to optimize the alignment and the structural models obtained. Forty structural models were generated with the final alignment and the model displaying the lowest value of the objective function was chosen as the final structural model for CotA.

Structure of CotA Mutants by Comparative Modeling Techniques—The same procedure used for the wild type was implemented to model the structures of the proteins bearing point mutations in the T1 center. The optimized alignment of the wild type was changed in the specified position of the mutation, and the model with the lowest value of the objective function was chosen from 40 generated structural models. Two mutants are described in this work, H479A and M502L, both affecting the type I copper site. For modeling the structure of the M502L mutant, mutants are described in this work, H479A and M502L, both affecting the type I copper site. For modeling the structure of the M502L mutant, the same procedure used for the wild type was implemented to model the structural model for CotA.

Briefly, the coat proteins were extracted from 2–580 units of purified plasmid pLOM8, pLOM9, and pLOM2 with selection for chloramphenicol resistance (Cm'). Transformants were expected to arise as the result of a single reciprocal recombination event (Campbell-type mechanism) between the cloned DNA and the corresponding region of homology in the host chromosome (see Fig. 1). For each cross, transformants were identified by the expected structure in the vicinities of the locus and expressed the wild type, cotA1477A, or cotA5060L, alleles, respectively (Table I).

Construction of a B. subtilis Strain Bearing a Multicopy Allele of cotA—Primers cotAa107D (5'-CCGGCTGACGACGAGAATTTTGTGG-3') and cotAa1892R (see above) were used to PCR-amplify a 1785-bp fragment extending 55 bp upstream of the cotA transcription initiation site. The resulting PCR product encompassed the entire cotA gene including its promoter flanked by engineered PstI and BglII sites (Fig. 1). The PCR product was digested and cloned between the PstI and BamHI sites of plasmid pMK3 (28), thereby creating the replicative plasmid pMK3 (28), thereby creating the replicative plasmid pTc68. This plasmid was introduced into the wild type strain MB24 by transformation following by selection for neomycin resistance. This produced strain AH2734, a congeneric derivative of MB24 that bears a multicopy allele of the cotA gene (Table I).

Purification of Spores and Extraction of Spore Coat Proteins—Male spores were harvested 24 h after the onset of sporulation and subjected to a step gradient of Renwal-76 (Bristol-Myers Squibb Co.) for purification (4). When specified, spores were harvested after treatment of the cultures with lysosome (12.5 units/ml for 10 min at 37°C). Occasionally a drop of chloroform was added to facilitate lysis of the mother cell. The analysis of the cotA gene and the coat proteins were done as previously described (4).

Briefly, the coat proteins were extracted from 2 A550 units of purified spores by boiling the suspension for 8 min in the presence of 125 mM Tris, 4% SDS, 10% (v/v) 2-mercaptoethanol, 1 mM dithiothreitol, 0.05% bromophenol blue, 10% glycerol at pH 6.8 (4). The extracted proteins were resolved on 15% SDS-PAGE. The gels were then stained with Coomassie Blue, destained, and scanned for analysis.

Overproduction and Purification of CotA—The cotA gene was amplified by PCR using oligonucleotides cotA159D (5'-CTATGCTAGTCTAGTTTGAAATTAG-3') and cotA1892R (see above). The 1733-bp-long PCR product was digested with BglII and SpeI and inserted between the BamHI and NheI sites of plasmid pET21a (+) (Novagen) to yield plasmid pLOM10. Introduction of pLOM10 into the E. coli strain Tuner (DE3) (Novagen) produced strain AH3517 (Table I) in which the cotA protein could be produced under the control of the T7lac promoter. Strain

**Bacillus subtilis CotA Is a Highly Stable Laccase**

AH3517 was grown in LB medium supplemented with 0.25 mM CuCl₂ at 30 °C. Growth was followed until the midlog phase (A600nm = 0.3), at which time 1 mM IPTG was added to the culture medium. Incubation was continued for further 3-4 h. Cells were harvested by centrifugation (8,000 × g, 15 min, 4 °C). The cell sediment was suspended in Tris-HCl (20 mM, pH 7.6) containing DNase I (10 µg/ml extract), MgCl₂ (5 mM), and a mixture of protease inhibitors (Complete™, mini EDTA-free tablets, Roche Molecular Biochemicals). Cells were disrupted in a French pressure cell (at 19,000 p.s.i.) followed by ultracentrifugation (40,000 × g, 1 h, 4 °C) to remove cell debris and membranes. The resulting soluble extract was loaded onto an ion exchange SP-Sepharose column (bed volume 25 ml) equilibrated with Tris-HCl (20 mM, pH 7.6). Elution was carried out with a two-step linear NaCl gradient (0–0.5 and 0.5–1 M) in the same buffer. Fractions containing laccase-like activity were pooled, concentrated by ultrafiltration (cutoff of 30 kDa), and equilibrated to 20 mM Tris-HCl (pH 7.6). The resulting sample was applied to a MonoS HR5/5 Column (Amersham Biosciences). Elution was carried out with a two-step linear NaCl gradient (0–0.5 and 0.5–1 M). The active fractions were pooled and desalted. After boiling for 10 min in loading dye (see also "Results"), a single protein band of 65 kDa was revealed by SDS-PAGE (12.5%). All purification steps were carried out at room temperature in a fast protein liquid chromatography system (Akta fast protein liquid chromatography, Amersham Biosciences).

**Enzyme Assays—Laccase activity was routinely assayed at 37 °C using the ABTS or SGZ substrates as follows.** (a) The assay mixture contained 1 mM ABTS and 100 mM citrate-phosphate buffer (pH 4). Oxidation of ABTS was followed by the absorbance increase at 420 nm (ε = 36,000 M⁻¹ cm⁻¹). (b) The assay mixture contained 0.1 mM SGZ (dissolved in ethanol) ABTS and 100 mM citrate-phosphate buffer (pH 6). Oxidation of SGZ was followed by the absorbance increase at 525 nm (ε = 65,000 M⁻¹ cm⁻¹). The copper requirement was tested by adding CuCl₂ (0–1 mM) to the standard assay mixtures. Enzyme activity measurements were performed either on a Beckman DU6500 spectrophotometer (Beckman Instruments) or on a Molecular Devices Spectra Max 340 microplate reader with a 96-well plate. All assays were performed in triplicate. Enzyme-specific activity was expressed in nmol or µmol of substrate (ABTS or SGZ) oxidized/min/mg of protein or I₅₈₀ of a spore suspension. The protein content was determined by the Bradford assay (29) using bovine serum albumin as a standard.

**EPR and UV-Visible Spectra of the CotA Protein**—The UV-visible absorption spectrum was obtained at room temperature in 20 mM Tris-HCl buffer (pH 7.6) using a Shimadzu UV 3100 spectrophotometer. EPR spectra were measured with a Bruker ESP-380 spectrometer equipped with an Oxford Instruments ESR-900 continuous-flow helium cryostat. EPR spectra obtained under non-saturating conditions were theoretically simulated using the Asa and Yamanag approach (30).

**Characterization of the CotA Protein**—The effect of pH on the activity of the enzyme was determined at 37 °C in 100 mM citrate-phosphate buffer (pH 3.0–7.0) and 100 mM potassium phosphate buffer (pH 7.0–8.0) for the ABTS or SGZ substrates, respectively. The temperature optimum for the activity was determined at temperatures ranging from 22 to 80 °C by measuring ABTS oxidation. Enzyme thermostability was measured at 80 °C by incubating an appropriate amount of purified enzyme (25 µg) in 20 mM Tris-HCl (pH 7.6) or 11.5 mmol units of a spore suspension in water. At appropriate times, samples were withdrawn, cooled, and examined for residual activity using the ABTS oxidation assay at 37 °C (see above). Kinetic parameters for the purified enzyme were determined at 37 °C by using different concentrations of ABTS (10–200 µM) or SGZ (1–100 µM). The reactions were initiated by the addition of 0.1 µg of purified CotA protein, and initial rates were obtained from the linear portion of the progress curve. Kinetic data were determined from Lineweaver-Burk plots assuming that simple Michaelis-Menten kinetics were followed.

**Other Methods**—The N-terminal amino acid sequence of purified recombinant CotA was determined on an Applied Biosystems protein sequencer (Model 477A) at the Instituto de Tecnologia Química e Biológica microsequencing facility. The copper content of purified recombinant CotA was measured by atomic absorption at the Instituto Superior Técnico (Technical University of Lisbon) chemical analysis facility. The molecular mass of the CotA protein was determined on a gel filtration Superose 6 HR 10/30 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl Ribonuclease (13.7 KDa), chymotrypsinogen A (25 KDa), ovalbumin (43 KDa), albumin (67 KDa), and aldolase (158 KDa) were used as standards. The isolectric point was evaluated in a Phast System (Amersham Biosciences) against broad pH standards following the manufacturer’s instructions.

**RESULTS**

CotA Resembles Ascorbate Oxidase and a Laccase—B. subtilis CotA is significantly similar at the primary structure level with multicopper oxidases, a protein family that includes the laccases (11, 31). Moreover, CotA shows similarity with the two members of this family whose structure is known, having 19.7% sequence identity and 36.6% similarity with zucchini ascorbate oxidase (22) and 22.4% identity and 39.3% similarity with laccase from C. cinereus (23). However, based on sequence comparisons, C. cinereus laccase and ZAO are more closely related to each other (30.6% identity and 50% similarity) than to CotA. Nevertheless, the comparison of the amino acid sequences between CotA and these members of the multicopper oxidase family showed that the copper ligands are conserved in CotA. Submission of the CotA sequence to GenTHREADER (32) revealed, with a confidence level above 99%, both ZAO and laccase as possible folds. Because they rely on various factors beyond sequence similarity, these threading methods reinforced the view that CotA is a member of the multicopper oxidase family. Consequently, the crystal structures of ZAO and laccase from C. cinereus were used to derive a structural model for CotA by comparative modeling techniques. The low overall similarity among CotA, ZAO, and the laccase posed several challenges for deriving its structural model. First, the initial 27 residues of CotA were excluded from the model because they could not be aligned with the sequences of ZAO and laccase (Fig. 2). Additionally, several parts of the CotA sequence correspond to insertions in the alignment and therefore, their final structure was not based on the structural information of the templates, e.g. a large segment of CotA, spanning from residue 83 to residue 98 (arrow on Fig. 3c). Nevertheless, the structure of these regions was still predicted on the basis of additional structural determinants considered by Modeler, even though the significance of the final structures of these
segments, especially the large segment referred above, may be low (Fig. 3, d-e). The analysis of protein molecular surfaces allows the comparison of the relative accessibility of their catalytic T1 copper sites (Fig. 4, d-e). The distance from the copper atom is mapped on the molecular surface as an estimate of the overall exposure of the site. CotA has the highest exposure of the center followed closely by ZAO and laccase structures. Methionine (Met-517) in ZAO is replaced by a leucine (Leu-462) in Bacillus subtilis CotA Is a Highly Stable Laccase (Fig. 4, c). The T1 copper site was modeled based on the structural data from ZAO alone. Note that the available laccase structure is a copper-depleted form in which the putative T2 copper atom is completely absent, possibly due to the use of EDTA; this has unknown consequences for the coordination of the remaining T3 copper atoms. Because CotA contains one methionine in the equivalent position (Fig. 4, b), the T1 copper site was modeled based on the structural data from ZAO alone. The results in Table II show that a suspension of wild type spores (strain MB24) oxidized about 24.5 nmol of ABTS/min/optical unit of spore suspension and about 9.5 nmol of SGZ/min/optical unit of spore suspension. The oxidation of SGZ or ABTS was about 2- and 4-fold stimulated (to 82.4 and 16.2 nmol/min/optical unit of spore suspension) when the spore suspensions were preincubated (to 82.4 and 16.2 nmol/min/optical unit of spore suspension). The ABTS- or SGZ-oxidizing activity was dependent on an insertional disruption of the cotA locus (Table II, strain AH76). The insertional mutant (strains MB24 and H11001) produced by a cotA insertion results in Enzyme Activity and Pigment Formation but Not the Assembly of CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in CotA to the endospore coat layers.
layers. Mutations in the reactive T1 copper center were designed (Fig. 4, d and e). Ligands involved in T1 copper binding are two histidines, a cysteine, and a fourth ligand that varies between the members of the multicopper oxidase family (36). Laccase of *C. cinereus* possesses a leucine (Leu-462) at this position, a residue that is not expected to coordinate with copper, thus leading to a tri-coordinate T1 planar center (Fig. 4b; Ref. 23). In ascorbate oxidase a methionine (Met-517) is present, forming a long S-Cu bond leading to a distorted tetrahedral coordination geometry (see Fig. 4a; Ref. 25). In the equivalent sequence position CotA has a methionine residue (Met-502) and, thus, presents a T1 copper site similar to that of ZAO but also to other fungal laccases (11) (Fig. 4c). The single amino acid substitution of histidine 497 to an alanine (H497A) should impair the copper coordination by the surface-exposed T1 center, thus altering drastically the enzymological properties of the protein (Fig. 4d). Because methionine was found to be a non-essential component of the blue copper site, the replacement of this residue at position 502 to a non-ligating leucine (M502L, Fig. 4e) was not expected to affect copper coordination. The mutations H497A and M502L were made in *vitro* in an integrational plasmid and transferred to the cotA locus by a single reciprocal crossover (Campbell-type recombination), as described under “Materials and Methods” (see also Fig. 1). To determine whether the cotA point mutations allowed normal synthesis and assembly of the CotAH497A and CotAM502L isoforms, we analyzed the coat polypeptide composition of purified spores produced by strains AH3512 (CotAH497A), AH3513 (CotAM502L), and AH3514 (CotAWT). CotA accumulated to wild type levels in spores produced by strains AH3512 and AH3514 (Fig. 5B, lanes 3–4) and to somewhat reduced levels in AH3513 spores, in which the CotAM502L form is produced (lane 5). None of the integrations into the cotA locus otherwise altered the pattern of extractable coat polypeptides (Fig. 5B). Strain AH3514 formed the characteristic dark brown colonies on DSM plates (Fig. 5A). However, strains AH3512 (CotAH497A) and AH3513 (CotAM502L) failed to develop the dark brown phenotype typical of the congenic wild type strain and were in that respect indistinguishable from the cotA null mutant. We then screened the spores for their capacity to oxidize ABTS or SGZ. The results in Table II show that strains AH3512 and AH3513 formed spores that are unable, like those of the cotA null mutant (AH76), to oxidize either substrate. We infer that CotA, and not a putative protein whose assembly could rely on cotA expression, is directly involved in pigment formation.

Overproduction and Purification of Recombinant CotA—Extraction of proteins from the endospore coat layers normally involves boiling of a spore suspension in the presence of SDS and high concentrations of reducing agents (2), which may interfere with the activity or otherwise modify the extracted polypeptides. Therefore, to analyze the properties of the CotA laccase, we first constructed an *E. coli* strain, AH3517, in which expression of cotA could be driven upon IPTG induction of the strong T7lac promoter. SDS-PAGE analysis of crude extracts from AH3517 revealed that the addition of IPTG to the culture resulted in the accumulation of a protein band at 65 kDa (Fig. 6A, lanes 3 and 4) that was absent in extracts prepared from

---

**FIG. 3.** Overall fold and metal centers of the proteins and their molecular surfaces. The folds are represented using *ribbons*, and the copper ions plus their non-protein ligands are represented by Corey-Pauling Koltun. Copper is colored *yellow*, and oxygen is colored *red*. The molecular surfaces are colored according to the distance from the type 1 copper atom. *Green zones* correspond to those close to this copper center. The folds and the molecular surfaces represent the proteins in the same orientation, with the type 1 copper center facing out. a, ascorbate oxidase fold; b, laccase fold; c, CotA fold; d, molecular surface of ZAO; e, molecular surface of laccase; f, molecular surface of CotA. The figures were prepared using Molscript (33), GRASP (34) and Raster 3D (35).
the strain AH3520, harboring the cloning vector pET21a(+) (Fig. 6A, lanes 1 or 2). The 65-kDa band was also absent from extracts of uninduced AH3517 (not shown).

Most of the overexpressed CotA protein was, however, found in the insoluble fraction obtained after centrifugation of the broken cell suspension, presumably in the form of inclusion bodies (Fig. 6A, lane 4). Attempts to recover the soluble protein from this fraction were unsuccessful. Consequently, work proceeded with the fraction containing the soluble protein, which contains about 10% of all the CotA produced by the recombinant E. coli strain (Fig. 6A, lane 3). Laccase activity in this fraction was shown to be dependent on the copper supplementation of the culture medium. In cell-free extracts of E. coli AH3517 grown in unsupplemented-copper LB medium, enzyme activity was only detected upon preincubation of the cell crude extract with copper (maximal activity of 0.197 \( \mu \text{mol min}^{-1} \text{mg}^{-1} \) of protein) in the presence of 0.25 \( \text{mM} \) CuCl\(_2\) (data not shown). In contrast, a significantly higher activity for the oxidation of ABTS (1.28 \( \mu \text{mol min}^{-1} \text{mg}^{-1} \)) was measured in cell-free extracts of the recombinant E. coli strain grown in the presence of exogenously added CuCl\(_2\) (optimal concentration 0.25 mM). The specific activity in these crude extracts was not affected by the addition of copper (tested in the range 0–1 mM) (data not shown).

CotA was purified using two chromatographic steps as described under “Materials and Methods.” A 195-fold purification

![FIG. 4. Close-up of the type 1 copper site in the different structures and mutants. Only the main fold of the protein (represented by a smooth Cα tracing), the copper atom, and its ligands are represented. In d and e, the mutated residues are labeled in red. a, ascorbate oxidase; b, laccase; c, CotA; d, CotA H497A mutant; e, CotA M502L mutant. The figures were prepared using Molscript (33), GRASP (34), and Raster 3D (35).](image)

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuner (DE3)</td>
<td>PT7lac promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>AH3520</td>
<td>Tuner (DE3) (pET21a(+))</td>
<td>This work</td>
</tr>
<tr>
<td>AH3617</td>
<td>Tuner (DE3) (pLOM10)</td>
<td>This work</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB24</td>
<td>trpC2 metC3/Spo(^+) (wild type)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>AH76</td>
<td>trpC2 metC3 cotA::cmr/Cmr</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>AH3512</td>
<td>MB24/pLOM8/Sp(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>AH3513</td>
<td>MB24/pLOM9/Sp(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>AH3514</td>
<td>MB24/pLOM2/Sp(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>AH2734</td>
<td>MB24 (pTC66)/Sp(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>AH2350</td>
<td>MB24 (pMK3)/Sp(^-)</td>
<td>Ref. 29</td>
</tr>
</tbody>
</table>

\(^a\) Replicative plasmids are shown in parenthesis.

\(^b\) The omega symbol (\(\omega\)) denotes that the plasmid was integrated into the B. subtilis chromosome by a Campbell integration event (single reciprocal crossover) in the cotA region of homology.
was achieved compared with the cell-free cytoplasmic extract, and CotA corresponded to ~2% of the total protein. The migration of purified CotA in SDS-PAGE varied with the heat treatment of the sample. Only an apparent 30-kDa form was seen if the purified protein was treated at room temperature in denaturing buffer containing SDS (1%) and 2-mercaptoethanol (2%) (Fig. 6B, lane 1). However, treatment of the purified protein in denaturing buffer for 5 min at 65, 80, and 100 °C caused the appearance of a 65-kDa species (Fig. 6B, lanes 2–4), which was the single form of CotA detected after a 10-min incubation of the sample at 100 °C (Fig. 6B, lane 5). The N-terminal sequence of both the 30- and 65-kDa forms was found to be identical to that of the B. subtilis native CotA (data not shown). The molecular mass deduced from the cotA gene sequence as assessed by its ability to migrate as a single band according to its predicted mass (65 kDa) in SDS-PAGE (Fig. 5), prompted us to examine its resistance to thermal inactivation. We examined the thermal denaturation profile of purified CotA as well as of a spore suspension of wild type spores. We found that purified recombinant CotA protein has a half-life of inactivation of about 112 min at 80 °C (Fig. 8A). Upon incubation of a suspension of wild type spores at 80 °C, an initial period of enzyme activation was detected (Fig. 8B). After the activation period, a deactivation following first-order kinetics was observed (insert in Fig. 8B). The rate constant was calculated (k = 0.0056 min⁻¹), and a half-life of inactivation of 124 min was determined; if the initial 100-min period of activation is considered, a t₁/₂ of 224 min can be estimated for the CotA coat-associated protein.

Overexpression of cotA in B. subtilis Results in Spores with Increased Levels of CotA—The remarkable thermostability of CotA and the fact that laccases have a wide range of potential biotechnological applications suggested to us that spores could be developed as biocatalysts. To determine whether the level of CotA protein assembled into the coats could be increased, we constructed a B. subtilis strain bearing a complete copy of the cotA gene in a replicative plasmid (AH2734, Table I). When

by these matrices. The T2 copper center is the most accessible to oxygen ligands and to anionic inhibitors and is reportedly a labile copper atom in the multicopper oxidases (23). The EPR CotA protein spectrum (Fig. 7B, trace a) shows resonances characteristic of T1 and T2 copper centers. The resonances were deconvoluted by theoretical simulation of the spectrum obtained under non-saturating conditions. The type I copper center has g values of 2.227, 2.059, and 2.033 and a hyperfine coupling constant (A₉) of 7.2 × 10⁻⁴ cm⁻¹ (Fig. 7B, trace c), whereas the type II center presents g values of 2.344 and 2.078 and A₉ = 102 × 10⁻⁴ cm⁻¹ (Fig. 7B, trace d). The two copper centers are present in a 1:1 ratio, as deduced by comparing the experimental spectrum with the sum of the individual spectral components (Fig. 7B, trace a and b). The type II center has a hyperfine coupling constant lower than those reported for similar centers. The integration of the EPR spectrum for CotA should be regarded as preliminary, since the recombinant protein has a sub-stoichiometric copper content. Both UV-visible and EPR features are typical of multicopper oxidases (15). Catalytic Properties of Recombinant CotA—The dependence of the rate on the substrate concentration followed Michaelis-Menten kinetics. From Lineweaver-Burk plots, Kₘ and Vₘₐₓ values were determined at 37 °C for the recombinant enzyme in 100 mM citrate-phosphate buffer toward ABTS (at pH 4.0) and SGZ (at pH 6.0). The kinetic values herein reported were determined in air-saturated solutions. With ABTS as substrate, apparent Kₘ values of 106 ± 11 μM and 22 ± 6 μmol min⁻¹ mg⁻¹ of protein were determined. For SGZ as substrate, these constants were 26 ± 2 μM and 4 ± 1 μmol min⁻¹ mg⁻¹ of protein, respectively. Assuming a molecular mass of 65 kDa, turnover numbers (Vₘₐₓ values) of 16.8 ± 0.8 and 3.7 ± 0.1 s⁻¹ were calculated for ABTS and SGZ, respectively. The CotA recombinant protein exhibited maximal activity for ABTS and for SGZ oxidation, as measured at 37 °C, at pH ≤ 3.0 and 7.0, respectively (data not shown). A similar pH profile was measured for the CotA assembled in spore coat of B. subtilis (data not shown). Thus, assembly of CotA into the endospore coat structure does not change its pH dependence for oxidation of ABTS or SGZ.

The temperature dependence activity for both CotA recombinant and the spore coat enzyme, measured between 25 and 80 °C, showed an optimum at 75 °C (Fig. 8A). The observation that purified CotA was partially resistant to heat denaturation, as assessed by its ability to migrate as a single band according to its predicted mass (65 kDa) in SDS-PAGE (Fig. 5), prompted us to examine its resistance to thermal inactivation. We examined the thermal denaturation profile of purified CotA as well as of a spore suspension of wild type spores. We found that purified recombinant CotA protein has a half-life of inactivation of about 112 min at 80 °C (Fig. 8B). Upon incubation of a suspension of wild type spores at 80 °C, an initial period of enzyme activation was detected (Fig. 8B). After the activation period, a deactivation following first-order kinetics was observed (insert in Fig. 8B). The rate constant was calculated (k = 0.0056 min⁻¹), and a half-life of inactivation of 124 min was determined; if the initial 100-min period of activation is considered, a t₁/₂ of 224 min can be estimated for the CotA coat-associated protein.

Overexpression of cotA in B. subtilis Results in Spores with Increased Levels of CotA—The remarkable thermostability of CotA and the fact that laccases have a wide range of potential biotechnological applications suggested to us that spores could be developed as biocatalysts. To determine whether the level of CotA protein assembled into the coats could be increased, we constructed a B. subtilis strain bearing a complete copy of the cotA gene in a replicative plasmid (AH2734, Table I). When

**Fig. 5.** Plate phenotype and profile of spore coat proteins extracted from different B. subtilis strains. A, pigmentation phenotype presented by strains bearing the following cotA alleles. 1, wild type (strain AH3514); 2, cotAΔ::cat (strain AH76); 3, cotAΔH₄⁴⁷Δ (strain AH3512); 4, cotAΔH₄⁴⁷ΔH₂⁴⁶ (strain AH3513); 5, wild type (strain AH2350). B, profile of spore coat proteins extracted from spores produced by strains bearing various cotA alleles. 1, wild type (strain MB24); 2, cotAΔ::cat (AH76); 3, wild type (AH3514); 4, cotAΔH₄⁴⁷Δ (AH3512); 5, cotAΔH₂⁴⁶Δ (AH3513). Strain AH3514 (wild type cotA) differs from MB24 in that although it expresses a wild type cotA allele, it results from the integration of pLOM2 into the cotA region (see also the Fig. 1). C, profile of proteins solubilized from the outer layer of spores produced by strains bearing a multicopy allele of the cotA gene (strain AH2734, lane 2) or the parental vector pMK3 (strain AH2350, lane 1). For panels B and C, the extracted coat proteins were resolved on 15% SDS-PAGE gels, as described under “Materials and Methods” section.
cultivated in DSM liquid media, wild type *B. subtilis* cells undergo lysis from 8 h after the initiation of sporulation onward, a process that releases the free mature spore into the environment (2). Strain AH2734 did not show significant numbers of free spores 24 h and even 48 h after the onset of sporulation (not shown). Presumably, overproduction of CotA causes copper depletion and interferes with the process of mother-cell lysis by an as yet unknown mechanism. Nevertheless, cells were harvested by centrifugation at 48 h and treated with lysozyme and chloroform, and the released spores were then purified on density gradients (see "Materials and Methods"). The SDS-PAGE analysis of the coat protein composition of AH2734 spores revealed higher amounts of CotA as compared with the wild type strain (Fig. 5C). However, the specific activity for the oxidation of ABTS was lower by using spores of this overproducing strain as compared with the wild type spores (Table III). These results led us to suspect that overproduced CotA was assembled in the spore coat in a copper-dependent and, therefore, inactive form. To test this hypothesis the spore suspension was preincubated with copper (0.25 mM CuCl₂) and then assayed for activity. This treatment resulted in 77-fold activation (as measured by ABTS oxidation), significantly higher than the activation (3-fold) observed for wild type spores subjected to the same treatment (Table III). Cultivation of strain AH2734 in a media supplemented with copper (0.25 mM) resulted in the efficient release of free spores 48 h after the onset of sporulation (not shown). When the cells were grown in the presence of copper, the ABTS-oxidizing activity was still higher for AH2734 spores as compared with the wild type, but further addition of copper to the spore suspension did not improve ABTS oxidation by AH2734 or wild type spores (Table III). This may be due to copper inhibition of the enzyme activity (38) or to a more complex interaction of copper with the coat structure. In any case, the results show that the coat system is permissible to the assembly of increased levels of CotA and that, under the appropriate conditions, these spores show increased laccase activity. Interestingly, expression of the cotA gene from the multicopy plasmid also resulted in sporulating colonies with a darker brown phenotype relative to wild type colonies on DSM plates supplemented with copper (50 μM CuCl₂; Fig. 5A, compare colonies labeled 5 and 6), an observation that reinforces the notion that CotA is directly involved in pigment formation.

**Table II**

<table>
<thead>
<tr>
<th>Strains</th>
<th>ABTS</th>
<th>SGZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 mm Cu²⁺</td>
<td>No Cu²⁺</td>
</tr>
<tr>
<td>MB24</td>
<td>82.4</td>
<td>24.5</td>
</tr>
<tr>
<td>AH3514</td>
<td>20.0</td>
<td>ND</td>
</tr>
<tr>
<td>AH76 (cotA::cat)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AH3512 (cotA^K631A)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AH3513 (cotA^K1121)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Not determined, ND, not detected.

![Figure 6](image)

**Fig. 6.** A, SDS-PAGE analysis of CotA overproduction in *E. coli*. Lane 1, crude extract of a *E. coli* AH3520 culture. Lane 2, crude extract of an IPTG-induced *E. coli* AH3517 culture. Lane 3, supernatant of a crude extract of an IPTG-induced *E. coli* AH3517 culture. The band indicated by the arrow is the CotA protein. B, SDS-polyacrylamide gel analysis of CotA purified from recombinant *E. coli* AH3517. Samples of purified CotA were incubated with an equal volume of SDS (1%) and 2-mercaptoethanol (1%) for 5 min at room temperature (lane 1), 65 °C (lane 2), 80 °C (lane 3), 100 °C (lane 4), and for 10 min at 100 °C (lane 5) before electrophoresis using 12.5% (w/v) acrylamide containing SDS (0.1%, w/v).

**DISCUSSION**

In this study we provide the first biochemical characterization of a bacterial laccase. Fungal and plant laccases have been extensively described and characterized (15). Several bacterial genomes encode predicted proteins with similarity to laccases (11) and, in addition to *B. subtilis* (Refs. 12, 13, 39, and this work), laccase-like activity has been reported in three other bacterial species (18–21). These observations suggest that laccases are widespread in bacteria (11), but to date no bacterial laccase has been examined in detail.

We offer evidence that the CotA protein of *B. subtilis*, which occurs as an abundant structural component of the endospore coat, shares strong structural features with multicopper oxides, as our modeling studies indicate that CotA is likely to have the same overall fold of zucchini ascorbate oxidase (22) and the laccase from the *C. cinereus* fungus (23). Wild type spores, but not those of a cotA deletion mutant, present full enzymatic activity toward the laccase substrates ABTS and SGZ upon copper addition to the enzymatic assay, indicating that copper is involved in the catalytic process. Single amino acid substitutions (H497A or M502L) in the T1 (blue copper center) of CotA completely abrogate this activity. The H497A and M502L single amino acid substitution was expected to abolish laccase activity, since the conserved His-497 directly participates in the coordination of the copper atom at the T1 center (22, 23). However, the reason for the lack of activity of the M502L form remains unclear, as leucine is found at the homologous position in other studied laccases (36). More detailed biochemical and structural studies will be required to understand this result. Because both mutant forms of CotA are still competent for assembly, we infer that the point mutations are unlikely to significantly interfere with the overall fold of the proteins and that CotA is the sole protein contributing to this oxidase activity in the spore coats. In addition, we observed that strains producing the mutated proteins fail to accumulate the dark brown pigment typical of *B. subtilis* colonies reaching the late stages of sporulation, in that respect indistinguishable from the cotA null mutant spores. These findings establish that CotA oxidase activity is directly required for formation of the spore...
pigment, which appears to be a melanin (13). Laccases have been implicated in melanin synthesis in a variety of fungal species (40–43), and the first reported bacterial laccase from the soil bacterium A. lipoferum, has also been linked to the production of a dark-brown pigment that could be a melanin (18, 44). The synthesis and accumulation of B. subtilis spore pigment, which requires CotA, is known to contribute to spore resistance against UV light and hydrogen peroxide since brownish pigmented spores were significantly more resistant than the cotA null mutant spores to H₂O₂, UVB, UVA, and simulated solar light (13). Additional biological functions can possibly be envisaged to CotA, since the broad substrate specificity of laccases is reflected in a variety of biological roles (15, 17). For example, when compared with C. cinereus laccase, CotA appears to have a more accessible catalytic T1 copper site, suggesting that CotA may use larger reduced substrates. One attractive possibility is that CotA promotes the oxidative cross-linking of other endospore coat structural components. In this regard, we note that o,o-dityrosine cross-links have been detected in purified coat material (1, 2) and oxidative cross-linking of the coat component, CotG, which is tyrosine-rich (11%), has already been proposed (45). In fact, in plants there is evidence for a potential role of laccases in the oxidative process of lignification (46), and in fungi a possible relation of laccase production to mycelial growth was also reported (17).
We found that CotA was properly synthesized and folded in E. coli, and the biochemical and kinetic properties determined (N-terminal amino acid sequence, molecular mass, enzymatic activity, and activity dependence on temperature and pH) were similar for the purified recombinant CotA enzyme and for the native coat-associated enzyme. Moreover, the properties of the purified recombinant enzyme are comparable with those described for well studied fungal laccases (15, 17). It presents the typical blue color that characterizes all the blue oxidases due to its absorbance at 600 nm and a band at 330 nm, presumably due to the T3 binuclear center. The EPR signature is similar to those of other laccases (15), namely showing the presence of a stoichiometric amount of T1 and T2 copper centers. Taking together the spectroscopic data strongly suggests the presence in CotA of all four copper (II) ions typical of laccases. CotA is a monomeric protein with a molecular mass of 65 kDa, and with few exceptions, all laccases that have been analyzed for this property consist of a single subunit with molecular masses ranging between 60 and 80 kDa (15, 17). Although significant differences exist among laccases with regard to thermodynamic and kinetic properties, the $K_m$ and $k_{cat}$ of CotA calculated toward ABTS and SGZ were within the ranges determined for fungal laccases (18, 52). CotA exhibits a neutral pI (7.7), whereas most of the laccases characterized showed a pI in a pH range from 3 to 5. The pH activity profiles for these two substrates, a monotonically decrease as pH increases for the ABTS and a bell-shape profile for SGZ oxidation, are consistent with those exhibited by well studied fungal laccases (47, 48).

A striking feature of this enzyme is the high optimal temperature for activity, ca. 75 °C. Fungal laccases usually have optimal temperatures for activity between 30 and 60 °C, the optimal temperature range for activity of a laccase produced from the thermophilic fungus Chaetomium thermophilum was 50–60 °C (49). However, the most remarkable property exhibited by CotA was its high intrinsic thermostability. Deletion of cotA does not change the heat resistance properties of the B. subtilis endospore (8). However, we reasoned that the assembly of CotA into the coat layers of the endospore, a structure inherently highly resistant to heat (1, 2), could change its ability to resist to thermal denaturation. We found half-lives of inactivation ($t_{1/2}$) of 112 min and 220 min at 80 °C for the purified and for the coat-associated protein, respectively, although the rate constants of thermal deactivation were identical in both cases. The half-life for the coat-associated enzyme is longer, since it includes the initial activation period (Fig. 8). Possibly, the immobilization of CotA in the coat structure impairs its activity, and the increase in temperature tends to loose the structure, allowing CotA to approach the properties of the enzyme in solution. These results clearly indicate that the remarkable thermostability of CotA is intrinsic to the protein and not a function of its immobilization within the coat layers. To our knowledge no other characterized laccase is capable of withstanding heat denaturation as CotA. For example, laccases purified from the thermophiles Myceliophthora thermophila and Scytalidium thermophilum did not resist a 1-h period of incubation at 80 °C (47), whereas the laccase purified from C. thermophilum is stable only 8 min after incubation at 80 °C (49).

Last, we have shown that the amount of CotA associated with the coat structure can be greatly increased by expressing the cotA gene from a replicative plasmid. It has been previously suggested that CotA is associated with the outer coat on the basis of its absence from the collection of polypeptides extracted from spores of a cotE mutant, which fail to assemble the outer coat structure (9). We found no SGZ- or ABTS-oxidizing activity in spores of a cotE mutant, confirming that CotA is exclusively localized in the outer coat layers of the spore (this work, results not shown). Hence, CotA and the laccase activity associated with wild type spores is localized to the surface layers of the endospore. Spores have been used for the presentation of biologically active heterologous proteins with applications in vaccine development (26). The possibility of manipulating the levels of CotA displayed at the surface of the B. subtilis endospore suggests that spores can be used as biocatalyst vehicles in biotechnological applications demanding high levels of immobilized thermostable laccase.

Acknowledgments—We thank Charles P. Moran Jr. for advice and continuous support of this work. We thank C. P. Moran and Karina B. Xavier for critically reading the manuscript. We also thank C. Peixoto for help with the determination of the pI of CotA.

REFERENCES