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

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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 mutant proteins into the coat and did not alter the pattern of extractable coat polypeptides. However, in contrast to a wild type strain, both mutants produced unpigmented colonies and spores unable to oxidize syringaldazine (SGZ) and 2′2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The CotA protein was purified to homogeneity from an overproducing *Escherichia coli* strain. The purified CotA shows an absorbance and a EPR spectra typical of blue multicopper oxidases. Optimal enzymatic activity was found at ≤pH 3.0 and at pH 7.0 for ABTS or SGZ oxidation, respectively. The apparent K_m values for ABTS and SGZ at 37 °C were of 106 ± 11 and 26 ± 2 μM, respectively, with corresponding k_{cat} values of 16.8 ± 0.8 and 3.7 ± 0.1 s^{-1}. Maximal enzyme activity was observed at 75 °C with ABTS as substrate. Remarkably, the coat-associated or the purified enzyme showed a half-life of inactivation at 80 °C of about 4 and 2 h, respectively, indicating that CotA is intrinsically highly thermostable.

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).

The structure of the coat results from a multistep assembly process that involves the temporally and spatially regulated synthesis of more than 30 different protein components ranging in size from about 6 to 70 kDa (1–3). Their order of assembly and final destination within the coat layers appears to rely on a complex pattern of specific protein-protein interactions as well as on a variety of post-translational modifications, including proteolytic processing, cross-linking, and glycosylation (1, 2). Several components of the *B. subtilis* spore coat are enzymes, with possible roles in the post-translational modifications that accompany the macromolecular assembly of the coat or in the final resistance properties of the spore structure. For example, a manganese-dependent catalase is a component of the inner coat layers (4, 5), and a transglutaminase associates with the outer coat layers to promote ε-(γ-Glu)Lys cross-linking of specific structural components (6, 7). Another enzyme that appears to associate with the outer coat layers is the 65-kDa product of the *cotA* gene (8–10). CotA belongs to a diverse group of multi-copper "blue" oxidases that includes the laccases (11). Purified wild type *B. subtilis* spores but not those of a *cotA* insertional mutant are able to oxidize the laccase substrates SGZ[1] and ABTS (12, 13). The absence of CotA has no detectable effect on lysozyme resistance or germination, but it does prevent the appearance of a brown pigment characteristic of colonies in the late stages of sporulation (8, 14), which appears to protect spores against UV light (13). Laccases (EC 1.10.3.2) catalyze the oxidation of a variety of aromatic compounds, in particular phenolic substrates, coupled to the reduction of molecular oxygen to water (15). Their catalytic centers consist of three structurally and functionally distinct copper centers. T1 copper ("blue copper"), is a mononuclear center involved in substrate oxidation, whereas both T2 and T3 form a trinuclear center involved in the oxygen reduction to water (15). Laccases are receiving increased attention as a model system for characterizing the structure-function relationship of copper-containing proteins because of their potential for biotechnological applications in fields such as delignification, plant fiber deri-

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[1] The abbreviations used are: SGZ, syringaldazine; ABTS, 2′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).

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 pigment. 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; PDB code 1AOZ) and laccase from *Coprinus cinereus* (Ref. 23; PDB 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 (see "Results").

The initial alignment of CotA was used to generate structural models that were then checked using two criteria, identification of zones displaying restrain 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, H497A and M502L, both affecting the type I copper site. For modeling the structure of the M502L mutant, the structure of the T1 center of laccase (with its ligands) was used.

Bacterial Strains and Culture Conditions—The *B. subtilis* strains used in this study are listed in Table I. They are congenic derivatives of the wild type MB24 strain (Table I). The *Escherichia coli* strain DH5α (laboratory stock) was used for routine cloning procedures, propagation, and amplification of all plasmid constructs. Overproduction of the CotA protein was performed in *E. coli* strain Tuner (DE3) (Novagen). The *E. coli* and *B. subtilis* strains were routinely grown and maintained in LB medium with appropriate antibiotic selection when needed. Sporulation of *B. subtilis* was induced by growth and exhaustion in Difco sporulation medium (4). Whenever required, $CuCl_2$ was added to LB or DSM liquid or solid media, as specified in the text.

Construction of Strains Containing Mutations in the CotA-coding Region—The 3′-end of the wild type *cotA* gene was PCR-amplified from chromosomal DNA of strain MB24 (Table I) using primers *cotA*-1189D (5′-CAGATGCATATATCATGCAATTCAGAGTC-3′) and *cotA*-1892R (5′-TCATGTAGATCTTGTGTGAGCATAAAAAGCAGCTCC-3′). The resulting 703-bp DNA fragment was purified, digested with *Nsi*I and *Bgl*II, and cloned between the same sites in pMS38[2] to produce pLOM2. Plasmid pLOM2 served as a template for site-directed mutagenesis. Single amino acid substitutions in copper center I (histidine 479 to an alanine, H479A, or of methionine 502 to a leucine, M502L) were created using the QuikChange system (Stratagene). Primers *cotA*-HAD (5′-GCCATATTCTAGAGGCGGAAGACTATGACATG-3′) and *cotA*-HAR (5′-CATGTCATAGTCTTCCGCCTCTAGAATATGGC-3′) were used to create the H479A mutation, whereas primers *cotA*-MLD (5′-GCATGAAGACTATGACCTGATGAGACCGATGG-3′) and *cotA*-MLR (5′-CCATCGGTCTCATCAGGTCATAGTCTTCATGC-3′) were used to generate the M502L mutation. The presence of the desired mutations in the resulting plasmids, pLOM8 (carrying the H479A allele) and pLOM9 (bearing the M502L mutation), and the absence of unwanted mutations in other regions of the insert or in pLOM2 (wild type *cotA* sequence) were confirmed by sequencing.

Competent cells of *B. subtilis* MB24 were transformed with plasmids pLOM8, pLOM9, and pLOM2 with selection for chloramphenicol resistance (Cm^r). 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 with the expected structure in the vicinity of the *cotA* locus, as determined by PCR with appropriate primers. Most of the crossovers were expected to occur upstream of the mutation, because the insert in the plasmid extends by about 500 bp upstream of this position and only some 200 bp downstream of this position (see Fig. 1). Crossovers upstream of the mutation would result in cells expressing only the mutant *cotA* allele. To establish that the crossover had generated a full-length mutated copy of the *cotA* gene, the presence of the correct mutations was confirmed by sequencing appropriate PCR fragments derived from the recombinant chromosomes. In addition, the *cotA* locus in cells resulting from integration of pLOM2 (which carries the wild type sequence) was also sequenced to confirm that no other mutations had been serendipitously introduced into the chromosome. Strains AH3512, AH3513, and AH3514 were the result of the Campbell integration of pLOM2, pLOM8, and pLOM9 into the *cotA* locus and express the wild type, $cotA^{H479A}$, or $cotA^{M502L}$ alleles, respectively (Table I).

Construction of a B. subtilis Strain Bearing a Multicopy Allele of cotA—Primers cotA107D (5′-CGGGCTGCAGCACGAAGATTTTTTG-3′) and *cotA*-1892R (see above) were used to PCR-amplify a 1785-bp fragment extending 55 bp upstream of the *cotA* transcription initiation (+1) site (27). The resulting PCR product encompasses the entire *cotA* gene including its promoter flanked by engineered *Pst*I and *Bgl*II sites (Fig. 1). The PCR product was digested and cloned between the *Pst*I and *Bam*HI sites of plasmid pMK3 (28), thereby creating the replicative plasmid pTC66. This plasmid was introduced into the wild type strain MB24 by transformation followed by selection for neomycin resistance. This produced strain AH2734, a congenic derivative of MB24 that bears a multicopy allele of the *cotA* gene (Table I).

Purification of Spores and Extraction of Spore Coat Proteins—Mature spores were harvested 24 h after the onset of sporulation and subjected to a step gradient of Renocal-76 (Bristol-Myers Squibb Co.) for purification (4). When specified, spores were harvested after treatment of the cultures with lysozyme (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 coat polypeptide composition in wild type and various mutant spores was done as previously described (4). Briefly, the coat proteins were extracted from 2 A_{580} 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% bromphenol 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′-CTATAGTACTAGTTTGGAAAATTTAG-3′) and *cotA*-1892R (see above). The 1733-bp-long PCR product was digested with *Bgl*II and *Spe*I and inserted between the *Bam*HI and *Nhe*I sites of plasmid pET21a(+) (Novagen) to yield 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 T7*lac* promoter. Strain

[2] M. Serrano, R. Fior, C. P. Moran, Jr., and A. O. Henriques, unpublished results.

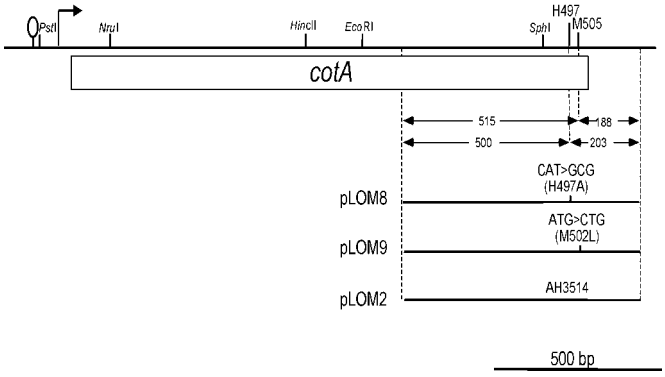

FIG. 1. **The *cotA* region of the *B. subtilis* chromosome.** Position of the *cotA*-coding sequence relative to a partial restriction map of the region. Only selected restriction enzyme sites are shown for reference. The stem and loop structure represented upstream of the *cotA* gene represents a putative transcription terminator for the gene upstream of *cotA*. The *cotA* promoter is represented by a *horizontal arrow*. Transcription of the *cotA* gene is from *left* to *right*. The position of the codons encoding His-497 and Met-502, which are part of the type I copper center, is indicated toward the 3′-end of the gene. The *lines* below the *box* representing the *cotA* open reading frame indicate the extent of the DNA fragments cloned in the indicated plasmids. Plasmid pLOM2 carries wild type *cotA* sequences. pLOM8 carries a CAT to GCG triple mutation that converts H at position 497 into an alanine, whereas pLOM9 carries an A to C transversion that converts methionine at position 502 to a leucine. The nature and position of the mutations is indicated in the *inset*. Plasmids pLOM2, pLOM8, and pLOM9 were transferred to the *B. subtilis cotA* locus via a single reciprocal crossover event (Campbell-type recombination) that placed a functional copy of the gene under the control of its natural promoter. The presence of the desired mutations or of the wild type sequence in the resulting strains was verified by cycle sequencing of appropriate PCR products.

AH3517 was grown in LB medium supplemented with 0.25 mM $CuCl_2$ at 30 °C. Growth was followed until the midlog phase (A_{600} = 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), $MgCl_2$ (5 mM), and a mixture of protease inhibitors (Complete™, mini EDTA-free protease inhibitor mixture 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 (Åkta 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 $CuCl_2$ (0–1 mM) to the standard assay mixtures. Enzyme activity measurements were performed either on a Beckman DU©70 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 /A_{580} 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 Aasa and Vänngard 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 A_{580} 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 mM). 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 Biosystem 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 isoelectric point was evaluated in a Phast System (Amersham Biosciences) against broad pI standards following the manufacture'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 on the alignment, and therefore, their final structure is 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. 3*c*). 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

FIG. 2. **Amino acid sequence alignment of CotA with *C. cinereus* laccase and zucchini ascorbate oxidase used to generate the structural model.** An alignment of the ZAO and laccase primary structures was generated that reflected the equivalence of residues in the two structures. The CotA sequence was then aligned against the primary alignment and used to generate structural models that were checked using Modeler and PROCHECK (Laskowski *et al*. (25); see "Materials and Methods"). The alignment was changed to correct for problems detected using the above-mentioned software. Several of these cycles were performed to optimize the alignment, and the structural models were obtained. The final alignment displayed in the figure was used to derive structural models for CotA, and an optimized model was chosen as the final structural model for CotA (see Fig. 3). *Two dots* indicate similarity, whereas an *asterisk* indicates identity. *Gaps* were introduced to maximize the quality of the final structural model for CotA.

segments, especially the large segment referred above, may be low (Fig. 3*c*). The T1 and T2 copper centers are different in the laccase and ZAO structures. Methionine (Met-517) in ZAO is replaced by a leucine (Leu-462) in *C. cinereus* laccase (Fig. 4, *a* and *b*), which confers a different geometry to the T1 copper center. Because CotA contains one methionine in the equivalent position (Fig. 4), the T1 copper site was modeled based on the ZAO structure 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 co-ordination of the remaining T3 copper atoms. Because the goal is to model CotA in the fully oxidized active state, these copper centers were modeled based on the structural data from ZAO alone. This was restricted to the copper ions and protein and non-protein ligands; the rest of the structure was modeled on the basis of the two x-ray structures.

The resulting model is presented in Fig. 3*c*. The overall fold of CotA is similar to the structure of both proteins but resembles more the structure of laccase (Fig. 3, *a–c*). The analysis of protein molecular surfaces allows the comparison of the relative accessibility of their catalytic T1 copper sites (Fig. 3, *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 laccase, whereas ZAO shows a substantially lower exposure of the center (Fig. 3, *d–e*).

cotA-dependent SGZ and ABTS Oxidation by Purified Spores—Recently Henriques *et al.* (12) and Hullo *et al.* (13) have shown that spore suspensions prepared from a wild type *B. subtilis* strain but not from a *cotA* deletion mutant were able to oxidize the phenolic and non-phenolic laccase substrates SGZ and ABTS in a copper-dependent manner. To quantify these activities in detail, we purified wild type spores and those produced by a *cotA* insertional mutant (strains MB24 and AH76, respectively; Table I) by density gradient centrifugation (see "Materials and Methods"). A protein of about 65 kDa previously identified as the CotA protein by N-terminal sequence analysis (8) accumulated in material purified from the coat layers of MB24 spores (Fig. 5*B*, *lane 1*) but not from AH76 spores (Fig. 5*B*, *lane 2*). 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, respectively) when the spore suspensions were preincubated with Cu^{2+} (optimal concentration 0.25 mM $CuCl_2$; data not shown) for a period no shorter than 15 min (Table II). All the ABTS- or SGZ-oxidizing activity was dependent on an intact *cotA locus* (Table II, strain AH76).

Mutations in the T1 Copper Center Abolish Spore-associated Enzyme Activity and Pigment Formation but Not the Assembly of CotA—The insertional disruption of the *cotA* gene results in unpigmented colonies, suggesting that CotA could be directly involved in pigment formation (Refs. 8 and 13; see also Fig. 5*A*). However, absence of pigmentation and decreased resistance could also be due to the absence of a protein that is recruited by CotA to the endospore coat layers. To distinguish between these possibilities, we sought mutations in *cotA* that would alter enzyme activity while not interfering with the overall fold of the protein and presumably with its assembly into the coat

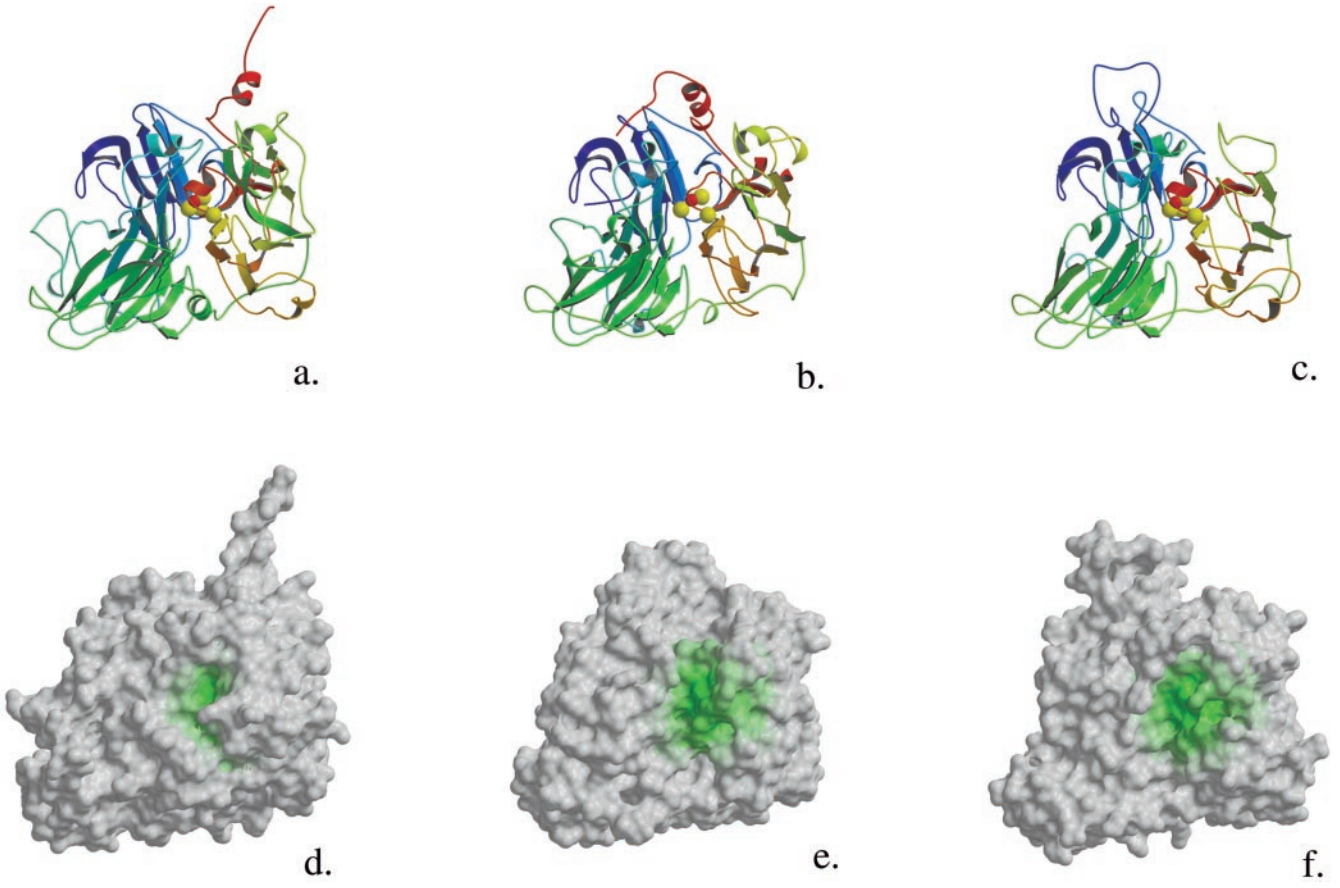

FIG. 3. **Overall fold and metal centers of the proteins and their molecular surfaces.** The folds are represented using *ribbons*, and the copper ions plus their non-protein ligands are represented by Corey-Pauling Koltun. Copper is colored *yellow*, and oxygen is colored *red*. The molecular surfaces 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).

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 co-ordinate with copper, thus leading to a tri-coordinate T1 planar center (Fig. 4*b*; Ref. 23). In ascorbate oxidase a methionine (Met-517) is present, forming a long S-Cu bond leading to a distorted tetrahedral co-ordination geometry (see Fig. 4*a*; 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. 4*c*). 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. 4*d*). Because methionine was found to be a non-obligatory component of the blue copper site, the replacement of this residue at position 502 to a non-ligating leucine (M502L, Fig. 4*e*) 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 CotA^{H497A} and CotA^{M502L} isoforms, we analyzed the coat polypeptide composition of purified spores produced by strains AH3512 (CotA^{H497A}), AH3513 (CotA^{M502L}), and AH3514 (CotA^{WT}). CotA accumulated to wild type levels in spores produced by strains AH3512 and AH3514 (Fig. 5*B*, *lanes 3–4*) and to somewhat reduced levels in AH3513 spores, in which the CotA^{M502L} form is produced (*lane 5*). None of the integrations into the *cotA* locus otherwise altered the pattern of extractable coat polypeptides (Fig. 5*B*). Strain AH3514 formed the characteristic dark brown colonies on DSM plates (Fig. 5*A*). However, strains AH3512 (CotA^{H497A}) and AH3513 (CotA^{M502L}) 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 T7*lac* promoter. SDS-PAGE analysis of crude extracts from AH3517 revealed that the addition of IPTG to the culture resulted in the accumulation of an extra band at 65 kDa (Fig. 6*A*, *lanes 3* and *4*) that was absent in extracts prepared from

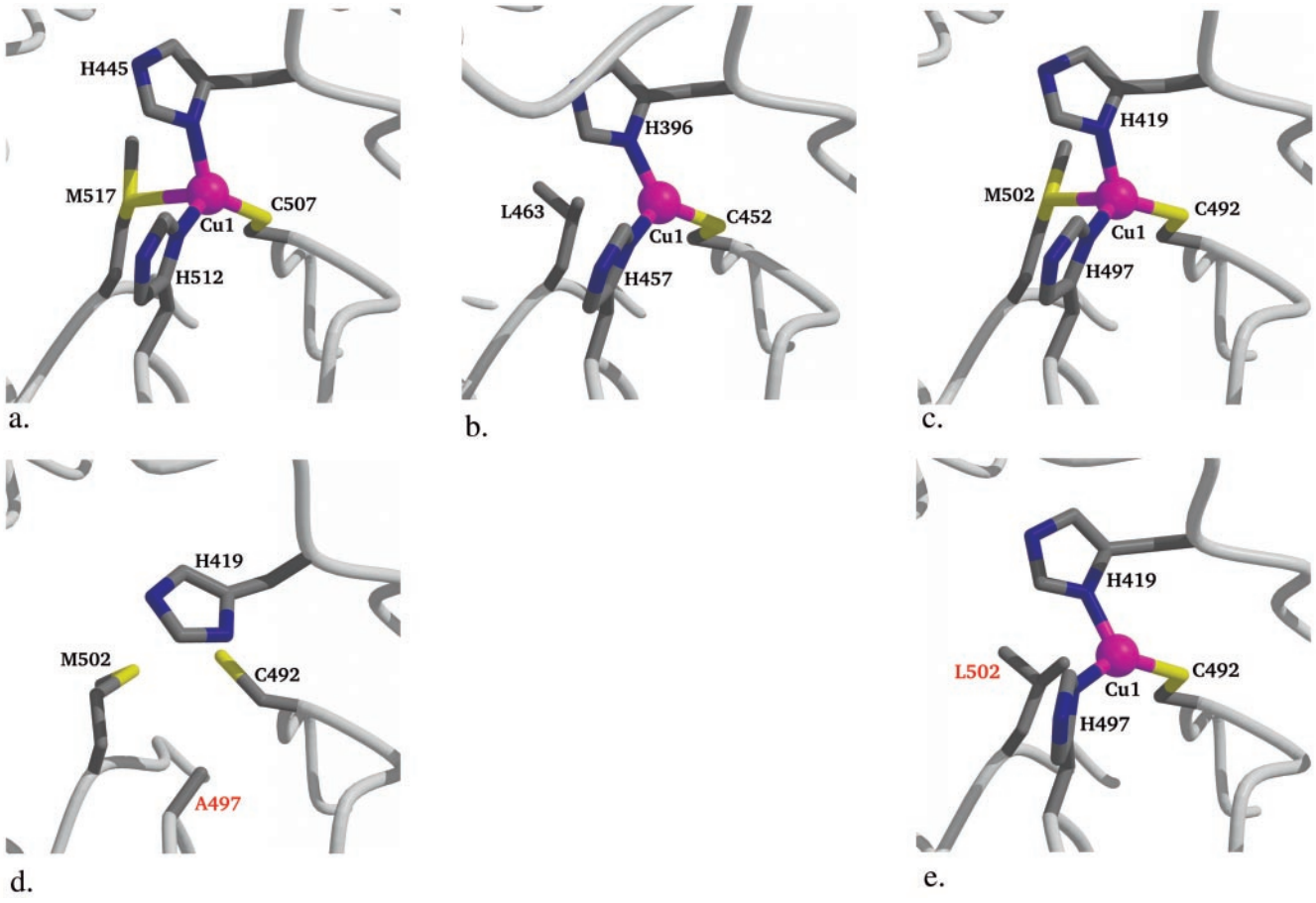

FIG. 4. **Close-up of the type 1 copper site in the different structures and mutants.** Only the main fold of the protein (represented by a smooth Cα tracing), the copper atom, and its ligands are represented. In *d* and *e*, the mutated residues are labeled in *red*. *a*, ascorbate oxidase; *b*, laccase;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

TABLE I
Bacterial strains used in this study

Strain or plasmid	Genotype or properties	Reference or source
E. coli		
Tuner (DE3)	*PT7lac* promoter	Novagen
AH3520	Tuner (DE3) (pET21a(+))[a]	This work
AH3517	Tuner (DE3) (pLOM10)	This work
B. subtilis		
MB24	*trpC2 metC3*/Spo$^+$ (wild type)	Laboratory stock
AH76	*trpC2 metC3 cotA*::*cm*/Cm^r	Laboratory stock
AH3512	MB24ΩpLOM8′[2]/Sp^r	This work
AH3513	MB24ΩpLOM9/Sp^r	This work
AH3514	MB24ΩpLOM2/Sp^r	This work
AH2734	MB24 (pTC66)/Sp^r	This work
AH2350	MB24 (pMK3)/Sp^r	Ref. 29

[a] Replicative plasmids are shown in parenthesis.
[b] The omega symbol (Ω) 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.

the strain AH3520, harboring the cloning vector pET21a-(+) (Fig. 6*A*, *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. 6*A*, *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. 6*A*, *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 μmol·min^{-1} mg of protein^{-1} in the presence of 0.25 mM $CuCl_2$) (data not shown). In contrast, a significantly higher activity for the oxidation of ABTS (1.28 μmol·min^{-1} mg of protein^{-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

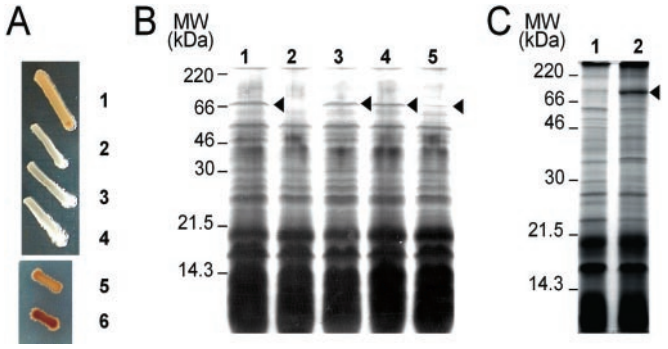

FIG. 5. **Plate phenotype and profile of spore coat proteins extracted from different *B. subtilis* strains.** *A*, pigmentation phenotype presented by strains bearing the following *cotA* alleles. *1*, wild type (strain AH3514); *2*, *cotA*::*cat* (strain AH76); *3*, *cotA*^{H497A} (strain AH3512); *4*, *cotA*^{M502L} (strain AH3513); *5*, wild type (strain AH2350); *6*, multicopy *cotA* (strain AH2734). *B*, profile of spore coat proteins extracted from spores produced by strains bearing various *cotA* alleles. *1*, wild type (MB24); *2*, *cotA*::*cat* (AH76); *3*, wild type (AH3514); *4*, *cotA*^{H497A} (AH3512); *5*, *cotA*^{M502L} (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 coat 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.

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. 6*B*, *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. 6*B*, *lanes 2–4*), which was the single form of CotA detected after a 10-min incubation of the sample at 100 °C (Fig. 6*B*, *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 well as the size of the protein previously identified as CotA on spore coat extracts is 65 kDa (8). Therefore we infer that the 65-kDa form represents the fully denatured protein (Fig. 6*B*) and suggest that the faster migrating 30-kDa species represents a partially unfolded form of the enzyme. Incomplete denaturation by SDS treatment has also been reported for some extremely thermostable enzymes purified from hyperthermophilic microorganisms (37), suggesting that CotA may be thermostable. Analysis of CotA by gel filtration (in the presence of 0.15 M NaCl) gave an M_r value of 53,000 ± 2,400. Together with the electrophoretic data, these results suggest that the enzyme is a monomeric protein, with a high degree of tolerance to heat denaturation. The isoelectric point (pI) of the purified protein was determined to be 7.7.

Spectroscopic Properties of Recombinant CotA—The purified protein exhibited the typical blue color of the multicopper oxidases. The UV-visible spectrum of the purified enzyme (Fig. 7*A*) showed a band at 600 nm (corresponding to the T1 or blue copper center) and a shoulder at ~330 nm (corresponding to the T3 binuclear copper center). A ratio of 1.3 mol of copper/mol of protein was found for recombinant CotA as determined by atomic absorption spectroscopy. This lower copper content may result from the chromatographic method employed for protein purification, as cation-exchange matrices were used, and labile copper atoms in the protein could be captured with high affinity 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. 7*B*, *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 ($A_\parallel$) of 72 × 10^{-4} cm^{-1} (Fig. 7*B*, *trace c*), whereas the type II center presents g values of 2.344 and 2.078 and $A_\parallel$ = 102 × 10^{-4} cm^{-1} (Fig. 7*B*, *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. 7*B*, *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, K_m and V_{max} 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 K_m and V_{max} values of 106 ± 11 μM and 22 ± 6 μmol min^{-1} mg of $protein^{-1}$ were determined. For SGZ as substrate, these constants were 26 ± 2 μM and 4 ± 1 μmol min^{-1} mg of $protein^{-1}$, respectively. Assuming a molecular mass of 65 kDa, turnover numbers (k_{cat} 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. 8*A*). 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. 8*B*). Upon incubation of a suspension of wild type spores at 80 °C, an initial period of enzyme activation was detected (Fig. 8*B*). After the activation period, a deactivation following first-order kinetics was observed (insert in Fig. 8*B*). 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 $t_{½}$ 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 thermotolerance 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

TABLE II
CotA laccase-like activity measured in spores suspensions of different B. subtilis strains grown in DSM media and harvested 24 h after the onset of sporulation

Strains	ABTS		SGZ	
	0.25 mM Cu^{2+}	No Cu^{2+}	0.25 mM Cu^{2+}	No Cu^{2+}
	Specific activity nmol min^{-1} OD580^{-1}			
MB24	82.4	24.5	16.2	9.5
AH3514	[a]	20.0	[a]	9.0
AH76 (cotA::*cat*)	ND[b]	ND[b]	ND[b]	ND[b]
AH3512 (*cotA*^{H497A})	ND[b]	ND[b]	ND[b]	ND[b]
AH3513 (cotA^{M502L})	ND[b]	ND[b]	ND[b]	ND[b]

[a] Not determined.
[b] ND, not detected.

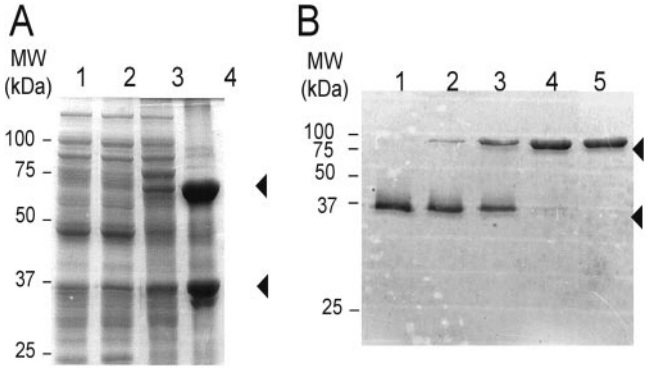

FIG. 6. *A*, SDS-PAGE analysis of CotA overproduction in *E. coli*. *Lane 1*, crude extract of a *E. coli* AH3520 culture. *Lane 2*, crude extract of an IPTG-induced *E. coli* AH3520 culture. *Lane 3*, supernatant of a crude extract of an IPTG-induced *E. coli* AH3517 culture. *Lane 4*, insoluble fraction 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 (8 μg) 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).

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. 5*C*). 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-depleted and, therefore, inactive form. To test this hypothesis the spore suspension was preincubated with copper (0.25 mM $CuCl_2$) 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 $CuCl_2$; Fig. 5*A*, compare colonies labeled *5* and *6*), an observation that reinforces the notion that CotA is directly involved in pigment formation.

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 oxidases, 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 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

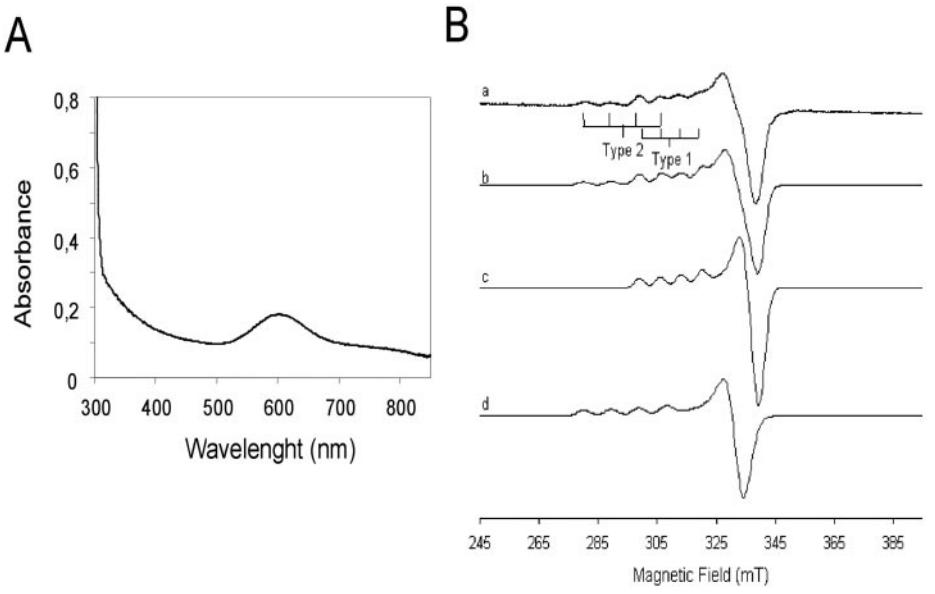

FIG. 7. **Spectroscopic properties of purified CotA.** *A*, UV-visible spectra. *B*: *a*, EPR spectra of the purified CotA; *b*, simulation of the total spectrum; *c* and *d*, deconvolution of the different components. In the simulation of type I center, *g* values of 2.227, 2.059, and 2.033 and a hyperfine coupling constant $A_{\parallel} = 72 \times 10^{-4}$ cm^{-1} were used, whereas for type II center, the *g* and $A_{\parallel}$ values used were 2.344 and 2.078 and 102×10^{-4} cm^{-1}. Microwave frequency, 9.64 GHz; microwave power: 2.4 mW, modulation amplitude: 0.9 millitesla, temperature 18 K.

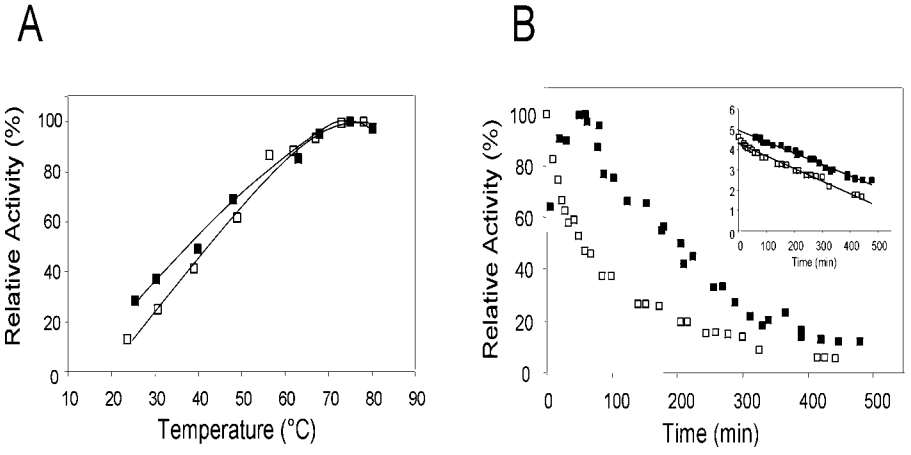

FIG. 8. **Temperature optimum and thermostability.** *A*, effect of temperature on the activity of recombinant purified CotA (*open symbols*) and in CotA assembled into the *B. subtilis* spore coat (*solid symbols*). The enzyme activity was assayed at each temperature in 100 mM citrate-phosphate buffer (pH 4.0) in the presence of 1 mM of ABTS. Activity was monitored at 420 nm. 100% activity in the spore suspension and in the purified protein sample equals 56 nmol ml^{-1} $A_{580}{}^{-1}$ and 62 μmol min^{-1} mg of protein^{-1}, respectively. *B*, thermal inactivation of purified recombinant CotA (*open symbols*) and CotA assembled in *B. subtilis* spore coat (*solid symbols*). Purified enzyme (25 μg/ml) and *B. subtilis* spores (11 A_{580} units) were incubated in vials at 80 °C. At the indicated times, samples (20 μl) were withdrawn and tested for laccase activity at 37 °C by using ABTS as substrate. 100% activity in the purified protein sample and in the spore suspension equals 28 μmol min^{-1} mg of protein^{-1} and 36 nmol ml^{-1} $A_{580}{}^{-1}$, respectively. The *inset* represents the first order kinetics of deactivation. The calculated half-life for thermal inactivation at 80 °C of the recombinant enzyme and the protein was 112 and 124 min, respectively. For the native spore-associated CotA, a $t_{1/2}$ of 224 min can be estimated, considering the initial 100-min period of activation.

TABLE III

CotA laccase-like activity measured in spore suspensions of different B. subtilis strains grown in DSM media supplemented or not with copper and harvested after 48 h after the onset of sporulation

Strains	Cu^{2+} in the culture media[a]	0.25 mM Cu^{2+}	No Cu^{2+}
		Specific activity, nmol min^{-1} $A_{580}{}^{-1}$	
MB24	−	8.0	2.6
MB24	+	7.2	13.0
AH2734	−	92.0	2.0
AH2734	+	65.0	135.0

[a] At a final concentration of 0.25 mM.

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 H_2O_2, 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 thermophilium* 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. thermophilium* 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.

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