

NOTES

CotA of *Bacillus subtilis* Is a Copper-Dependent Laccase

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The spore coat protein CotA of *Bacillus subtilis* displays similarities with multicopper oxidases, including manganese oxidases and laccases. *B. subtilis* is able to oxidize manganese, but neither CotA nor other sporulation proteins are involved. We demonstrate that CotA is a laccase. Syringaldazine, a specific substrate of laccases, reacted with wild-type spores but not with $\Delta cotA$ spores. CotA may participate in the biosynthesis of the brown spore pigment, which appears to be a melanin-like product and to protect against UV light.

The spore-forming bacterium *Bacillus subtilis* synthesizes and deposits a protein coat around the developing endospore during differentiation (8). The spore coat consists of at least 25 different polypeptides of 5 to 65 kDa, and some of them are highly cross-linked. These proteins are assembled into a lamella-like inner coat and an electron-dense outer coat, which protects the spore from a diverse range of stresses (8).

The *cotA* gene codes for a 65-kDa protein belonging to the outer spore coat of *B. subtilis*. It corresponds to the former *pig* locus (7, 21) and was one of the first *cot* genes to be cloned (7). The *cotA* gene is expressed under the control of sigma K, with GerE acting as a transcriptional repressor instead of its more usual function of transcriptional activator (24). The absence of CotA has no apparent effect on spore resistance, but it results in the loss of the usual brownish pigmentation of the colonies. Moreover, wild-type spore-forming colonies are darker at higher manganese concentrations (13). This is similar to marine *Bacillus* sp. strain SG1, which has spores that express manganese oxidase (30).

The CotA protein displays similarities with multicopper oxidases (for a review, see reference 28). In particular, it contains the four copper-binding sites, the type I blue copper center (T1) and the T2/T3 trinuclear cluster, which differ in their spectroscopic features (Fig. 1). The multicopper oxidase family includes ascorbate oxidase (EC 1.10.3.3), ceruloplasmin (EC 1.16.3.1), laccase (EC 1.10.3.2), various manganese oxidases, and other enzymes. Among the proteins most closely related to CotA are the manganese oxidase of *Leptothrix discophora* (4) and the CumA protein of *Pseudomonas putida* (1), which is essential for manganese oxidation. CotA also shares similarities with a polyphenoloxidase of *Acremonium murorum* and, with much lower similarity scores, laccases including those of *Neurospora crassa* (9), *Agaricus bisporus* (18), and *Marinomonas mediterranea*—formerly an *Alteromonas* species (MMB1) (23). Laccase was first found in the sap of the Japanese lacquer

tree (hence its name). Laccases are widespread in plants, where they are involved in lignin biosynthesis, and in fungi, where they are involved in ligninolysis, development-associated pigmentation, detoxification, and pathogenesis (28, 29). Laccases are polyphenoloxidases which oxidize a wide range of polyphenols, methoxy-substituted phenols, and diamines. Substrate specificity differs from one laccase to another, but most laccases do not oxidize tyrosine, in contrast to the classic tyrosinase (EC 1.14.18.1) (for a review, see reference 29). Tyrosinase is also a polyphenoloxidase, but it contains only one coupled binuclear copper center (type III; T3).

Despite similarities with the multicopper oxidases, it is not known if CotA has any oxidase activity in *B. subtilis*. We tested whether CotA is a manganese oxidase or a laccase with or without tyrosinase activity. We report that CotA is a classical laccase.

To identify the function of CotA, a *cotA::aphA3* mutant was constructed. A DNA fragment including the *cotA* gene was amplified by PCR using chromosomal DNA as the template. Oligonucleotides allowing the creation of a *Hind*III site 294 bp upstream from the start codon of *cotA* and an *Eco*RI site 114 bp downstream from the stop codon were used. The resulting fragment was ligated between the *Eco*RI and *Hind*III sites of pUC18. A kanamycin cassette was then inserted between a *Pst*I site (95 bp upstream of the translation start) and an *Xba*I site (54 bp upstream from the stop codon), deleting the whole coding sequence except for the last 18 amino acids. The resulting plasmid was linearized and used to transform *B. subtilis* strain 168 to give the *cotA::aphA3* mutant ($\Delta cotA$).

All experiments were performed in accordance with European regulation requirements concerning the contained use of genetically modified organisms from group 1 (regulation no. 2735).

To define the relationship between spore pigmentation and manganese, the formation of the brownish color was studied in several culture conditions and in various mutants. Precultures were diluted to an optical density at 600 nm of 0.1. Using a home-made replicator (for details, see <http://locus.jouy.inra.fr/genmic/madbase/mutant/home.gif>), the bacteria were streaked on SP agar medium (pH 7.0) (26) in square Falcon plates (Fig.

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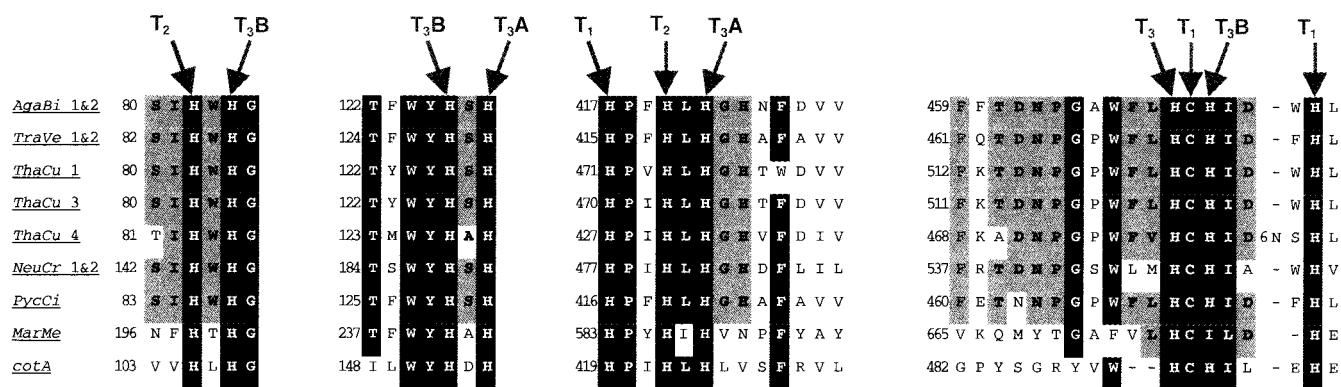


FIG. 1. The four copper-binding sites in CotA and in some fungal laccases. Abbreviations: AgaBi, *A. bisporus*; TraVe, *Trametes versicolor*; ThaCu, *Thanatephorus cucumeris*; NeuCr, *N. crassa*; PycCi, *Pycnoporus cinnabarinus*; MarMe, *M. mediterranea*. There are two laccases (laccases 1 and 2) which have identical copper-binding sites in *A. bisporus*, *T. versicolor*, and *N. crassa*. Conserved amino acids are shaded in black (90% conservation or more) or in grey (70 to 90% conservation). T₁, T₂, T₃A, and T₃B are the type 1, 2, and 3 copper centers, which differ by their spectroscopic features (T₃ center contains two copper atoms called A and B). For each copper center, arrows point to the copper-binding residues, which upon folding of the protein come into close proximity and coordinate copper.

2A). The bacteria were grown for 48 h at 37°C. To modify the pH, a Whatman paper (8 by 8 cm) was then laid in the plate lid and soaked with 1.5 ml of either distilled water (Fig. 2A, plate I) or 3 M sodium acetate, pH 4.8 (Fig. 2A, plate II). After 24 h, the pH was checked and found to be 8 in plate I and 3.5 in plate II. Small pieces of Whatman paper containing 10 μ l of 5 M MnCl₂ were then placed at one end of the streaks. After 48 h, a brown pigmentation appeared near the site of MnCl₂ application in plate I, but not in plate II (Fig. 2A). There was no pigmentation on plates similarly treated with manganese in the absence of bacteria (data not shown). Thus, the appearance of brown pigmentation in the agar around the colonies depends on the presence of *B. subtilis* and manganese and also on the pH of the plates. Similar results were obtained with strain 168, a Δ sigF mutant which is blocked early in the sporulation process, and the Δ cotA mutant (Fig. 2A). This pigmentation found at high pH is therefore independent of sporulation and particularly of CotA.

To determine whether the observed brown color was due to manganese oxide, leucoberbelin blue, which specifically reacts with MnIII to MnVII, was tested with the browned agar (14). For each strain, dark blue (tube I, corresponding to plate I) and pale blue (tube II, corresponding to plate II) staining revealed the presence of manganese oxide on plate I but not on plate II (Fig. 2B). Therefore, *B. subtilis* is able to oxidize manganese, but neither CotA nor sporulation is involved. Colonies transferred to nylon filters did not react with the leucoberbelin blue (data not shown). This indicates that the bacterial spore pigment is not manganese oxide, which agrees with the report of van Waasbergen and coworkers (31). Manganese oxide in the agar was also detected when the plates were autoclaved before the addition of manganese (data not shown) and is therefore not produced by enzymatic activity. It is probably formed by the alkalization of the medium, resulting from bacterial growth (5).

So, the CotA-dependent pigmentation remains to be explained. Four copper-binding sites are present in CotA and in laccases (Fig. 1). In spite of the overall low similarities, we then tested whether CotA could be a laccase. A substrate specific

for laccase, syringaldazine [*N,N'*-bis(3,5-dimethoxyhydroxybenzylidenehydrazine)], was used (10). Spore suspensions were incubated with 50 μ M syringaldazine and 10 μ M CuSO₄ in 100 mM phosphate buffer. A purple color developed with 168 spores, but not with Δ cotA spores, indicating that CotA may be a laccase (Fig. 2C). The optimal pH and temperature for this reaction were pH 7 and 45°C (data not shown). To confirm the laccase activity, the CotA protein was overproduced in *Escherichia coli*. The cotA gene (nucleotides -1 to +1656 from the start codon) was cloned under the control of the strong inducible T7 promoter in the pET20b+ vector (Novagen). The pET20b+cotA plasmid was transformed into *E. coli* strain BL21(DE3) (Novagen). Cells were grown at 37°C in Luria-Bertani medium until they reached an optical density at 600 nm of 3. IPTG (isopropyl- β -D-thiogalactopyranoside) (1 mM) was then added to induce cotA expression. Cells were harvested 2 h later, resuspended in 100 mM phosphate buffer (pH 7), and treated with toluene. After the addition of 50 μ M syringaldazine and 10 μ M CuSO₄, the purple color appeared at 45°C. No color appeared with pET20b+ alone. This demonstrates that CotA is a laccase.

Since fungal laccase synthesis is often induced by the copper cofactor or by their substrates, the expression of a transcriptional cotA-lacZ fusion (19) was tested on Luria-Bertani plates enriched with various concentrations of copper or with putative substrates. Neither copper, *p*-anisidine, veratric acid, resorcin, *p*-toluidine, *p*-coumaric acid, liginosulfonic acid, orcinol, ferulic acid, *p*-xylydine, nor liginosulfonic acid elicited any induction. In addition, no liginolytic activity was found with liginosulfonic acid (data not shown).

As some *Bacillus* strains produce pigment on agar media supplemented with tyrosine and copper salts (27), the CotA protein could be a laccase with tyrosinase activity, like the multipotent polyphenoloxidase of *M. mediterranea* (22). The addition of 1 mM tyrosine to SP medium did not modify the color of the colonies. In contrast, addition of 10 μ M CuSO₄ to SP medium enhanced the difference in the spore pigmentation between strain 168 and the Δ cotA mutant (Fig. 2D, plate III). This substantiates the role of copper in the enzymatic activity

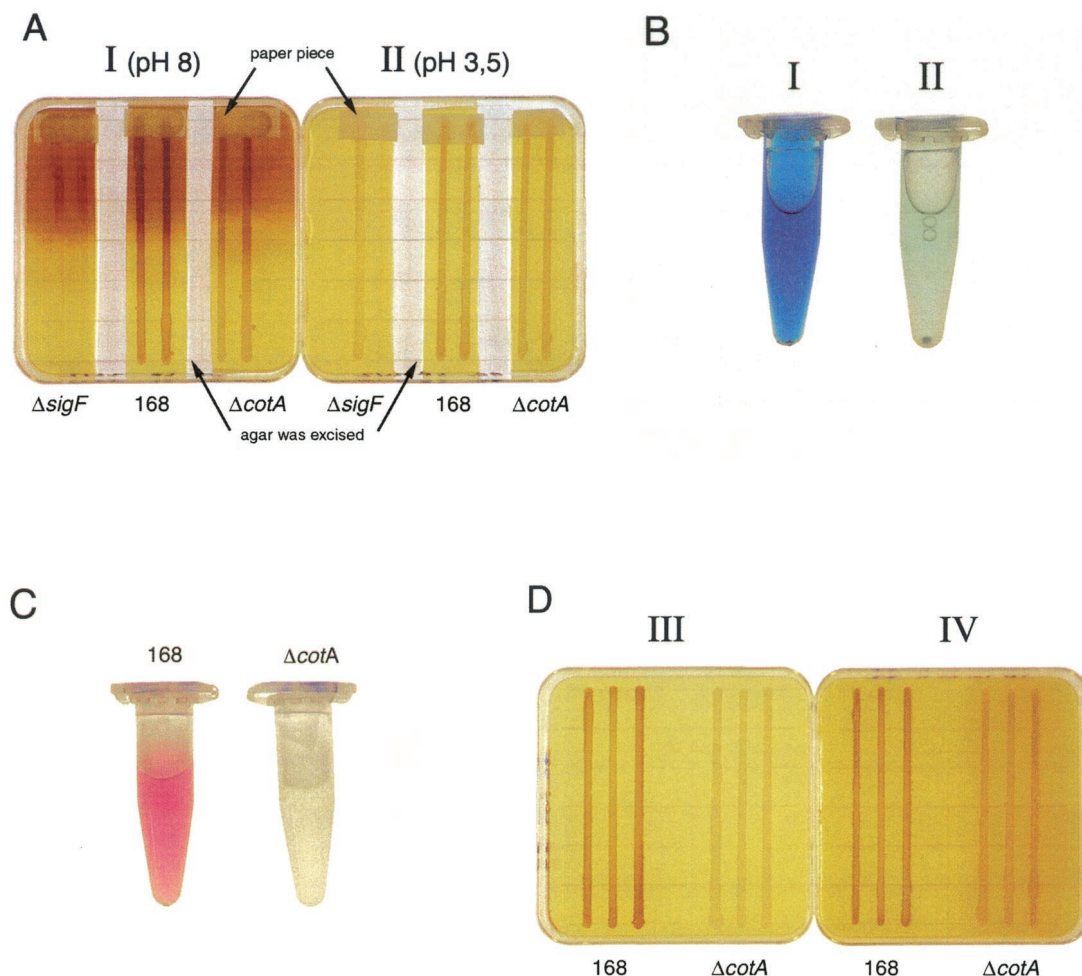


FIG. 2. *B. subtilis* pigmentation in several culture conditions. (A) Manganese oxidation on plates by wild-type, *sigF*, and $\Delta cotA$ strains. Ten microliters of 5 M $MnCl_2$ in Whatman paper was deposited at the top of the plates. An agar band was excised to separate each strain from its neighbors on the plate. (B) Leucoberbelin blue test. For each strain, a piece of 0.15 g of browned agar was excised from plates I and II shown in panel A near the paper band containing $MnCl_2$. After being melted in 800 μl of water acidified by the addition of 400 μl of 3 M sodium acetate (pH 4.8), 40 μl of leucoberbelin blue solution was added. Tubes I and II correspond to plates I and II of panel A, respectively. All three strains gave the same result on plate I, on the one hand, and on plate II, on the other hand. (C) The syringaldazine test was performed using a spore suspension obtained by the water-washing method (20). (D) Production of spore pigment on SP plates supplemented with 10 μM $CuSO_4$ and with (plate IV) or without (plate III) 1 mM $MnCl_2$.

of CotA. Further addition of 1 mM tyrosine did not modify this result, suggesting that CotA has no tyrosinase activity (data not shown). The color of the CotA-dependent pigment and that of manganese oxide are quite similar. The effect of Cu and Mn on the pigmentation of 168 and $\Delta cotA$ strains was then tested. $MnCl_2$ (1 mM) completely masked the difference between both strains on SP plates and reduced it on SP plates with 10 μM $CuSO_4$ (Fig. 2D, plate IV). This may explain why the spore pigmentation has been thought to be manganese dependent (13).

Laccase activity has been detected in *Bacillus sphaericus*. It correlates closely with spore formation and the appearance of dipicolinic acid (3). It has also been found in *Streptomyces galbus* (15), in *Azospirillum lipoferum* (6), and in *M. mediterranea* (22), all of which produce a melanic pigment. This suggests a possible link between laccase activity and melanin production, at least in *S. galbus* and *A. lipoferum*, as tyrosinase is

responsible for pigmentation in *M. mediterranea* (23). This is reminiscent of some fungal laccases involved in melanization (2). CotA participates in the biosynthesis of the brown spore pigment, which is also thought to be a melanin-like product (25). A few bacilli, among them a strain of *B. subtilis*, produce a black melanic pigment (11, 12, 17, 25).

To test whether the pigment produced by *B. subtilis* has some properties of melanin, spores of strains 168 and $\Delta cotA$ were purified following Riesenman and Nicholson (20) after growth on SP plates with and without 10 μM $CuSO_4$. The effect of 15% H_2O_2 was tested on the brownish spores of strain 168. The spore pigment was immediately bleached by this treatment, as would be expected for melanin. The spore coat has been previously shown to protect against hydrogen peroxide (20). The role of CotA in the resistance of spores to H_2O_2 was therefore tested. The 168 and the $\Delta cotA$ strains were treated 1 h in 5% H_2O_2 as described by Riesenman and Ni-

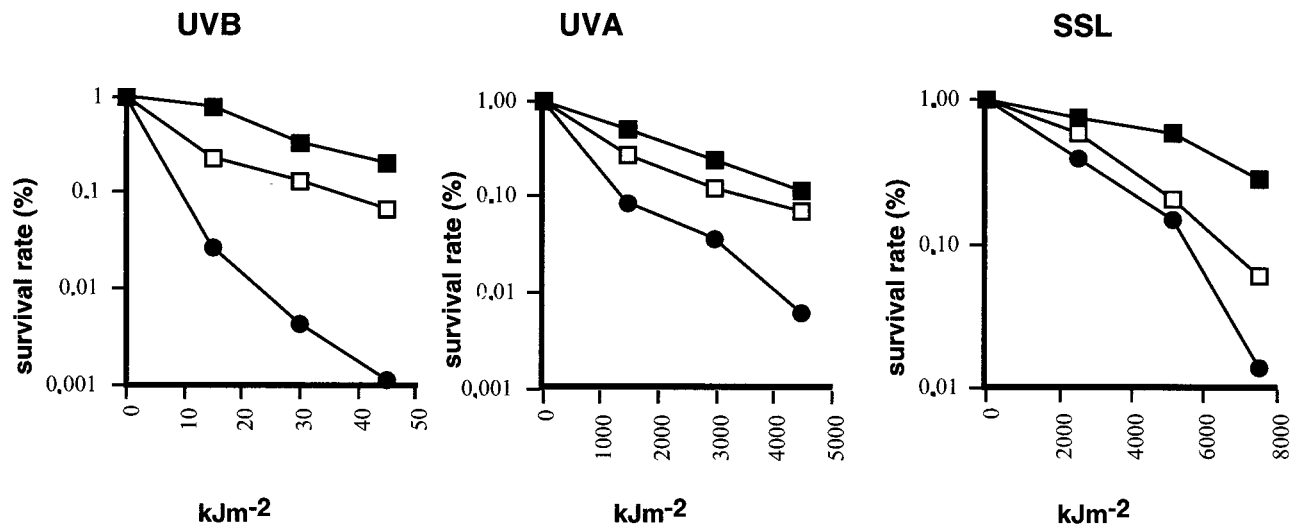


FIG. 3. UV light resistance of wild-type and $\Delta cotA$ strains. The UVB source was a Fluolink TFL-35 M (Vilber Lourmat, Torcy, France). The UVA source was a Supersun 5000 lamp (Mutzhas) emitting only UVA₂ radiation ($\lambda > 340$ nm). Intensity was $1,130 \text{ J m}^{-2}$ as measured by a VLX 3W radiometer equipped with interferential filters (Vilber Lourmat). For modeling the environmental solar radiation, a Kratos solar simulator equipped with a 2,500-W xenon compact arc lamp (Conrad-Hanovia, Inc., Newark, N.J.) was used in conjunction with a Schott WG 320 cutoff filter (3 mm) to eliminate UVC and shorter UVB radiation. The fluence rate was $1,700 \text{ J m}^{-2} \text{ s}^{-1}$, measured with a YSI Kettering 65a thermopile (Yellow Spring Instruments). Each experiment was done with two different batches of spores. ■, strain 168 grown on SP plus $10 \mu\text{M}$ CuSO_4 ; □, strain 168 grown on SP without CuSO_4 supplementation; ●, $\Delta cotA$ grown on SP plus $10 \mu\text{M}$ CuSO_4 .

cholson (20). A total of 2.2×10^{-3} cells survived in the 168 strain versus 10^{-8} cells in the $\Delta cotA$ strain. Weakly pigmented 168 spores obtained on SP plates were as resistant as brownish 168 spores obtained on SP plates supplemented with $10 \mu\text{M}$ CuSO_4 . This shows that the CotA protein and/or the pigment is involved in protection against hydrogen peroxide.

Melanin shields against radiation (16), and the spore coat confers resistance to UV light (20). The effects of UVB, UVA, and simulated solar light were therefore tested on spore survival following Riesenman and Nicholson (20). At the highest level of irradiation energy applied, the brownish spores of strain 168 were 177-, 18-, and 19-fold more resistant than the $\Delta cotA$ spores to UVB, UVA, and simulated solar light, respectively (Fig. 3). The weakly pigmented spores of strain 168 obtained in the absence of copper display an intermediate protection (Fig. 3). This suggests a possible correlation between the protective effect and the amount of pigment.

To our knowledge, this is the first time that an enzymatic function is attributed to a spore coat protein. CotA seems to be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide. This effect is probably at least partly mediated by the spore pigment. However, identification of this pigment as a melanin would require further study.

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