A Thermostable Laccase from *Streptomyces lavendulae* REN-7: Purification, Characterization, Nucleotide Sequence, and Expression

Takashi SUZUKI,1 Kohki ENDO,1 Masaaki ITO,1,† Hiroshi TSUJIBO,2 Katsushiro MIYAMOTO,2 and Yoshihiko INAMORI2

1Central Laboratory, Rengo Co., Ltd., 4-1-186 Ohhiraki, Fukushima-ku, Osaka 553-0007, Japan
2Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

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We found a polyphenoloxidase (PPO) in the cell extract of *Streptomyces lavendulae* REN-7. About 0.8 mg of purified PPO was obtained from 200 g of the mycelia with a yield of 9.0%. REN-7-PPO showed broad substrate specificity toward various aromatic compounds. Moreover, this enzyme was capable of oxidation of syringaldazine, which is a specific substrate for laccase. Interestingly, REN-7-PPO retained its original activity after 20 min of incubation at even 70°C. The gene encoding the PPO was cloned. Four copper-binding sites characteristics of laccases were contained in the deduced amino acid sequence. We constructed a high-level expression system of this gene in *Escherichia coli*. The properties of the recombinant enzyme were identical that of wild-type. In conclusion, this PPO is a thermostable laccase.

**Key words:** polyphenoloxidase; laccase; *Streptomyces*; thermostable laccase

Polyphenoloxidases (PPOs) are a group of copper enzymes, classified into three types of activity:1) cresolase (EC 1.14.18.1, monophenol monooxygenase), catechol oxidase (EC 1.10.3.1, o-diphenol: oxygen-oxidoreductase), and laccase (EC 1.10.3.2, p-diphenol: oxygen-oxidoreductase). Tyrosinases have both cresolase and catechol oxidase activity, and are widely distributed from bacteria to mammals. These enzymes are involved in melanin formation,2,3) as they catalyze o-hydroxylation of L-tyrosine into L-DOPA and its subsequent oxidation to L-dopaquinone. Another important kind of PPO is laccases. Laccases have been found in higher plants4) and numerous fungi.5,6) In some bacteria, laccase activity has been described.7–9) Fungal laccases are involved in pigmentation of fruiting bodies, sexual differentiation, lignonolysis, resistance to cell lysis, and detoxification.10)

Although tyrosinases and laccases are able to oxidize an overlapping range of diphenolic compounds, these enzymes have been distinguished on the basis of substrate specificity and sensitivity to specific inhibitors.10) The most important difference is that tyrosinases show cresolase activity and only laccases are able to oxidize methoxy-activated phenols such as syringaldazine. Concerning inhibitors, tyrosinases are strongly inhibited by tropolon and cinnamic and salicylhydroxamic acids,11,12) whereas these agents barely affect laccases.13) Since laccases have three spectroscopically different copper ions (Type I, II, and III), they are classified in multi-copper blue enzymes.14) On the other hand, tyrosinases have only a pair of type III copper ions.

Recently, PPOs have received great attention in biotechnological applications. Especially, it has been proposed that laccases are suitable for some applications, such as biodegradation of xenobiotic compounds, pulp delignification, textile dye bleaching, and others.15–17) In this connection, we screened for microorganisms producing laccase(s). We have isolated a PPO from the cell extract of *Streptomyces lavendulae* REN-7. Based on the substrate specificity and amino acid sequence, it was concluded that this enzyme is a laccase, and named it *Streptomyces* thermostable laccase (STSL).

**Materials and Methods**

**Materials.** *Pycnoporus coccineus* laccase (p-lac) was purchased from Koken. DEAE-cellulose and hydroxyapatite were bought from Wako Pure Chemical Industries, Ltd. Sephadex G-150, QAE-Sephadex A-25, and AlkPhos Direct Labeling and Detection System were from Amersham Biosciences. The BCA (bicinchoninic acid) Protein Assay Kit was purchased from Pierce Chemical Co. Restriction enzymes and DNA-modifying enzymes were bought from Nippon Gene and Toyobo. The Big Dye
Terminator Cycle Sequencing Ready Reaction was from Applied Biosystems.

Isolation and identification of the organism. *Streptomyces lavendulae* REN-7 was isolated from a soil sample collected in Sakyo-ku, Kyoto. Identification of the microorganism was done by NCIMB Japan.

**Assay for polyphenoloxidase activity.** Polyphenoloxidase activity was assayed by oxygen-uptake and colorimetric methods.

In the oxygen-uptake method, oxygen uptake coupled to the oxidation of substrate was measured using YSI Model 5300 Biological Oxygen Monitor. After 50 mM sodium acetate buffer, pH 4.5, containing various substrates had been allowed to equilibrate with the air by being stirred at 30°C for 10 min, the reaction was started by addition of the enzyme solution. The final volume of the reaction mixture was 5.0 ml. The rate of oxygen uptake was recorded and enzyme activity was calculated from the initial slope. One unit was defined as the amount of enzyme required for uptake of 1 μmol of O₂ per min under these conditions.

In the colorimetric method, the reaction was started by the addition of the enzyme solution to 50 mM sodium acetate buffer, pH 4.5, containing 5 mM catechol. The final volume of the reaction mixture was 5.0 ml. The increase of absorbance at 475 nm caused by the oxidation of catechol at 30°C was measured, and one unit of the activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per liter of reaction mixture per min. No autoxidation was detected during the reactions by the oxygen-uptake and the colorimetric methods.

**Protein assays.** The protein concentration of fractions collected during column chromatography was monitored by absorbance at 280 nm. For accurate measurement of protein concentrations, a BCA protein assay kit was used with bovine serum albumin as a standard.

Cultivation of *Streptomyces lavendulae* REN-7. The mycelia of *Streptomyces lavendulae* REN-7 were inoculated into 100 ml of a medium consisted of 0.5% polypepton, 0.1% yeast extract, 0.001% FeSO₄·7H₂O, and 0.0001% CuSO₄·7H₂O at pH 7.0 in a 500-ml flask at 30°C for 72 h with shaking (110 strokes/min). After the cultivation, mycelia were collected by suction filtration. 200 g of mycelia from 20 liters of the broth were suspended in 800 ml of 50 mM sodium phosphate buffer, pH 7.0 (buffer A). The suspension was disrupted by sonication. The sonicate was centrifuged (25,000 × g for 30 min), and the supernatant was used for purification of the enzyme.

**Purification of STSL.**

**Step-1.** The supernatant was treated at 70°C for 20 min. Denatured materials were removed by centrifugation (25,000 × g for 30 min).

**Step-2.** The sample was fractionated with ammonium sulfate (20% to 50% saturation). The precipitate was dissolved in 50 mM sodium phosphate buffer, pH 6.0 (buffer B), and dialyzed against the same buffer.

**Step-3.** The dialyze was put on to a column of DEAE-cellulose (50 × 400 mm) equilibrated with buffer B. The column was washed with the same buffer, and the enzyme was eluted with a 0 to 1.0 M NaCl linear gradient. Fractions containing PPO activity were pooled and precipitated with ammonium sulfate (80% saturation). The precipitate was collected by centrifugation (30,000 × g for 20 min). The precipitate was dissolved in buffer A. The sample was dialyzed against 1 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM CaCl₂ (buffer C), and dialyzed against the same buffer.

**Step-4.** The dialyze was put on to a column of hydroxyapatite (26 × 220 mm) equilibrated with buffer C. The column was washed with the same buffer, and the enzyme was eluted with a 400-ml linear gradient of buffer C to 500 mM sodium phosphate buffer, pH 6.0. Fractions containing PPO activity were pooled and concentrated with ultrafiltration.

**Step-5.** The concentrate was put on to a column of Sephadex G-150 (10 × 450 mm) equilibrated with buffer A and eluted with the same buffer at a flow rate of 10 ml/h. Fractions containing PPO activity were pooled and stored at −80°C until use.

**Analysis of N-terminal amino acid sequence.** The purified enzyme was electrophoresed on SDS polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane. The protein on the membrane was stained with Coomassie brilliant blue R-250. The protein band was sequenced with an ABI Procise 491HT protein sequencer by the method of Matsudaaira.³⁸

**Kinetic analyses.** Kinetic analysis was done by the oxygen-uptake method by using various concentrations of catechol and 50 mM sodium acetate buffer, pH 4.5. The fastest initial rate of oxygen-uptake coupled to the oxidation of substrate was measured to give the initial velocity. The $K_m$ and $V_{max}$ were estimated by Lineweaver-Burk plotting, with at least six initial substrate concentrations. The $k_{cat}$ was obtained with the equation: $V_{max} = k_{cat}[E]$, where [E] is the enzyme concentration.
Cloning of the STSL gene. In order to obtain the partial gene fragment, the polymerase chain reaction (PCR) was done with KOD plus DNA polymerase (Toyobo) using primers designed from the amino acid sequence around copper-binding sites of laccases. The amplified DNA fragment by PCR using 1F (5′-TGG TAT/C CAT/C CAT/C CAT/ C GCC/G ATG-3′) as a sense primer and 2R (5′-AA/GA/G TGA/G/T ATA/G TGC ATC/G GGA/G TG-3′) as an antisense primer (denaturation at 97°C for 15 sec, annealing at 60°C for 15 sec, and extension at 68°C for 30 cycles) was labeled with alkaline phosphatase with the Alkphos Direct Labeling and Detection System, and used as a probe for hybridization. Genomic DNA of Streptomyces lavendulae REN-7 was digested with some restriction enzymes and analyzed by Southern blotting with the probe. The specific activity of STSL was 7.3 U/mg. The purified enzyme showed a single protein band on 10.0% polyacrylamide gel electrophoresis (SDS-PAGE)

Results

Purification of STSL

Table 1 summarizes the purification of STSL. About 0.8 mg of purified enzyme was obtained from 200 grams of mycelia with a yield of 9.0%. The specific activity of STSL was 7.3 U/mg. The purified enzyme showed a single protein band on 10.0% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular mass of about 73,000 Da (data not shown).

N-terminal amino acid sequence of STSL

The N-terminal amino acid sequence of the enzyme was NH2-Ala-Pro-Ala-Ala-Ala-Gly-Glu-Leu-Thr-Pro-Tyr-Ala-Ala-Pro-Leu-Thr-Val- by a gas-phase sequenator.

Effects of pH on STSL activity and stability

Effects of pH on STSL activity and stability were investigated by the oxygen-uptake method using 5 mM catechol as substrate in the following buffers: sodium acetate buffer (pH 3.5 to 6.0), sodium phos-
Polyphenoloxidase activity was measured by the oxygen-uptake method using catechol as the substrate at pH 4.5. (A) Thermal stability of STSL (open circle) and p-lac (solid circle). These enzymes (100 μg/ml) were incubated for 10 min at each temperature in sodium phosphate buffer, pH 7.0. After the incubation, residual activity was measured at 30°C. (B) Time course of inactivation of STSL. Purified STSL (100 μg/ml) was incubated at 70°C. At the indicated times, a sample was withdrawn and residual activity measured at 30°C.

### Table 2. Substrate Specificity

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<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<td></td>
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<tr>
<td>Catechol</td>
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<tr>
<td>Resorcinol</td>
<td>7.1</td>
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<td>Hydroquinone</td>
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<tr>
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<td>o-Cresol</td>
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<tr>
<td>m-Cresol</td>
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<td>p-Cresol</td>
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</tr>
<tr>
<td>Guaiacol</td>
<td>12.9</td>
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<tr>
<td>p-Phenylenediamine</td>
<td>48.6</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>2.9</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>2.4</td>
</tr>
<tr>
<td>l-DOPA</td>
<td>37.1</td>
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<tr>
<td>L-Ascorbic acid</td>
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Activity was measured by the oxygen-uptake method described in Materials and Methods. Concentration of each substrates was 5 mM.

### Table 3. Effects of Inhibitors and Metal Ions

<table>
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<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Remaining activity (%)</th>
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<tr>
<td></td>
<td>STSL</td>
<td>p-lac</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>1.0</td>
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<td>Arbutin</td>
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<td>L-Cysteine</td>
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<tr>
<td>DL-Dithiothreitol</td>
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<td>Sodium azide</td>
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<td>DDCa</td>
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<tr>
<td>NaCl</td>
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</table>

Activity was measured by the oxygen-uptake method using 5 mM as substrate.

Effects of temperature on STSL activity and stability

The effects of temperature on STSL activity were investigated by the colorimetric method. STSL was most active around 50°C when catechol was used as the substrate (data not shown). Effects of temperature on the stability of STSL and *Pycnoporus coccineus* laccase (p-lac) were also studied. The enzyme solution was kept for 10 min at various temperatures and the remaining activity was measured. Under these conditions, STSL was stable up to 70°C, but p-lac was unstable above 60°C (Fig. 1A). Figure 1B shows the course of STSL activity when it was incubated at 70°C. STSL retained its original activity for up to 20 min of treatment and had half-lives of 100 min. These results indicate that STSL is a thermostable enzyme.
Fig. 2. Nucleotide Sequence and the Deduced Amino Acid Sequence of STSL.

The termination codon is indicated by an asterisk. In the protein sequence, the putative signal peptide region of STSL is underlined.
Substrate specificity toward aromatic compounds

The substrate specificities of STSL and p-lac toward various aromatic compounds are summarized in Table 2. These enzymes were able to catalyze oxidation of a wide range of aromatic compounds and showed similar specificities. Although laccases preferentially act on \( p \)-diphenols, STSL preferred \( \alpha \)-diphenol (catechol). In addition, guaiacol, which is good substrate for laccases, was not oxidized efficiently by STSL.

Since this enzyme oxidized guaiacol, syringaldazine, which is a specific substrate for laccase,\(^{19}\) was tested. A purple color developed with this enzyme (data not shown). STSL oxidized \( L \)-tyrosine, but did not show any cresolase activity. These observations indicate that the PPO is a laccase.

Kinetic analyses

Kinetic analysis for STSL was done by oxygen-uptake method using catechol, hydroquinone, or guaiacol as substrate. This enzyme was shown to oxidize catechol most effectively among these substrates tested. The kinetic parameters of catechol for STSL were \( K_m = 0.043 \text{ mM}, k_{cat} = 10.9 \text{ s}^{-1}, \) and \( k_{cat}/K_m = 253 \text{ mM}^{-1} \text{s}^{-1} \), respectively. These values were different from those for p-lac (\( K_m = 1.0 \text{ mM}, k_{cat} = 103 \text{ s}^{-1}, \) and \( k_{cat}/K_m = 103 \text{ mM}^{-1} \text{s}^{-1} \)). The \( K_m \), \( k_{cat} \), and \( k_{cat}/K_m \) values toward other substrates were found to be 0.039 mM, 5.3 s\(^{-1}\), and 136 mM\(^{-1}\)s\(^{-1}\) (hydroquinone), and 0.058 mM, 1.6 s\(^{-1}\), and 27.6 mM\(^{-1}\)s\(^{-1}\) (guaiacol), respectively. These values were in good agreement with the results for substrate specificity.

Effects of inhibitors and metal ions

The effects of some putative laccase inhibitors and metal ions are summarized in Table 3. STSL was completely inhibited by 1.0 mM \( L \)-cysteine, DL-dithiothreitol, and sodium azide. Kojic acid was also an effective inhibitor (about 70% inhibition). The sensitivity of STSL toward these compounds was very similar to that of p-lac. \( \text{Ca}^{2+} \), and \( \text{Mn}^{2+} \) ions slightly activated STSL, but not p-lac. On the other hand, \( \text{Cu}^{2+} \) ion stimulated activity of p-lac, but not STSL.

Cloning of the STSL gene

In order to clarify that the PPO from \( S. \ lavendulae \) REN-7 belongs to a laccase, the gene was cloned. Oligonucleotide primers were synthesized from the consensus copper binding sites among laccases. A 1.0-kbp DNA fragment was amplified by PCR using the genomic DNA of \( S. \ lavendulae \) REN-7 as a template (data not shown). The fragment was labeled with alkaline phosphatase and used as a probe for hybridization. Genomic DNA of \( S. \ lavendulae \) REN-7 was digested with some restriction enzymes and analyzed by Southern blotting with the probe. A 2.3-kbp fragment of \( SmaI \)-digested DNA was hybridized with the probe (data not shown). \( SmaI \)-digested 2.3-kbp fragments were purified and self-ligated with Ligation High. Then, inverse PCR was done, and the sequence of the amplified DNA fragment was analyzed (Fig. 2, DDBJ accession No. AB092576). An open reading frame consisted of 1896 nucleotides. The deduced amino acid sequence consisted of 631 amino acid residues with a molecular weight of 68,743 (Fig. 2). The N-terminal amino acid sequence of the wild-type enzyme was started at position of Ala20. This result suggests that the STSL gene consists of two regions: a signal region (19 amino acid residues) and a mature region (612 amino acid residues, MW = 66,771). The deduced amino acid sequence showed high identity (76%) to those of the phenoxazinone synthase (PHS) from \( S. \ antibioticus \) (data not shown).\(^{20}\) The amino acid sequence of STSL contained four copper-binding sites characteristic of the blue multicopper proteins, including all laccases (Fig. 3). Thus, it was concluded that STSL is a thermostable laccase.

Expression and purification of the recombinant STSL gene

A high-level expression system for STSL was constructed using the pET20b (+) vector. Recombinant STSL was successfully expressed in \( E. \ coli \) (about 30 mg/1 liter broth). All the activity was found in the bacterial sonicate. About 10 mg of purified STSL was obtained from 1 liter of broth with the yield of 30%. The specific activity of recombinant STSL was 7.0 U/mg. Recombinant STSL showed the same enzymatic properties (optimum temperature, thermal stability, and kinetic parameters) of the wild-type enzyme (data not shown), but the N-terminal amino acid sequence of recombinant STSL was \( \text{NH}_2\text{-Thr-Asp-Ile-Ile-Glu-} \).

Discussion

A PPO from \( S. \ lavendulae \) REN-7 was capable of oxidizing syringaldazine, which is specific substrate for laccases. In addition, the sensitivity toward laccase inhibitors was almost identical to the classical one (Table 3). To confirm the PPO is a laccase, the gene encoding this enzyme was cloned. All four copper-binding sites characteristic of laccases were contained in the deduced amino acid sequence of the PPO (Fig. 3). Thus, the enzyme should be classified as a laccase.

The enzymatic properties of the PPO were investigated. The molecular mass was estimated to be 73,000 Da by SDS-PAGE. This enzyme was most active at around pH 4.5 and 50°C using catechol as substrate. These values were similar to those of fungal laccases.\(^{20}\) Interestingly, the PPO showed high thermal stability. After treatment at 70°C for 10 min, its original activity was fully retained (Fig. 1A).
Moreover, even after treatment at 80°C, about 35% of the activity remained while almost all fungal laccases were completely inactivated. Few papers have been published on thermostable laccases. They are the thermophilic fungus Chaetomium thermophilium laccase\(^{25}\) and \textit{B. subtilis} cotA.\(^{26}\) To our knowledge, this is the first report of a thermostable laccase from Actinomyces, and named it Streptomyces thermostable laccase (STSL).

Since laccases catalyze the oxidation of a variety of aromatic compounds, they have received great attention in biotechnological applications. But the productivity of STSL was very low (about 4.5 mg/100 g of mycelia, Table 2). To obtain more abundant STSL, 2,5-xylidine, which is an effective inducer of fungal laccases,\(^{27}\) was added to the medium described under “Materials and Methods”. Although the productivity of laccases was stimulated about 10-fold higher in some fungi, this had no influence in the productivity of STSL (data not shown). Next, we attempted construction of recombinant expression system of this gene in \textit{E. coli}. When the open reading frame of STSL gene was ligated to the \textit{Nde}I-\textit{Bam}HI site of pET20b (+), it was effectively expressed (30 mg/1 liter broth), but the activity was very low (0.1 mg/1 liter broth) before dialysis against Tris-HCl buffer containing CuSO\(_4\). This clearly showed that STSL is a copper-dependent enzyme. Recombinant STSL had an aggregative nature. Due to aggregation, it was difficult to purify the STSL. This problem was cleared up by the addition of urea and 2-mercaptoethanol, and purified recombinant STSL tended to suppress aggregation. Recombinant STSL probably aggregated with intrinsic \textit{E. coli} protein.

Alexandre \textit{et al.} has supposed that laccases are widespread in bacteria, and that they are excreted,\(^{29}\) but all the bacterial laccases were found in the cell extract. Multipotent PPO from \textit{Marinomonas mediterranea} was mainly bound to the membrane fraction.\(^{29}\) Both \textit{B. subtilis} cotA\(^{30}\) and \textit{S. antibioticus} PHS existed in their spores.\(^{31}\) In the case of STSL, all of the activities were found in the cell extract, similar to the case of other bacterial laccases.

Although the functions of fungal laccases have been well studied, those of bacterial laccases were hardly identified. \textit{M. mediterranea} contains two PPOs, a tyrosinase-like PPO and multipotent PPO showing all the activities of tyrosinase and laccase.\(^{32}\) In this strain, a tyrosinase-like PPO is required for melanogenesis.\(^{32}\) But the function of multipotent PPO has so far been unclear. \textit{Azospirillum lipoferum} laccase was identified to be involved in melanin formation.\(^{33}\) \textit{B. subtilis} cotA is expressed during sporulation.\(^{30}\) Quite recently, it was clarified that cotA is directly required for the formation of spore pigment.\(^{30}\) It was assumed that PHS from \textit{S. antibioticus} was involved in the biosynthesis of actinomycin,\(^{34}\) but a disrupted mutant of the \textit{phsA} gene produced slightly more actinomycin than wild-type, so it was concluded that PHS is not required for actinomycin biosynthesis.\(^{31}\) Since PHS is a component of \textit{S. antibioticus} spores and has sequence similarity with cotA, it is thought that PHS as well as cotA may be involved in the process of spore formation.\(^{31}\)

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**Fig. 3.** Alignment of the Putative Copper-binding Motifs of STSL and Other Laccases.

The amino acid residues corresponding to potential copper binding sites of three recognized types are shown as follows: type I, T\(_1\); type II, T\(_2\); type III, T\(_3\). Highly conserved amino acid residues that are common in more than four laccases among the six aligned laccases are shaded. Abbreviations: 

- \textit{S. laven.}, Streptomyces lavendulae REN-7 STSL (this paper);
- \textit{S. antib.}, \textit{Streptomyces antibioticus} PHS;
- \textit{B. subti.}, \textit{Bacillus subtilis} cotA;
- \textit{M. medit.}, \textit{Marinomonas mediterranea} multipotent PPO;
- \textit{C. subve.}, \textit{Ceriporiopsis subvermispora} laccase 1;
- \textit{T. versi.}, \textit{Trametes versicolor} laccase A.

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<th>T(_2)</th>
<th>T(_3)</th>
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2173
function(s) of STSL have been quite unknown. It requires further study.

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References


