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Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*

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Summary. A newly identified extracellular laccase produced by *Streptomyces ipomoea* CECT 3341 (SilA) was cloned and overexpressed, and its physicochemical characteristics assessed together with its capability to decolorize and detoxify an azo-type dye. Molecular analysis of the deduced sequence revealed that SilA contains a TAT-type signal peptide at the N-terminus and only two cupredoxine domains; this is consistent with reports describing two other *Streptomyces* laccases but contrasts with most laccases, which contain three cupredoxine domains. The heterologous expression and purification of SilA revealed that the homodimer is the only active form of the enzyme. Its stability at high pH and temperature, together with its resistance to high concentrations of NaCl and to typical laccase inhibitors such as sodium azide confirmed the unique properties of this novel laccase. The range of substrates that SilA is able to oxidize was found to be pH-dependent; at alkaline pH, SilA oxidized a wide range of phenolic compounds, including the syringyl and guayacil moieties derived from lignin. The oxidative potential of this enzyme to use phenolic compounds as natural redox mediators was shown through the coordinated action of SilA and acetosyringone (as mediator), which resulted in the complete detoxification of the azo-type dye Orange II. [**Int Microbiol** 2009; 12(1):13-21]

Key words: *Streptomyces ipomoea* · laccases · azo-dye detoxification

Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are simple multicopper oxidases, most of which have a similar structure based on three cupredoxine-type domains. These enzymes are of low substrate specificity and oxidize a broad group of phenolic compounds, including monophenols, polyphenols, and methoxy-substituted phenols as well as aromatic amines and metallic ions [16,22,42,47]. Laccases are widely distributed in nature and have been described in fungi [3,8], plants [30], insects [10], and, more recently, in bacteria [2,14,26,29]. The biological role of fungal and plant laccases has been exhaustively studied and shown to be related to the degradation and synthesis of lignin, respectively [23]. Among the increasing number of bacterial laccases reported, several with distinctive functions have been described, including roles in morphogenesis and sporulation processes, pigment production, and resistance to copper and phenolic compounds [14,40,42,46,48].

Research on laccases has intensified in recent years, with particular focus on their catalytic versatility and oxidative activity in the absence of any requirement for additional reactive compounds (in contrast to peroxidases). In addition,

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the possibility to clone these enzymes and to expand their range of oxidation through redox mediators offers considerable biotechnological potential. The use of low-molecular-weight compounds as mediators has particular merit becasue once they are oxidized by laccases to stable radicals, these radicals may continue oxidizing other compounds, including those not used directly as substrates by the enzyme. The application of laccases to the textile industry is particularly important. In fact, 90% of reactive textile dyes entering activated sludge sewage treatment plants pass through unchanged and are discharged into rivers [1]. Although several combined oxic and anoxic treatments have been reported to enhance the degradation of textile dyes, the generation of carcinogenic amines from azo dyes (the most widely used dyes in the industry) through anoxic processes poses a serious health hazard. In addition, the colored industrial effluents significantly reduce oxygen solubility in receiving waters and are thus an important environmental hazard. Laccases and laccase-mediator systems therefore offer the potential to oxidatively degrade a wide range of aromatic compounds, providing an alternative to conventional treatments [20]. Fungal laccases combined with synthetic [36,43] or natural [6] mediators have been reported to decolorize several groups of textile dyes. Recently, a *Streptomyces* laccase that decolorizes an indigoid dye was described, opening up the possibility to use bacterial laccases for new purposes [11]. However, the suitability of these enzymatic systems to decolorize and detoxify azo-type dyes has yet to be fully evaluated.

This work describes the molecular and physicochemical characterization of a laccase produced by *Streptomyces ipomoea* CECT 3341, together with its heterologous expression and its effectiveness in decolorizing and detoxifying an azo dye.

Materials and methods

Bacterial strains, growth conditions, plasmids, and oligonucleotides. The characteristics of *Streptomyces ipomoea* and *Escherichia coli* strains as well as those of the plasmids and oligonucleotides used in this study are summarized in Table 1-SI. To obtain chromosomal preparations, *S. ipomoea* was grown in YEME medium [21] at 28ºC with shaking at 180 rpm. *E. coli* strains were grown at 37ºC in LB medium, which was supplemented with ampicillin (50 μg/ml), kanamycin (25 or 50 g/ml) or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) as required.

Laccase production and N-terminal amino-acid sequence determination. Active supernatant containing laccase activity was obtained after 4 days of incubation of *S. ipomoea* in a minimal basal medium supplemented with 1% (w/v) galactomannan and 0.2 % (w/v) asparagine [31]. The supernatant was 10-fold concentrated in a Macrosep 3K ultrafiltration system (Pall Corporation). The concentrated broth (1 ml) was electrophoresed by SDS-PAGE under native conditions. Gels were immersed in a solution containing 5 mM ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma) in 100 mM acetate buffer pH 5 to reveal the laccase activity band. The active band was excised and the protein electroeluted by dialysis (10–12 kDa nominal cut-off). The content of the dialysis bags containing the gel fragment was subjected to electrophoresis (Bio-Rad) at 90 V for 30 min at 4ºC. Once electroeluted, the proteins were concentrated in a 3K Nanosep ultrafiltration system (Pall Corporation). This concentrate was re-electrophoresed using a 15% polyacrylamide gel under native conditions. Laccase activity was detected as previously described and proteins bands were stained with Coomasie brilliant blue R-250 (Bio-Rad). Active bands were excised and electroeluted as above and, after concentration, the proteins were denatured by heating at 100ºC in the presence of 5 % β-mercaptoethanol (v/v). Molecular mass of purified protein was estimated by comparison with molecular mass markers (Bio-Rad). Finally, denaturing PAGE sheets of purified laccase were transferred to a polyvinylidene difluoride membrane (PVDF-Immobilon-P, 0.45 μm; Millipore) using a Trans-Blot electrophoretic transfer cell (Bio-Rad). The band, after staining with 0.1% (w/v) amido black, was excised and sequenced using the degradation method [12] in a Procise Protein Sequencer (Applied Biosystems) according to the manufacturer's instructions.

DNA technology. *Streptomyces ipomoea* chromosomal DNA was obtained using standard method [21,39]. Plasmids were prepared using the High Pure Plasmid Isolation Kit (Roche) or the Wizard Plus SV Midipreps DNA Purification System (Promega). PCR products were purified by the High Pure PCR Product Purification Kit (Roche). Routine techniques for restriction, digestion, ligation, and transformation were used [39].

Cloning of the laccase gene *silA*. Two oligonucleotides were designed on the basis of the N-terminal amino acid sequence of the native protein and the second copper-binding region of laccases from *S. coelicolor* and *S. griseus*. The PCR product was labeled with digoxigenin-dUTP; Southern hybridization was then carried out at 42ºC with *S. ipomoea* chromosome digested with different endonucleases. Color detection was performed with an antibody anti-digoxigenin conjugated with alkaline phosphatase using nitro blue tetrazolium and BCIP. All three methods were carried out according to the recommendations provided with the DIG DNA Labeling and Detection Kit (Roche). Fragments of appropriated size were purified from an agarose gel by electroelution and ligated into linearized pBSK. *E. coli* JM109 was transformed with the ligation mixture, and the transformants were selected on LB plates supplemented with ampicillin and X-Gal. Colonies containing a positive plasmid were identified by colony hybridization with the previously described probe.

The deduced protein sequence was compared with sequences in the databases using BLAST software from Expasy Proteomics Server [http://www.expasy.org/tools/blast/]. The presence of signal peptide was verified with the prediction servers SignalP [http://www.cbs.dtu.dk/services/SignalP/] and TatP [http://www.cbs.dtu.dk/services/TatP/]. InterPro Scan [http://www.ebi.ac.uk/InterProScan/] analysis was used to determine the presence of conserved domains.

Heterologous expression and purification of the recombinant laccase. The DNA fragment encoding SilA, including the region for the signal sequence, was generated by PCR with pBSK-SIL2 plasmid as template and the primers EXP-ATC and EXP-CT. The first primer introduced a *Nde*I site at the start codon, and the second a *Bam*HI site after the stop codon. The *Nde*I/*Bam*HI-digested amplicon was cloned into pET28a(+) (Novagen), generating pET-ATC, which was used to transform *E. coli* BL21(DE3) competent cells. Transformants were selected on LB plates containing 25 μg kanamycin/ml.

For the production of SilA, 2 l of LB liquid medium containing 50 μg kanamycin/ml was inoculated with 40 ml of an exponential-growth-phase culture obtained in the same medium (OD_{600} 0.5–1.0). When exponential growth had resumed, the temperature was reduced to 28ºC, and SilA expression induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma). After 2.5 h, cells were harvested by centrifugation at 4ºC for 10 min

at 12,000 ×*g*, the pellet was washed twice with PBS buffer [39] and stored at –20ºC until processed. To obtain the recombinant protein, cells were suspended in chilled 10 mM phosphate buffer (pH 7) and completely disrupted in a French press (Sim-Aminco) after two passes at 1500 PSIG. The insoluble fraction was discarded after centrifugation (10 min at 15,000 ×*g* and 4ºC), and the soluble fraction containing the protein was collected. To activate the enzyme, this fraction was incubated for 3 h on ice with 1 mM $CuSO_4$, dialyzed at 4ºC overnight against 5 l of 10 mM phosphate buffer (pH 7), and stored at –20ºC until processing.

The His-tagged recombinant laccase was purified using a fast protein liquid chromatography (FPLC) system (Pharmacia). The sample was loaded onto a HisTrap HP column (GE Healthcare) equilibrated with 20 mM sodium phosphate buffer, 0.5 M NaCl, and 10 mM imidazole (pH 7.4). Prior to application to the column, the samples were mixed with an equal volume of the mobile phase (2-fold concentrated). The protein was eluted with the same buffer but containing 750 mM imidazole and the active fractions were dialyzed against PBS buffer in a PD-10 desalting column (GE Healthcare) to remove imidazole. The amount of protein in the different samples was determined by the Bradford method [5] using the Bio-Rad Protein Assay; the efficiency of expression and purification was verified by native and denaturing gel electrophoresis (PAGE). The samples were boiled for 10 min with 5% (v/v) β-mercaptoethanol for denaturation.

Enzyme assays. Laccase activity was routinely determined at room temperature by measuring the oxidation of 5 mM ABTS in 50 mM acetate buffer (pH 5). The increase in the absorbance at 436 nm was monitored considering a molar extinction coefficient of 29,300 M^{-1} cm^{-1} for oxidized ABTS. When 2,6-DMP (2,6-dimethoxyphenol) was used as substrate, the assay was carried out with 5 mM 2,6-DMP in 50 mM phosphate buffer (pH 8) and the absorbance increase was monitored at 469 nm, based on a molar extinction coefficient of 27,500 M^{-1} cm^{-1} [28].

Recombinant laccase characterization. The optimal temperature for enzyme activity was determined using a temperature range of 25–85ºC. Thermal stability was estimated by incubating the enzyme with 25 mM phosphate buffer (pH 7) at a temperature range of 30–60ºC and subsequently measuring activity. Optimal pH was determined with different substrates in a pH range from 3 to 10 with 50 mM Britton-Robinson buffer [49]; pH stability was estimated by incubation in the same buffer and pH range as above. All these assays were carried out using ABTS as substrate. The molecular mass of the protein was determined by SDS-PAGE, comparing the bands with those of molecular mass markers (Bio-Rad). The isoelectric point of the protein was determined using IEF gels on a Phast System (Pharmacia) with broad pI standards. The UV-visible absorption spectrum of the purified laccase was assessed between 200 and 800 nm using a Hitachi U-2001 spectrophotometer. The sample was concentrated and completely dialyzed against 0.1 M phosphate buffer (pH 7) with a Nanosep system until the absorbance ratio A_{280}/A_{585} was <10.5. The effect of several compounds (EDTA, citric acid, oxalic acid, $FeSO_4$, $CuSO_4$, $MgSO_4$, $ZnSO_4$, $CoSO_4$, $MnCl_2$, $CaCl_2$, NaCl, sodium azide, potassium cyanide, thioglycolic acid, dithiothreitol, and tropolone) at different concentrations on laccase activity was determined using 5 mM ABTS at pH 5 and 10 mM 2,6-DMP at pH 8 as substrates.

The substrate specificity of the recombinant laccase was examined against 36 different putative substrates by a qualitative assay that monitored the changes in the absorption spectrum after a 24-h incubation. All compounds were assayed at 500 µM at pH 5 and pH 8 (20 mM phosphate buffer). The compounds assayed were: phenolic compounds [tyrosine, phenol, 2-methoxyphenol, 3-methoxyphenol, 4-methoxyphenol, 2,6-dimethoxyphenol, 3,5-dimethoxyphenol, catechol, resorcinol, hydroquinone, 2-methylhydroquinone, 2-methoxyhydroquinone, *p*-nitrophenol, 2-chlorophenol, 4-chlorophenol, 2,4,6-trimethylphenol, 2-aminophenol, 4-aminophenol, 4-hydroxybenzyl alcohol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-hydroxyacetophenone, vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone), protocatechuic acid]; aromatic amines (aminobenzene, *p*-diaminobenzene, *N,N*-dimethyl-*p*-phenylenediamine, 4-methoxyaniline); other aromatic compounds (syringaldazine, ABTS), and non-aromatic compounds (potassium ferrocyanide).

Steady-state kinetic constants for the purified laccase were estimated from Lineweaver-Burk plots. When ABTS or 2,6-DMP was used as substrate, the determinations were carried out as described above using a concentration range from 0 to 6.25 and 0 to 50 mM, respectively. With *p*-diaminobenzene, the reactions were monitored at 520 nm in 50 mM phosphate buffer (pH 6.5). To calculate k_{cat}, the molecular mass of the protein, as estimated electrophoretically, was used.

Decolorization assays and toxicity determination. Decolorization assays were carried out at 35ºC for 24 h. The reaction mixture contained 50 mM phosphate buffer (pH 8), 400 mU purified laccase/ml, 50 µM Orange II (Sigma), and 100 µM acetosyringone as mediator. Decolorization was monitored by measuring the absorbance at 485 nm. The molar extinction coefficient for Orange II at this wavelength and at pH 8 was 23,352 M^{-1} cm^{-1}.

After decolorization, toxicity was determined by the bioluminescent bacterium *Vibrio fischerii* according to the ISO 11348-3 and using the Microtox method. For these assays, the initial dye concentration was 170 µM. Samples were serially diluted in 2% NaCl (w/v) and this solution was used as negative control. Each assay was performed in duplicate at pH 8, 15ºC. An AZUR Environmental M500 Analyzer with MicrotoxOmni software was used to measure luminescence at 490 nm and to determine acute toxicity (5- and 15-min incubation times), expressed as the EC_{50}. The same dye dilutions used for toxicity test were spectrophotometrically measured at 490 nm. The absorbance values were used for the color correction, as recommended by the manufacturer.

Nucleotide sequence accession numbers. The nucleotide sequence of the DNA fragment containing the *silA* gene was deposited in the GenBank database under accession no. DQ832180.

Results

N-terminal amino-acid sequence of the native laccase. Gels stained with ABTS and Coomassie blue verified that a unique protein band was present after two steps of electrophoresis and electroelution. The molecular mass of this native laccase produced by *S. ipomoea* was estimated to be 77 kDa. However, after denaturing the enzyme with β-mercaptoethanol, a single band with a molecular mass of around 33 kDa was obtained. Comparison of the experimental N-terminal amino acid sequence (GGEVRHIKMCAEKLP) with other sequences deposited in the databases revealed a high similarity with two laccases from *Streptomyces* (85% with SLAC from *S. coelicolor* and 73% with EpoA from *S. griseus*).

Cloning, sequencing and characterization of the *silA* gene. Analysis of the obtained sequence showed a high percentage of identity with the above-mentioned *Streptomyces* laccases (94% and 79%, respectively). A 7.5-kb fragment from the chromosome digested with *Bam*HI was identified, which may have contained the gene for the lac-

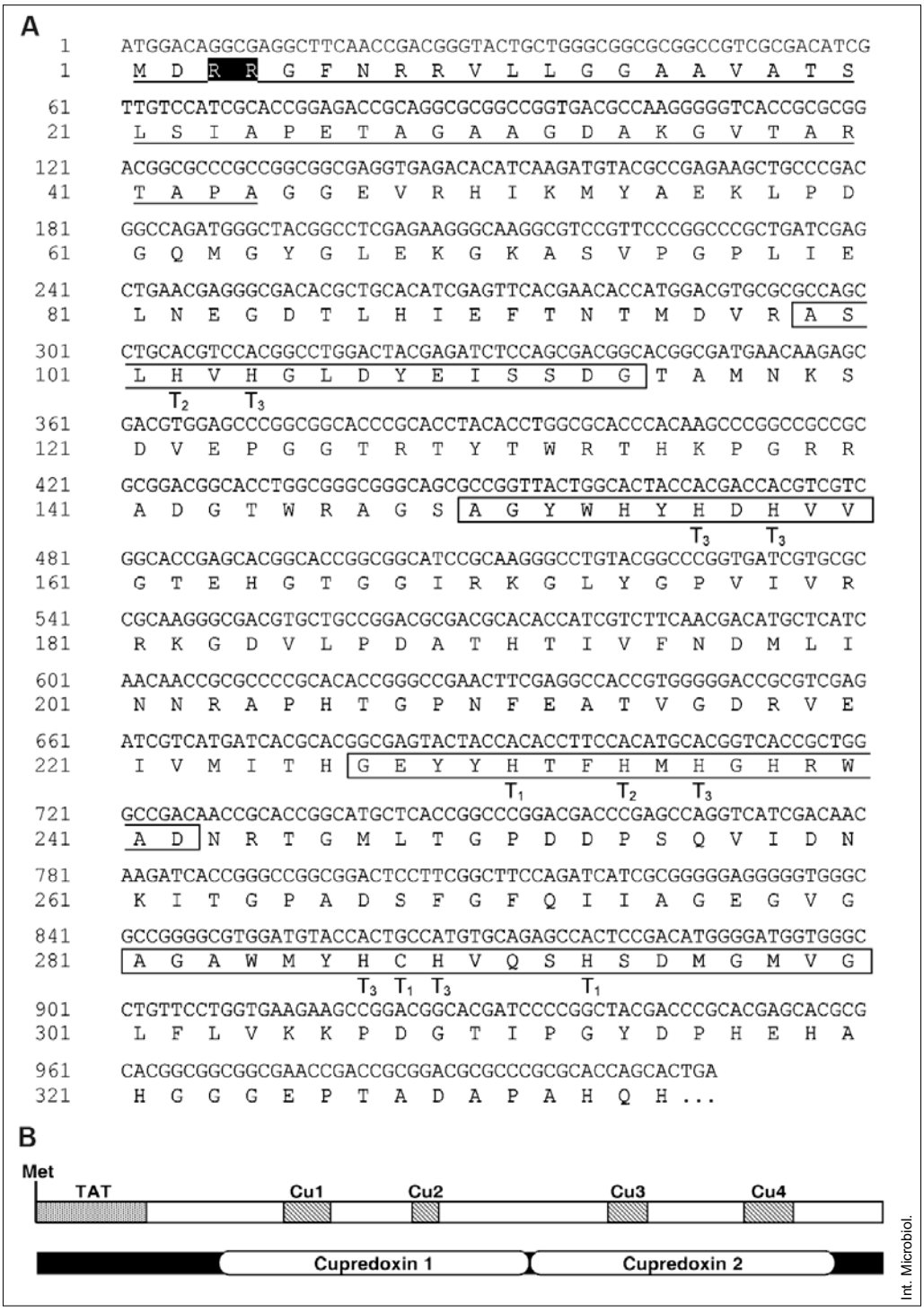

Fig. 1. Sequence and organization of the laccase SilA from *S. ipomoea*. (**A**) Nucleotide sequence of the *silA* gene and amino acid translation. The underlined sequence corresponds to the signal peptide. Twin arginines belonging to the TAT system are shown in a black box. Boxed amino acids correspond to those comprising the four copper-binding regions. Histidine and cysteine residues involved in the coordination of copper atoms are marked according to the copper type. (**B**) Schematic representation of SilA. The upper scheme shows the location and size of the four copper regions and the leader sequence. The lower scheme indicates the positions of the two cupredoxine domains.

case. The nucleotide sequence of this 2-kb insert confirmed the presence of a 1008-bp open reading frame (ORF) corresponding to the laccase gene, designated *silA*. The deduced amino acid sequence of SilA had 90% identity with SLAC and 73% with EpoA laccases. Manual analysis of the entire sequence of the protein showed the presence of the four His-rich regions involved in copper binding (Fig. 1). SilA contains a signature sequence in the N-terminal portion (RRGF) followed by the twin arginine translocation (TAT system). Furthermore, InterProScan analysis revealed the presence of two cupredoxine domains, in the amino acids intervals 73–195 and 196–316.

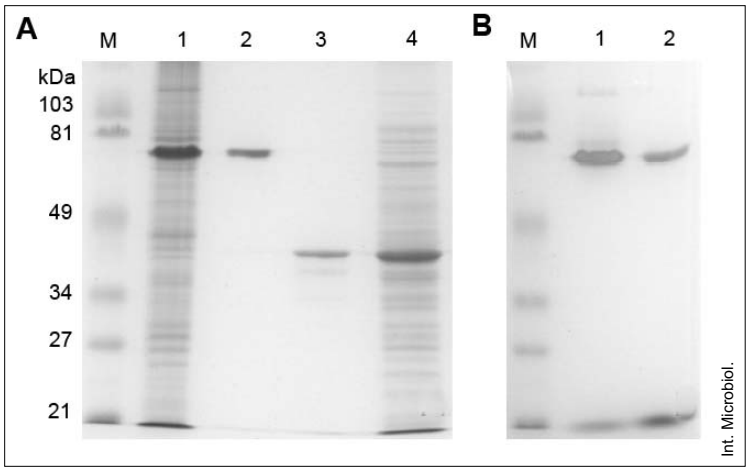

Fig. 2. Verification of SilA purification efficiency and laccase activity by zymogram. (**A**) SDS-PAGE of crude extract and the purified laccase stained with Coomasie Brilliant Blue R-250. (**B**) Zymogram of laccase activity, using 2,6-DMP as substrate. In both images; 1, crude extract after copper incubation and dialysis; 2, purified laccase; 3, purified laccase denatured with β-mercaptoethanol; 4, crude extract denatured with β-mercaptoethanol.

Heterologous expression and purification of the recombinant laccase. *silA*, including the TAT-leader sequence, was cloned into pET28a and the final construct was used to transform *E. coli* BL21(DE3), yielding a N-terminal His-tagged recombinant protein after induction with IPTG. The recombinant protein was always found in the cytoplasmic fraction. When the cells were cultured at 37ºC, a fraction of the protein was produced in insoluble form. This problem was avoided by growing the cells at 28ºC, after induction with IPTG. The purification process resulted in a purified laccase that had a specific activity of 13.60 U/mg protein, representing a 4.8-fold purification, and corresponded to a final yield of 85.6%. SDS-PAGE revealed that the molecular mass of samples denatured with β-mercaptoethanol was half that of non-denatured samples, indicating the homodimeric nature of the protein (Fig. 2).

Characterization of the purified recombinant laccase. The optimum pH for activity of the purified laccase was found to be substrate-dependent, with an optimum pH of around 5 for ABTS, 6.5 for an aromatic amine, and 8 for a phenolic compound. In terms of stability, the enzyme retained up to 100% of its initial activity after 36 h of incubation at pH values between 5 and 9. In contrast, the enzyme lost around 50% of its activity when incubated at pH 3–4. The optimum reaction temperature for the purified recombinant laccase was 60ºC, with the enzyme retaining up to 100% of its activity after incubation at 40ºC for 24 h at pH 7 and more than 50% of its activity after incubation at 60ºC for the same period. The molecular mass of the dimeric form of this laccase, based on SDS-PAGE, was 79 kDa, and the molecular mass of the monomeric form 44.7 kDa (Fig. 2). The isoelectric point for this laccase was 6.18. The UV-visible spectrum of the purified laccase had an absorption peak at 585 nm and a shoulder at around 330 nm (data not shown); these peaks correspond to type 1 and type 3 copper, respectively [44], enabling the classification of the protein as a multicopper oxidase.

A qualitative study of substrate specificity revealed that not all phenolic compounds act as substrates for this enzyme (Table 2-SI). SilA was unable to oxidize tyrosine, distinguishing this enzyme from tyrosinases. In general, the laccase oxidized phenolic compounds containing methoxyl and amino groups under alkaline pH conditions. Although aromatic amines were oxidized at acidic and basic pH values, optimum activity was detected at alkaline pH. However, ABTS and ferrocyanide were oxidized only under acidic pH conditions.

Table 3-SI shows the effect of several compounds on the laccase activity at acidic and basic pH conditions. The addition of either citric or oxalic acid resulted in a decrease in laccase activity. Other common chelating agents such as EDTA inhibited activity only at pH 8 and at high concentrations (100 mM). The addition of ferrous iron resulted in the strongest inhibition of the laccase. Copper ions also had a noticeable inhibitory capacity, especially at acidic pH. SilA showed exceptional resistance to sodium chloride, maintaining 100% activity at high concentrations of this salt (1 M) at pH 8. In addition, the enzyme maintained 64% of its activity at pH 5. SilA also showed an unusual tolerance to sodium azide (10 mM), retaining more than 60% at pH 5 and more than 94% at pH 8.

The kinetic constants of the purified laccase were determined with ABTS, 2,6-DMP, and *p*-diaminobenzene, under optimal pH conditions in each case. For ABTS, the K_m was 0.40 mM; V_{max}, 7.59 mU/μg; k_{cat}, 9.99 s^{-1}; and k_{cat}/K_m, 2.50×10^4 M^{-1} s^{-1}. With 2,6-DMP, the kinetic constants were as follows: K_m, 4.27 mM; V_{max}, 3.19 mU/μg; k_{cat}, 4.20 s^{-1}; and k_{cat}/K_m, 0.98×10^3 M^{-1} s^{-1}. In the case of *p*-diaminobenzene, the absorption spectrum changed significantly with pH. At the optimum pH for this

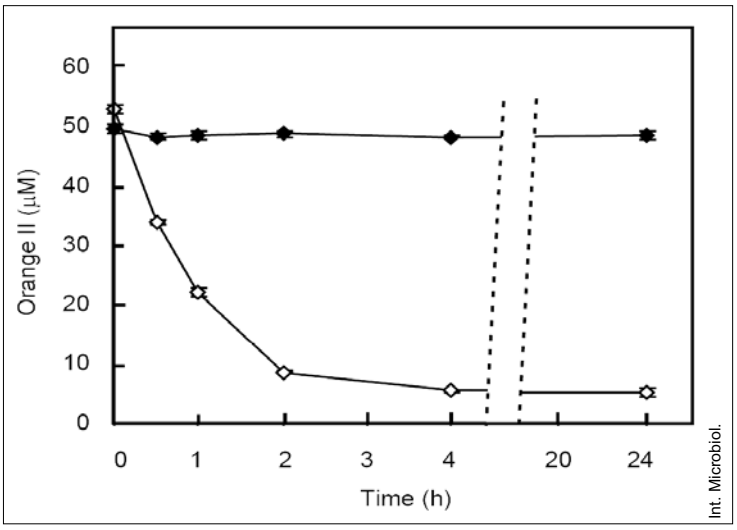

Fig. 3. Decolorization pattern of Orange II in the presence of laccase (closed diamonds) and the laccase-acetosyringone system (open circles).

substrate, maximum absorption was reached at 520 nm, but at this wavelength the molar extinction coefficient for this substrate could not be calculated, while the K_m was determined to be 1.41 mM.

Decolorization of Orange II and toxicity determination. The recombinant laccase alone could not decolorize the azo-type dye Orange II. However, the addition of a redox mediator, acetosyringone, resulted in a 90% decrease in the dye's color after 4 h of incubation, which corresponded to a decrease in the dye concentration from 50 to 5 μM (Fig. 3). No color reversion or new color formation was detected after 24 h of incubation. For toxicity determination, a higher concentration of Orange II (170 μM) was used, enabling detection in the Microtox system. At this dye concentration, the acute toxicity, expressed as the EC_{50}, was 32 ± 1.50 μM. When Orange II was incubated with the laccase-acetosyringone system under the same conditions as in the previous experiment, complete detoxification of the dye occurred after a 4-h incubation.

Discussion

The paucity of information on the molecular and physiological characteristics of bacterial laccases encourages the screening of new enzymes in order to increase our knowledge of their functional and biotechnological perspectives. Sequencing of the N-terminal region of a *S. ipomoea* protein with activity against ABTS allowed us to clone a gene encoding a new bacterial laccase, designated SilA. Analysis of the deduced sequence of *silA* showed that the protein is secreted by the TAT pathway, which transports fully folded proteins with their cofactor bound before translocation [4]. An association with this export system, which is especially common in *Streptomyces* [9], has been reported in other multicopper oxidases, such as CueO from *E. coli* [35] and CuoB from *Myxococcus xanthus* [42].

SilA contains the copper-binding motifs typical of multicopper oxidases, i.e., ten histidines and one cysteine [25,44]. Note that only two cupredoxine domains were detected in the SilA sequence, as was previously described for the SLAC protein of *S. coelicolor* [26]; this is in contrast to laccases containing three cupredoxine domains. Based on this evidence, the presence of only two cupredoxine domains could be considered as a feature common to certain *Streptomyces* laccases. The absence of a third cupredoxine domain might explain why SilA has the smallest number of amino acids (335) among all laccases described so far. Heterologous expression of SilA in *E. coli* led to the production of the recombinant laccase, which was obtained mainly in the soluble fraction simply by reducing the incubation temperature of *E. coli* after IPTG induction.

Among the physicochemical characteristics of SilA, its response to pH is of great interest. In fact, the range of compounds that this enzyme can oxidize was found to be pH-dependent. The optimal pH for ABTS oxidation was 5, which is similar to that described for the laccase of *S. cyaneus* [2] and higher than the average pH (3) among fungal laccases [3]. However, the optimum pH for the oxidation of 2,6-DMP was around 8. This alkaline pH for laccase activity against a phenolic compound is only found in a few bacteria and fungi [26,38]. As expected, the study of substrate specificity at pH 5 and pH 8 (Table 2-SI) revealed that the enzyme was able to oxidize phenolic compounds only at pH 8. In addition, SilA activity was strongly affected by the nature, position, and number of ring substituents. In general, the presence of elec-

tron-donor substituents (i.e., methoxyl and hydroxyl groups) in the *ortho*- and *para*- positions conferred increased enzyme activity. However, SilA was unable to oxidize phenolic compounds when these substituents were in the *meta*-position; this negative effect on activity was also shown for aromatic compounds containing electron-withdrawing substituents (e.g., NO_2^-, Cl^-).

The activity shown by SilA against lignin-related compounds, such as syringaldehyde, and acetosyringone (derived from syringyl units) and vanillyl alcohol (derived from guaiacyl units), is noteworthy as it allows their exploitation as redox mediators in order to extend the oxidative range of SilA to compounds not directly oxidized by the enzyme. SilA was also able to oxidize, at the two pH values assessed, all the assayed aromatic amines except aminobenzene, probably due to the requirement for additional activating groups in the ring to be oxidized.

The activity of SilA at alkaline pH is similar to that previously described for the laccases produced by *S. coelicolor* [11,26] and is in contrast to most laccases, which are only active at acid pH. This unusual property may be due to the resistance of some *Streptomyces* laccases to hydroxide, which has been described as a laccase inhibitor in that it blocks electron flux in the trinuclear copper center [49]. In addition, the increase in activity with increasing pH has been attributed to a decrease in the substrate reduction potential, which favors electron transfer to the type 1 copper site [49]. On the other hand, the presence of a highly conserved aspartic acid residue in the multicopper oxidases of eukaryotes has been shown to correspond with O_2 reduction when this amino acid is protonated [34]. However, although SilA conserves the aspartate near the first Cu-binding region, the alkaline pH activity of this enzyme cannot be attributed to the same mechanism. Taking into account that the redox potential of substrates whose oxidation does not involve protons (i.e., ABTS and ferrocyanide) is therefore pH-independent [49], it is surprising that SilA was able to oxidize both substrates only at acid pH.

The study of the electrophoretic mobility of native and recombinant SilA in SDS-PAGE showed that the enzyme did not lose activity in the presence of SDS. Further denaturation of both proteins with β-mercaptoethanol revealed the enzyme's dimeric nature. The difference between the molecular mass of the monomeric forms of native (33 kDa) and recombinant (44 kDa) laccase could be attributed to the presence of a His-tag in the latter and/or to any slight modificacould reduce the electrophoretic mobility of proteins. Many bacterial laccases are multimeric, although their monomeric constituents also show activity [7]. However, the laccase from *S. ipomoea* was only active in the dimeric form, as has been described for other *Streptomyces* laccases with two cupredoxine domains [14,26]. A polypeptide chain with only two cupredoxine domains may not be enough to provide for the correct configuration of the trinuclear center of Cu (T_2-T_3) [32], explaining why a dimeric form is necessary for activity. The unique characteristics of these laccases have led some authors to consider laccases with only two domains as the ancestral link of the remaining multicopper oxidases.

The behavior of SilA against a range of putative inhibitors was also found to be pH-dependent. The high resistance shown by this enzyme to EDTA (100 mM) at acidic pH can be compared to that described for fungal laccases [24,27]. In contrast, at acidic pH, *Streptomyces* laccases are commonly inhibited by EDTA at a concentration of 1 mM [13,45]. However, when inhibited at alkaline pH, SilA activity was detected at the lowest concentration of EDTA assayed. In terms of inhibition by metal ions, Fe^{2+} strongly inhibited the enzyme. The inhibitory effect of Fe^{2+} has been described also for laccases from *S. cyaneus* [2] and some fungal laccases [3]. This iron ion may be a competitive substrate for laccase activity in those enzymes that also show ferroxidase activity [26]. Enzyme activity was also inhibited in the presence of high concentrations of Cu^{2+}; this has been described also for the laccase of *Trametes versicolor* [24], even though a positive effect of this ion on the activity of the laccase produced by *S. cyaneus* also has been reported [2].

SilA showed a differential response to the presence of two laccase activity inhibitors, potassium cyanide and sodium azide. A total loss of activity was detected with 1 mM potassium cyanide; as has been described for other bacterial laccases [13,37]. However, SilA showed partial resistance to sodium azide, losing only 40% of its activity in the presence of 10 mM of this compound. This result distinguishes this enzyme from other laccases, such as those produced by *T. versicolor*, *S. lavendulae*, and *Rhus vernicifera*, which are inhibited, either totally or partially, in the presence of 100 μM, 1 mM, and 2.5 mM sodium azide, respectively [18,19,45]. Considering that sodium azide binds to copper atoms in the trinuclear center of laccases, the high resistance of SilA to this compound and its high tolerance to hydroxide suggest that the configuration of the enzyme's active center differs from that of other laccases. The high sensitivity shown by SilA against reducing agents (thioglycolic acid and dithiothreitol) should not be considered as true inhibition, because the reducing action on the oxidized products could mask the effect. The high resistance of SilA to high concentrations of sodium chloride is also of interest, with the enzyme maintaining 100% activity in the presence of 1 M sodium chloride at pH 8, and losing just 35% activity at the same concentration at pH 5. This unusual tolerance makes SilA one of the

most halo-resistant laccases [50]. Among bacterial laccases, halide tolerance has been described in the laccase of the marine bacterium *Marinomonas mediterranea* [17] and, more recently, in *S. coelicolor* [11].

One of the main bottlenecks that must be overcome in order to use laccases for biotechnological and environmental applications is their loss of effectiveness under extreme conditions. In this work, we examined the potential usefulness of SilA to degrade and detoxify an azo-type textile dye. Although SilA was not able to oxidize the azo dye Orange II directly, the action of this enzyme when combined with a phenolic compound such as acetosyringone (acting as a redox mediator) resulted in 90% dye decolorization. This level of decolorization is comparable with the best results obtained with fungal laccases degrading this type of dye [6,43]. However, most studies failed to evaluate the toxicity of either the dyes and/or the reaction products. In fact, the degradation of azo dyes could result in the production of compounds of increased toxicity [15]. Toxicity studies carried out during these studies evaluated the broader effect of SilA-acetosyringone system on the azo dye. Microtox analysis confirmed that this system was able not only to degrade the azo dye but also to detoxify it completely at alkaline pH. To our knowledge, this is the first description of a bacterial laccase able to degrade and detoxify azo dyes.

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Table 1-SI. Bacterial strains, plasmids and oligonucleotides used in this study

Bacterial strain	Description	Reference
Escherichia coli		
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F′[*traD*36 *proAB*+ *lacI*q l*acZ* ΔM15]	[51]
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	[33]
S. ipomoea		
CECT 3341		[31]
Plasmids	**Relevant features and/or genes**	**Reference**
pBSK$^+$	Ap^r; cloning vector	Stratagene
pBSK-SIL1	Ap^r; vector containing DNA probe	This study
pBSK-SIL2	Ap^r; vector containing chromosomal 7 kb fragment	This study
pBSK-SIL3	Ap^r; vector containing chromosomal 2 kb fragment	This study
pET28a	Km^r; general expression vector	Novagen
pET-ATC	Km^r; pET28a carrying *silA* gene	This study
Oligonucleotide	**Sequence (5′-3′)**[a]	**Purpose**
SipoMCOP1BamH1F	CCCGGATCCGARGTSCGSCACATCAAGATG	Synthesis of probe
SipoMCOP1BamH1R	CCCGGATCCGTGGTCGTGGTAGTGCCAGTA	Synthesis of probe
SecRev2	GCGAGGTGAGACACATCAAG	Amplification inside *silA*
SecUni2	GGGAGACGGTCGCCCGGGAC	Amplification inside *silA*
SecRev3	GCGTCGAGATCGTCATGATC	Amplification inside *silA*
SecUni3	CGTGATCATGACGATCTCGA	Amplification inside *silA*
SecRev4	GTGAACGGCGCGCAAGGCGT	Amplification inside *silA*
SecUni4	TCTTGATGTGTCTCACCTCG	Amplification inside *silA*
SecRev5	CCGACGGGTACTGCTGGGCG	Amplification inside *silA*
SecUni5	TCGCCGTGCCGTCGCTGGAG	Amplification inside *silA*
EXP-ATC	TTCCATATGGACAGGCGAGGCTTCA	Expression of SilA
EXP-CT	CCGGGATCCTCAGTGCTGGTGCGCGGGC	Expression of SilA

[a]Underlined nucleotides indicate the restriction sites added to the sequences.

Table 2-SI. Qualitative analysis of recombinant laccase activity on different types of substrates

Assay No.	Substrate	pH 5	pH 8	Assay No.	Substrate	pH 5	pH 8
1	tyrosine	–	–	19	4-hydroxybenzyl alcohol	–	–
2	phenol	–	–	20	4-hydroxybenzoic acid	–	–
3	2-methoxyphenol (guayacol)	–	+	21	4-hydroxybenzaldehyde	–	–
4	3-methoxyphenol	–	–	22	4-hydroxyacetophenone	–	–
5	4-methoxyphenol	–	+	23	vanillyl alcohol	–	+
6	2,6-dimethoxyphenol	+	+[a]	24	vanillin	–	–
7	3,5-dimethoxyphenol	–	–	25	vanillic acid	–	–
8	catechol (1,2-benzenediol)	–	+	26	syringaldehyde	–	+
9	resorcinol (1,3- benzenediol)	–	–	27	syringic acid	–	+
10	hydroquinone (1,4-benzenediol)	–	+	28	acetosyringone	–	+
11	2-methylhydroquinone	–	+	29	protocatechuic acid	–	+
12	2-methoxyhydroquinone	–	+[a]	30	aminobenzene	–	–
13	*p*-nitrophenol	–	–	31	*p*-diaminobenzene	+	+[a]
14	2-chlorophenol	–	–	32	N,N-dimethyl-*p*-diaminobenzene	+	+[a]
15	4-chlorophenol	–	–	33	4-methoxyaniline	+	+
16	2,4,6-trimethylphenol	–	–	34	potassium ferrocyanide	+[a]	–
17	2-aminophenol	–	+[a]	35	syringaldazine	-	+[a]
18	4-aminophenol	+	+[a]	36	ABTS	+[a]	–

+: detection of changes in the absorption spectra.

–: no changes were detected.

[a]With these compounds, changes were detected in the absorption spectra before 20 min.

Table 3-SI. Effect of different compounds on the activity of the purified laccase

Compound	Concentration	Activity (%)[a]		Compound	Concentration	Activity (%)[a]	
		pH 5	pH 8			pH 5	pH 8
EDTA (disodium salt)	100 mM	104.9	0.0	$CoSO_4$	10 mM	119.1	[b]
	10 mM	101.1	36.8		1 mM	97.5	99.6
	1 mM	96.2	84.6	$CaCl_2$	10 mM	88.5	[b]
Citric acid	1 mM	87.4	97.9		1 mM	95.0	100.0
Oxalic acid	1 mM	60.8	96.3	NaCl	1 M	64.4	107.5
$FeSO_4$	1 mM	0.0	1.4		100 mM	77.6	100.2
	100 µM	13.6	70.9		10 mM	96.1	101.7
	10 µM	93.3	94.4		1 mM	98.3	111.1
$CuSO_4$	100 mM	13.9	[b]	Sodium azide	10 mM	60.3	94.9
	10 mM	23.7	[b]		1 mM	80.8	86.4
	1 mM	38.4	62.3[c]		100 µM	81.9	90.0
$MgSO_4$	100 mM	137.7	[b]	Potassium cyanide	10 mM	0.5	0.0
	10 mM	106.0	97.2		1 mM	7.6	0.0
	1 mM	90.7	100.0		100 µM	34.1	45.8
$ZnSO_4$	100 mM	87.5	[c]	Thioglycolic acid	100 µM	0.0	8.5
	10 mM	71.4	[c]		10 µM	91.2[d]	53.4
	1 mM	72.4	111.0	Dithiothreitol	100 µM	0.0	0.0
$MnCl_2$	100 mM	83.9	[b]		10 µM	27.1[d]	41.8
	10 mM	90.2	[b]	Tropolone	10 mM	13.5	38.7
	1 mM	95.1	34.4		1 mM	84.3	91.5
					100 µM	95.1	100.0

[a]Relative activity (%) compared with the initial activity of the purified laccase.

[b]In these cases, a precipitate was formed, which prevented spectrophotometric determination.

[c]At this pH, these metallic ions oxidized 2,6-DMP, which prevented spectrophotometric determination.

[d]Values correspond to the first minute of reaction.