BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A bacterial laccase from marine microbial metagenome exhibiting chloride tolerance and dye decolorization ability

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Abstract Laccases are blue multicopper oxidases with potential applications in environmental and industrial biotechnology. In this study, a new bacterial laccase gene of 1.32 kb was obtained from a marine microbial metagenome of the South China Sea by using a sequence screening strategy. The protein (named as Lac15) of 439 amino acids encoded by the gene contains three conserved Cu^{2+}-binding domains, but shares less than 40% of sequence identities with all of the bacterial multicopper oxidases characterized. Lac15, recombinantly expressed in *Escherichia coli*, showed high activity towards syringaldazine at pH 6.5–9.0 with an optimum pH of 7.5 and with the highest activity occurring at 45 °C. Lac15 was stable at pH ranging from 5.5 to 9.0 and at temperatures from 15 to 45 °C. Distinguished from fungal laccases, the activity of Lac15 was enhanced twofold by chloride at concentrations lower than 700 mM, and kept the original level even at 1,000 mM chloride. Furthermore, Lac15 showed an ability to decolorize several industrial dyes of reactive azo class under alkalescent conditions. The properties of alkalescence-dependent activity, high chloride tolerance, and dye decolorization ability make the new laccase Lac15 an alternative for specific industrial applications.

Keywords Bacterial laccase · Chloride tolerance · Dye decolorization · Marine microbial metagenome · Multicopper oxidase

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Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC1.10.3.2), a family of blue multicopper oxidases, are capable of oxidizing a wide range of aromatic compounds, with concomitant reduction of molecular oxygen to water (Hoegger et al. 2006). The substrate range for laccases is greatly broadened by low-molecular-weight (MW) redox mediators, such as 2,2′-azino-bis (3-ethylbenzothazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT). In the presence of appropriate mediators, laccases can degrade several hardly degraded compounds, e.g., nonphenolic compounds (Bourbonnais and Paice 1990), polycyclic aromatic hydrocarbons (Pickard et al. 1999), and dye pollutants (Arora and Sharma, 2010). Laccases are thus regarded as potential candidates for biotechnological applications in industrial effluent detoxification, herbicide or pesticide elimination (Murugesan 2003; Wesenberg et al. 2003), organic synthesis (Mikolasch and Schauer 2009; Plonka and Grabacka 2006), and wood-based material production (Rodríguez Couto and Toca Herrera 2006; Witayakran and Ragauskas 2009), etc.

Laccases exist widely in higher plants, fungi, bacteria, and arthropods (Hoegger et al. 2006), and most of the industrial laccases, such as the DeniLite and the Zylite, are from fungi, especially basidiomycetes (Rodríguez Couto and Toca Herrera 2006). Unfortunately, nearly all fungal laccases lose their activities under alkaline conditions (Sharma et al. 2007), though they can recover to some extent under acidulous conditions after alkaline treatment (Xiao et al. 2003). Furthermore, fungal laccases are sensitive to chloride and lose most of the laccase activities at concentrations higher than 100 mM (Jimenez-Juarez et al. 2005). These disadvantages of fungal laccases make it an important work to screen for laccases bearing activity at high pH and high concentrations of chloride. Bacteria-derived laccases may be such ideal alternatives.

Analysis on prokaryotic genomes suggested that laccase-like enzymes may be widespread in bacteria (Alexandre and Zhulin 2000; Sharma et al. 2007). Laccase-like genes or activity have been found in several bacterial strains, including *Bacillus halodurans* (Ruijssenaars and Hartmans 2004), *Bacillus licheniformis* (Koschorreck et al. 2008), *Bacillus subtilis* (Martins et al. 2002), *Escherichia coli* (Roberts et al. 2001), γ-proteobacterium (Singh et al. 2007), *Thermus thermophilus* (Miyazaki 2005), and strepto-mycetes (Arias et al. 2003; Endo et al. 2003). However, few bacterial laccases have been biochemically characterized, and little is known about their applications in biotechnology industries (Arias et al. 2003; Koschorreck et al. 2009), albeit their applications in industrial processes are so potent. Identifying and characterizing new laccases from bacteria and evaluating their application potential will greatly help us better use them in industrial processes.

To obtain new laccases with ideal characteristics, a marine microbial metagenome of the South China Sea was constructed and screened using sequence strategy. A new bacterial laccase with alkalescence-dependent activity and excellent chloride tolerance was obtained and characterized. The potential of the enzyme in decolorization of industrial dyes was also evaluated.

Materials and methods

Microorganisms and agents

Escherichia coli EPI300 and pIndigoBAC-5 vector were obtained from Epicentre (Madison, WI, USA); *E. coli* DH5α and *E. coli* BL21(DE3) were from TransGen (Beijing, China); ABTS, 2,6-dimethoxyphenol (DMP) and syringaldazine were from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents were of analytical grade.

Construction of bacterial artificial chromosome (BAC) library and screening for laccase genes

Microbes were collected from the surface water of the South China Sea. High-MW DNA was prepared according to the protocol of Chu et al. (2008). DNA was partially digested with *Hin*dIII (NEB, Beijing, China) and separated by pulse field gel electrophoresis (Bio-Rad, Hercules, CA, USA). DNA fragments of 50–150 kb were recovered and ligated into pIndigoBAC-5 vector. The recombinant plasmids were transformed into *E. coli* EPI300 according to the manufac-turer's protocol. The clones obtained were then placed in 384-well plates and stored at −70 °C.

A sequence screening strategy, based on the conserved region of laccases in copper-binding sites, was adopted to clone laccase genes as follows: Firstly, 384 clones in the same plate were cultured in a bottle of Luria–Bertani (LB) liquid medium,

and mixed plasmids were extracted from the total culture and used as polymerase chain reaction (PCR) templates. Degenerate PCR primers of Cu1F (ACMWCKGTTCAYTGG-CACGG) and Cu4R (TGNTCNAGNAWGTGRCARTG), which were designed based on the relatively conserved sequences coding for the copper binding sites I and IV in bacterial laccases (Fig. S1), were used to amplify DNA fragments of potential laccases (Hoegger et al. 2006). Plates with a fragment about 1,100 bp in PCR products were considered as positive plates. Subsequently, the same PCR reaction was performed for every clone of positive plates and those with a fragment of about 1,100 bp in PCR product were considered as positive clones.

Inverse PCR was used to obtain the full length laccase genes. Briefly, the positive plasmids in positive clones were fully digested with *Nde*I (TaKaRa, Dalian, China), self-ligated by T4 DNA ligase (TaKaRa) and then used as templates. The flanking sequences of the laccase DNA fragments were amplified using LA-Taq (TaKaRa) with inverse primers of D15IF (TACACTTGTGGATGCGGGTGAGAC) and D15IR (AGACCCTTCGCAACTTGTTCCCAT). The PCR products were ligated into pMD18-T vector (TaKaRa) and sequenced.

Sequence analysis of *lac15*

The ORF of *lac15* was determined using the ORF Finder provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Similar sequence searching was performed using BlastP at NCBI. The module structure of the enzyme was analyzed with simple modular architecture research tool SMART (http://smart.embl-heidelberg.de/). The presence and location of the signal peptides in *lac15* was estimated using the Neural Networks and Hidden Markov models trained on Gram-negative bacteria with SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al. 2004). Multiple sequence alignment of *lac15* with other related laccase sequences was performed using Clustal X 2.0 and GENEDOC.

Heterologous expression of *lac15* in *E. coli*

The open reading frame (ORF) of *lac15* was amplified using the primer pair of AAACATATGAACAGGCGA-GACTTCCTGG (*Nde*I site italicized) and AAA CTCGAGTGCGACCTCCACCCAGGTCT (*Xho*I site italicized). Construction of plasmid pET22b–*lac15* was performed according to Koschorreck et al. (2008). *Escherichia coli* BL21(DE3) cells carrying pET22b–*lac15* were grown at 16 °C in 200 mL of LB medium containing 100 μg/mL ampicillin. Isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM was added into the culture of $OD_{600} \approx 0.6$ to induce enzyme expression. After an additional incubation for 16 h, the cells were

collected by centrifugation. The pellets were resuspended in cold 20 mM Tris–HCl buffer (pH 7.9) containing 500 mM NaCl and 5 mM imidazole, disrupted by sonication, and then centrifuged at 30,000×g for 30 min. The supernatant was applied to Ni–NTA (Novagen, Darmstadt, Germany) affinity chromatography to purify the recombinant Lac15 (Lac15). The purified protein was stored at 4 °C.

Characterization of Lac15

The assay mixture consisted of 10 μL of appropriately diluted protein stock and 990 μL of 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ buffer (pH 7.5) containing 100 μM syringaldazine (ε_{525}= 65,000 M^{-1} cm^{-1}) and 100 μM CuSO$_4$. The reaction was initiated by adding 10 μL syringaldazine into the solution. The mixture was incubated at 45 °C for 5 min and then transferred into ice-water bath for 30 s. One activity unit (U) was defined as the amount of Lac15 required for oxidizing 1 μmol of syringaldazine per minute. Alternative substrates for measurement of laccase activity were ABTS (ε_{420}= 36,000 M^{-1} cm^{-1}) and DMP (ε_{468}=49,600 M^{-1} cm^{-1}) at final concentrations of 0.5 and 2 mM, respectively. Reactions with heat-treated Lac15 were used as controls.

Protein concentration of Lac15 was determined following the Bradford method. The MW of Lac15 was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The pH optimal for activity was evaluated at 45 °C in 50 mM sodium citrate buffer (4.5–5.5), Na$_2$HPO$_4$–KH$_2$PO$_4$ buffer (5.5–8.0), and Tris–HCl buffer (8.0–9.0). The stability against pH was determined by measuring the residual activities of Lac15 after incubation at 4 °C for 1 h in the aforementioned buffer at different pH values. Effect of temperature on the enzyme activity was measured by incubating Lac15 at pH 7.5 and a temperature range from 15 to 55 °C. Thermostability was determined by incubating Lac15 at various temperatures (15 to 55 °C) at pH 7.5 for 15 min.

Effects of Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, K$^+$, NaN$_3$, SDS, and EDTA on Lac15 activity were investigated by incubating Lac15 with each effector for 15 min at 4 °C prior to substrate syringaldazine addition. The laccase assays were carried out under the aforementioned conditions. Chloride effect was determined at concentrations ranging from 1 to 2,000 mM. Control was carried out under the conditions with Na$_2$SO$_4$, KCl, or without NaCl in the normal manner.

Decolorization of dyes

Two anthraquinone dyes: Reactive Brilliant Blue X-BR (maximal absorbance at 600 nm) and K-GR (600 nm) and four azo dyes: Reactive Deep Blue M-2GE (620 nm), Reactive Brilliant Orange K-7R (490 nm), Reactive Red KM-8B (520 nm), and KD-8B (550 nm) were representatively selected to evaluate the ability of Lac15 to decolorize industrial dyes. Enzymatic treatment of the dyes was performed at 45 °C in the presence or absence of 100 μM mediator (ABTS, methyl-syringate, or syringaldehyde) in 1 mL Na$_2$HPO$_4$–KH$_2$PO$_4$ buffer (pH 7.5) containing 50 μM of dyes and 5 U/L or 10 U/L Lac15. The decolorization ability of Lac15 was determined spectrophotometrically as the relative decrease of absorbance at each maximal absorbance wavelength of the dyes.

Nucleotide sequence accession number

The nucleotide sequence data has been disposited in the GenBank database with accession number of HM623889. The corresponding protein ID is ADM87301.

Results

Cloning of a bacterial laccase gene

The marine microbial metagenome library obtained consisted of about 20,000 clones, harboring 1.4 Gb DNA. To search for new laccase genes, we screened for DNA fragments of laccase genes by PCR based on conserved sequences of copper binding sites I and IV in laccases (Fig. S1). A fragment (designated as *lac15p*) about 1.1 kb in length was amplified from one colony named as pSB46D15 using primers of Cu1F and Cu4R.

Inverse PCR was adopted to obtain the flanking sequences adjacent to *lac15p*. A fragment of about 5 kb (designated as *lac15rp*) was amplified from the self-ligated products of the *Nde*I-treated pSB46D15 BAC plasmids. The full-length laccase ORF of 1,320 bp (*lac15*) was thus obtained by integrating *lac15p* with *lac15rp*.

Analysis of *lac15* sequence

The ORF of lac15 encodes a putative protein of 439 amino acids with a predicted MW of 47,860 Da. The deduced amino acid sequence of Lac15 is the most similar to an uncharacterized hypothetical multicopper oxidase deduced from the genome of *Roseobacter* sp. MED193 (ZP_01057124) with 84% identity. Further analysis indicated that Lac15 has low sequence identities of less than 40% to all of the bacterial multicopper oxidases already characterized (Table S1), with 22% identity to the typical bacterial laccase from *B. subtilis* (CAB12449), 24% to that from *E. coli* (BAB96698), 26% and 21% to those from marine bacteria *Marinomonas mediterranea* (AAF75831), and *Oceanobacillus iheyensis* (BAC13302), respectively. Lac15 also shares low identity to the bacterial laccases of RL5 from a bovine rumen metagenome (CAK32503, 7%) and Lac591 from a mangrove soil metagenome (ACV83921, 25%). Potential bacterial laccase relatives for Lac15 were also found by searching

against the database of environmental samples at NCBI using BlastP, and all of the hits were uncharacterized hypothetical proteins from marine metagenomes (Yooseph et al. 2007).

Module analysis suggested that Lac15 possesses three conserved copper-oxidase domains (Fig. 1), which are characteristics of laccases (Hoegger et al. 2006), with the Pfam database accession numbers of PF07732, PF00394, and PF07731. The first 22 amino acid residues (MNRRDFLVTTSAATLFPRIALA) at the N-terminus were recognized as a Tat (twin arginine translocation; Pfam: PF10518) signal sequence.

Heterologous expression of Lac15

To analyze the biochemical properties of Lac15, its ORF absent of the 22-amino-acid-residue signal peptide was cloned into the plasmid pET-22b(+) and heterologously

expressed in *E. coli* BL21(DE3). Lac15 was expressed mostly in inclusion body when induced at 30 °C, but partly soluble when induced at 16 °C. However, no soluble protein was obtained even at 16 °C when the signal peptide was retained. The pure protein showed an apparent MW of 49 kDa as determined by SDS-PAGE (Fig. 2), in accordance with the value of 47.9 kDa calculated based on the amino acid sequence with the His_6 tag taken into account.

Characterization of Lac15

Lac15 was stable at pH values ranging from 5.5 to 9.0 and was the most stable at pH 7.0 (Fig. 3a). Specifically, it displayed the maximum activity at pH 7.5 (Fig. 3a) when using syringaldazine as substrate. On the other hand, the enzyme exhibited the highest activity at 45 °C and remained active even at 15 or 55 °C (Fig. 3b). The half-time of Lac15 was 72 min when incubated at pH 7.5 and 45 °C.
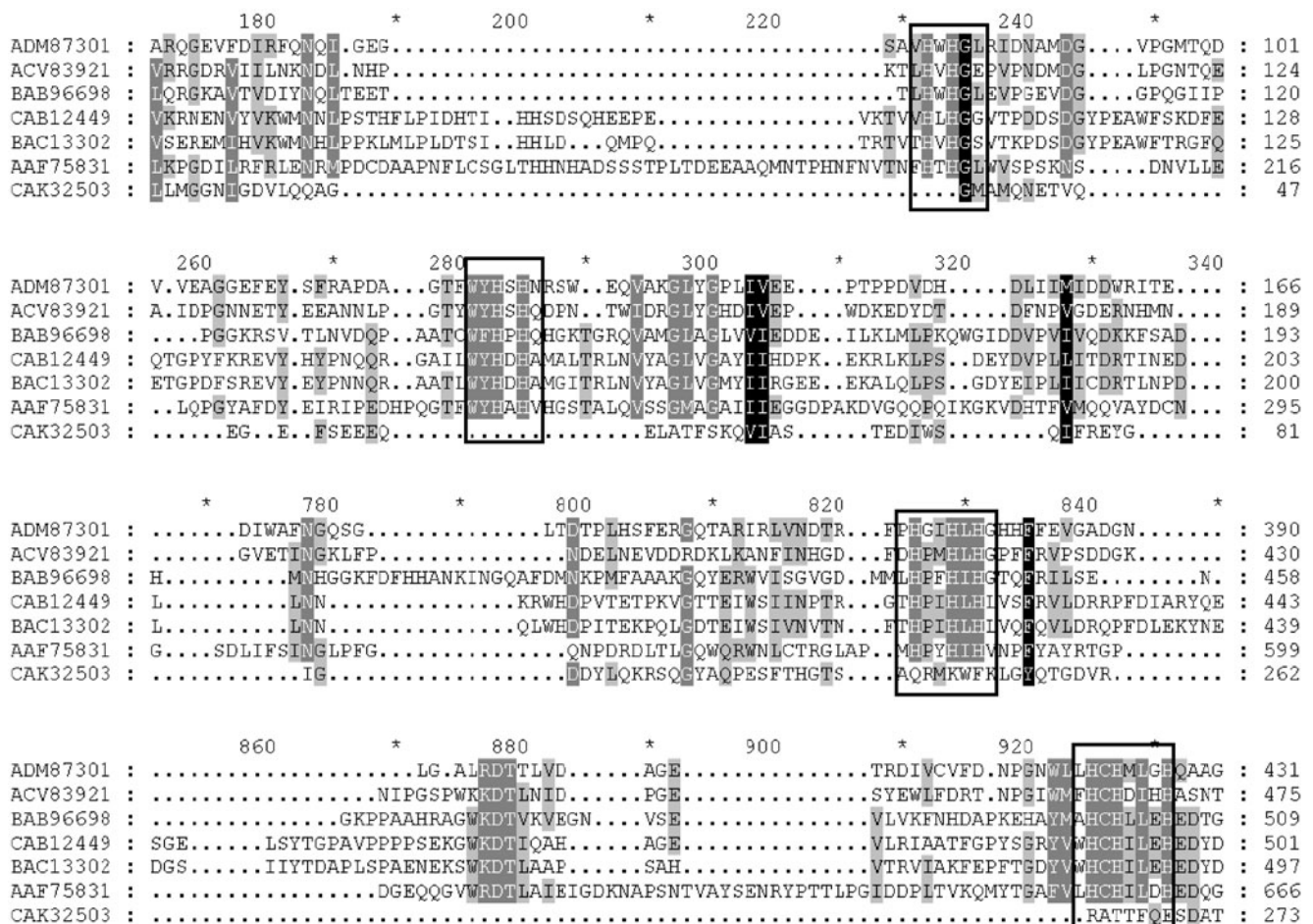
Fig. 1 Multialignment of Lac15 with some other bacterial laccases. The protein sequences were retrieved from GenBank with the following accession numbers: uncultured bacterium of this study (ADM87301), *B. subtilis* (CAB12449), *E. coli* (BAB96698), *M. mediterranea* (AAF75831), *O. iheyensis* (BAC13302), RL5 from a

bovine rumen metagenome (CAK32503), and Lac591 from a mangrove soil metagenome (ACV83921). Sequence alignment was performed using Clustal X 2.0 and GENEDOC. Four histidine-rich copper binding domains were indicated by *full-length vertical boxes*
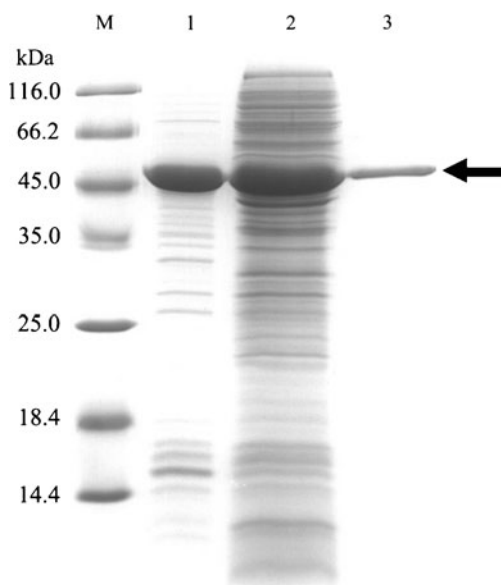
Fig. 2 SDS-PAGE of overexpressed and Ni^{2+}-NTA purified Lac15 without signal peptide. *Lane 1*, precipitate of the sonication product after centrifugation; *lane 2*, supernatant of the sonication product after centrifugation; *lane 3*, purified Lac15

Effects of several potential inhibitors on Lac15 activity were also investigated. Under the test conditions, Lac15 was inactive in the presence of 10 μM EDTA, but retained 50% of the original activity in 2.5 mM NaN$_3$. In contrast, SDS at a low concentration of 50 μM stimulated the enzyme activity up to 127%. The activity was gradually decreased as SDS concentration further increased, and retained 50% of the original value in the presence of 0.5 mM SDS.

Effects of cations on Lac15 activity were also assayed (Fig. 4). In the presence of 1 mM Mg^{2+} or Mn^{2+}, the activity was stimulated up to 146% and 130%, respectively, compared to controls without the corresponding metal ions. Ca^{2+} and K$^+$ had no obvious effect on Lac15 activity, whereas addition of Zn^{2+} or Co^{2+} dramatically reduced enzyme activity to 60% and 51%, respectively.

Since chloride affects fungal laccase activities dramatically (Xu 1996), the effect of chloride on Lac15 activity was determined using NaCl as the chloride donor. Interestingly, the activity was enhanced to about 200% by NaCl at concentrations from 100 to 700 mM. Furthermore, the activity retained the original level even in the presence of 1,000 mM NaCl. As the NaCl concentration further increased, Lac15 activity decreased slowly, with the I_{50} being 1,500 mM (Fig. 5).

The specificities of Lac15 towards syringaldazine and ABTS, under the corresponding optimum conditions, were 1.0 and 0.5 U mg^{-1}, respectively. The K_m and V_{max} of Lac15 were 4.5 μM and 18.5 μmol min^{-1} mg^{-1}, 123 μM, and 6.7 μmol min^{-1} mg^{-1}, for syringaldazine and ABTS, respectively. However, no activity was detected for guaiacol.

Decolorization of artificial dyes

Six industrial dyes, including two of anthraquinone class and four of azo class, were used to evaluate the dye decolorization ability of Lac15. Under the test conditions, Lac15 was unable to decolorize two anthraquinone dyes either with or without the low MW mediators. In contrast, decolorization of the azo dyes was enhanced by addition of the mediators (except for K-7R and KM-8B, with ABTS as the mediator). Methyl-syringate was the best mediator for Lac15 to decolorize azo dyes. In the presence of methyl-syringate, 5 U/L decolorized 70% of K-7R and 60% of M-2GE after 1 h at 45 °C. However, less than 10% of K-7R was
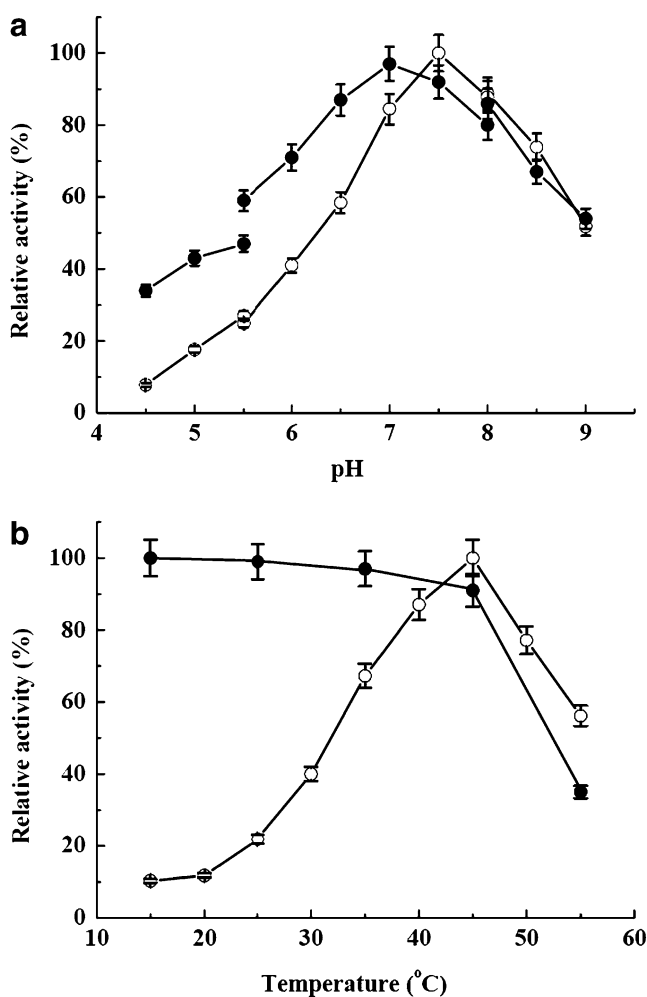
Fig. 3 **a** Optimum pH (*white circle*) and pH stability (*black circle*) of Lac15 were investigated by measuring the enzyme activity at 45 °C with syringaldazine as substrate. **b** Optimum temperature (*white circle*) and themostability (*black circle*) for enzyme activity were measured in 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ at pH 7.5, with syringaldazine as substrate. The results presented in the figures are average values calculated from triplicate technical repeats of measurements. *Error bars* represent±5 standard deviations
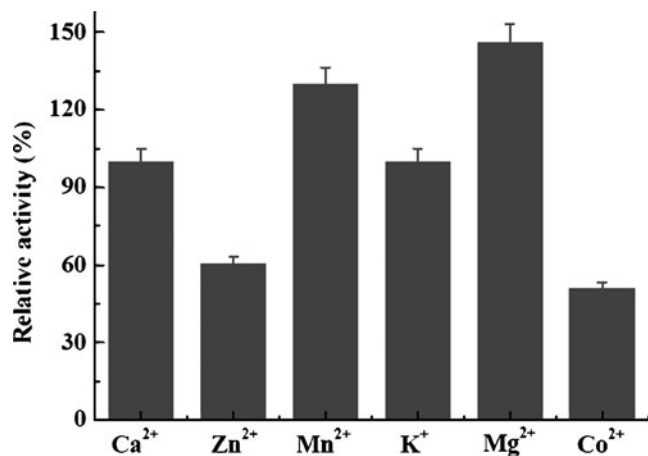
Fig. 4 Effects of metal ions on the activity of Lac15. Sulphates are the donators of the mental ions. The activity was measured in 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ at pH 7.5, supplemented with 0.1 mM CuSO$_4$, at 45 °C with syringaldazine as substrate. The results presented in the figure are average values calculated from triplicate technical repeats of measurements. *Error bars* represent±5 standard deviations
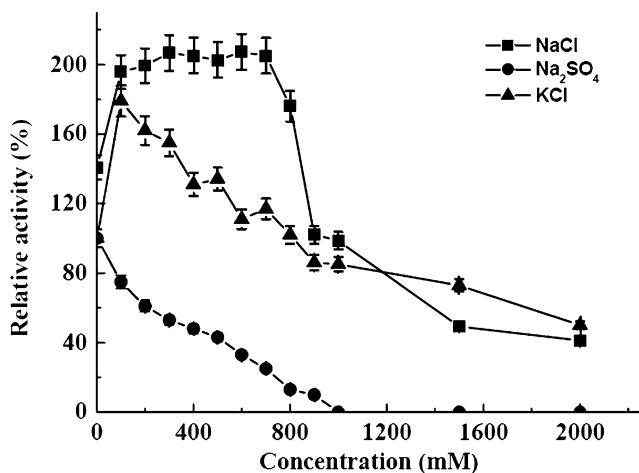
decolorized with ABTS as the mediator or without mediator (Fig. 6). K-7R and M-2GE in concentrations of 50 mM can be completely decolorized by 10 U/L Lac15 after 1 h at 45 °C.
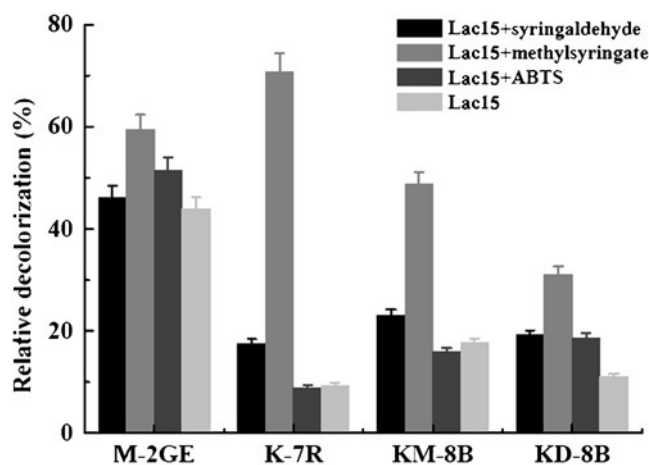
Discussion

Fungal laccases have been used commercially in textile industries (Rodríguez Couto and Toca Herrera 2006) and possess potential in more industrial applications. Compared

to fungal laccases, bacteria-derived ones have some unique characteristics, such as activity and stability at high pH values (Sharma et al. 2007), which make bacterial laccases alternatives for some special fields where fungal laccases are inactive. Studies in recent years have suggested that laccases are widespread in the bacterial kingdom (Alexandre and Zhulin 2000; Sharma et al. 2007), but only a few bacterial laccases have been characterized so far. The diversity of marine microbes and the unique environment properties of the South China Sea potentially contribute to the diversity, novelty, and uniqueness of laccase genes. Laccases from marine microbes may exhibit some characteristics valuable for specific biotechnological application. Thus, we constructed and screened the microbial metagenomic library of the South China Sea to obtain new bacterial laccases.

A function-driven screening method based on enzymatic activity against chromogenic substrates is usually adopted to screen new laccases from microbial cultures. Recently, two bacterial laccases, RL5 and Lac591, were obtained by using this method from a bovine rumen microflora metagenome expression library (Beloqui et al. 2006) and a microbial metagenomic library from mangrove soil (Ye et al. 2010), respectively. Unfortunately, no laccase activity was detected from the microbial metagenomic library we constructed with syringaldazine or guaiacol as substrate (data not shown), which might be due to the failure of potential laccase genes in the library to express under the test conditions. Alternatively, a sequence screening strategy using conserved degenerate primers based on the copper-binding sites of laccases was adopted to amplify laccase genes in the library, and a new laccase gene (*lac15*) was screened out. Lac15 has low sequence identities (less than

Fig. 5 Effect of chloride on the activity of Lac15. The activity was measured in 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ at pH 7.5, supplemented with 0.1 mM CuSO$_4$, at 45 °C with syringaldazine as substrate. The results presented in the figure are average values calculated from triplicate technical repeats of measurements. *Error bars* represent±5 standard deviations

Fig. 6 Decolorization of different reactive azo dyes by Lac15. The assay was measured in 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ at pH 7.5, supplemented with 0.1 mM CuSO$_4$, at 45 °C for 1 h, with ABTS, methyl-syringate, or syringaldehyde as mediator. The results presented in the figure are average values calculated from triplicate technical repeats of measurements. *Error bars* represent±5 standard deviations

40%) with all of the bacterial laccases characterized, though it harbors four conserved copper-binding domains characteristic of laccases. In addition, Lac15 showed excellent chloride tolerance but no guaiacol oxidizing activity compared to the two metagenome-derivered bacterial laccases mentioned above (Beloqui et al. 2006; Ye et al. 2010). The success of the sequence screening strategy demonstrated that it is an effective way to mine new bacterial laccase genes from microbial metagenomic libraries.

It is well known that fungal laccases catalyze the typical substrate syringaldazine at optimum pH around 5 (Jimenez-Juarez et al. 2005) and lose their activities at pH over 7. However, most bacterial laccases are active at pH 7.5–8.5. Recently, a bacterial laccase RL5 from a bovine rumen microflora metagenome expression library revealed a multipotent capacity to oxidize a wide range of substrates over an unusually broad range of pH values from 3.5 to 9.0, with the pH optimum at 4.0–5.0 for syringaldazine oxidation (Beloqui et al. 2006). Different from RL5, Lac15 showed excellent syringaldazine-catalyzing activity under alkalescent conditions and kept most of the highest activity even at pH 9. The alkalescence-dependent activity for Lac15 is similar to the bacterial laccase Lac591, which was stable in the pH range of 7.0–10.0, with the pH optimum at 8.0 for syringaldazine oxidation (Ye et al. 2010), and other bacterial laccases reported (Ruijssenaars and Hartmans 2004; Jimenez-Juarez et al. 2005; Singh et al. 2007). In addition, potential inhibitors exerted various effects on Lac15 activity. The chelating agent EDTA inhibited the activity, indicating that Lac15 is a metalloprotein, in accordance with the deduction that the metal ion Cu^{2+} is essential for bacterial laccase activity (Solano et al. 2001). In contrast, SDS at low concentrations enhanced Lac15 activity, similar to the laccases from Sinorhizobium meliloti (Castro-Sowinski et al. 2002) and Azospirillum lipoferum (Diamantidis et al. 2000). The activity enhancement might be attributed to a limited conformational change, exerted by SDS, capable of inducing a latent enzymatic form of more activity.

One of the major obstacles that have prevented rapid progress in the practical application of laccases in biotechnique industries is the requirement for activity under high concentrations of chloride (Jimenez-Juarez et al. 2005; Xu 1996). Recently, the activity of laccase Lbh1, found in B. halodurans C-125, was reported to be stimulated by NaCl at concentrations of 100–450 mM (Ruijssenaars and Hartmans 2004). The laccase PPO1 from M. mediterranea was also tolerant to NaCl at pH 5 with an I_{50} value of 547 mM, and was entirely insensitive to 100 mM NaCl at pH 7 (Jimenez-Juarez et al. 2005). In the present study, Lac15 activity was enhanced twofold by NaCl at less than 700 mM and kept the original level even in the presence of 1,000 mM NaCl, with an I_{50} of 1,500 mM. Thus, Lac15 showed excellent chloride

tolerant ability compared to other bacterial laccases, not to mention fungal laccases. This advantage highlights the potential for Lac15 in several applications, such as biobleaching of paper pulp and dyestuffs processing, where most fungal laccases are unsuitable (Singh et al. 2009).

Laccases from fungi are well known to be capable of decolorizing synthetic dyes, but little is known about the decolorization potential of bacterial laccases. It has been reported that laccase-containing spores of B. subtilis SF and the corresponding immobilized spores could decolorize textile dyes such as Mordant Black 9 (Held et al. 2005). The laccase-positive bacterium Stenotrophomonas maltophilia AAP56 was also proven to be capable of decolorizing several synthetic dyes at some degrees (Galai et al. 2009). In this study, we demonstrated that the pure laccase Lac15, other than the laccase-containing strain, could completely decolorize reactive azo dyes K-7R and M-2GE, which unambiguously provided proof for the dye decorization ability of bacterial laccases. However, the laccase purified from a typical laccase-producing fungus, Ganoderma sp. 77002, could only decolorize 80% M-2GE and 63% K-7R in the optimal condition with HBT as mediator (Lin Zhu, personal communication).

In conclusion, we obtained a new bacterial laccase (Lac15) from a marine microbial metagenomic library and showed that Lac15 had an alkalescence-dependent activity, chloride tolerance, and an ability of dye decolorization.

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