HEINRICH HEINE
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# Identification and optimization of novel bacterial laccases

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# 1 Zusammenfassung

Laccasen (EC 1.10.3.2) gehören zu den blauen Multikupferoxidasen. Sie verknüpfen die Oxidation von vier Substratmolekülen durch Abstraktion jeweils eines Elektrons mit der Vier-Elektronen-Reduktion von molekularem Sauerstoff. Dabei akzeptieren Laccasen eine Vielzahl unterschiedlicher phenolischer und nicht-phenolischer Aromaten als Substrate. Als Coprodukt entsteht lediglich Wasser. Dadurch können Laccasen in etlichen oxidativen „grünen“ Prozessen genutzt werden. Aktuell werden ausschließlich Laccasen aus Pilzen für industrielle Zwecke eingesetzt, obwohl bakterielle Laccasen eine Reihe von Vorteilen bieten. Diese zeigen meist Aktivität im neutralen bis alkalischen pH-Bereich, sind stabil bei erhöhten Temperaturen und unempfindlicher gegenüber inhibierenden Substanzen. Zudem können sie in prokaryotischen Wirtsorganismen hergestellt werden, was hohe Ausbeuten und eine einfache genetische Manipulation verspricht. Dass sie aktuell nicht in industriellen Prozessen verwendet werden, liegt zum einen an der relativ geringen Zahl charakterisierter bakterieller Laccasen. Zum anderen weisen sie in der Regel niedrige Redoxpotentiale auf. Da die Aktivität von der Redoxpotentialdifferenz zwischen Laccase und Substrat abhängt, sind die Anzahl möglicher Substrate und die spezifische Aktivität bakterieller Laccasen beschränkt. Zum dritten sind heterolog exprimierte bakterielle Laccasen häufig kupferdefizient und erreichen dementsprechend nicht ihre volle Aktivität. In der vorliegenden Arbeit sollte die Nutzbarkeit bakterieller Laccasen verbessert werden, um so in Zukunft einen Einsatz in industriellen Prozessen zu ermöglichen.

Um die Anzahl verfügbarer Enzyme zu erhöhen, wurde die Laccase Ssl1 aus *Streptomyces sviceus* kloniert und charakterisiert. Ssl1 gehört zu den sogenannten kleinen Laccasen, die sich durch eine Architektur aus zwei Domänen auszeichnen. Es besitzt mit phenolischen Substraten Aktivitätsoptima im alkalischen pH-Bereich. Die gemessenen Optima bei pH 9 für Guaiacol und 2,6-Dimethoxyphenol gehören zu den höchsten bekannten Optima von Laccasen. Nicht nur die Aktivität, auch die Stabilität im alkalischen Bereich ist bemerkenswert hoch. So waren nach sieben Tagen

bei pH 11 immer noch 75 % Restaktivität vorhanden. Die Stabilität von Ssl1 wurde durch den Zusatz von organischen Lösemitteln und Detergenzien kaum beeinflusst. Die Aktivität von Ssl1 sank durch den Zusatz zwar ab, aber selbst bei einem Anteil von 40 % wassermischbarem organischen Lösemittel konnten mindestens 20 % Aktivität nachgewiesen werden, mit Detergenzien mindestens 60 %. Der Zusatz von 10 mM Natriumazid, einem bekannten Laccaseinhibitor, hatte keinen messbaren Effekt auf die Aktivität oder Stabilität von Ssl1. Auch die Thermostabilität von Ssl1 ist beachtlich. Bei 60 °C lag die Halbwertszeit der Aktivität bei 88 min.

Ssl1 hat ein Redoxpotential von 375 mV und muss daher als Laccase mit niedrigem Redoxpotential eingeordnet werden. Um das Redoxpotential von Ssl1 gezielt zu erhöhen, wurde Ssl1 kristallisiert und seine Struktur durch Röntgenkristallographie aufgeklärt. Das ermöglichte die Auswahl und den Austausch von Aminosäuren die das Redoxpotential beeinflussen könnten. Auf diese Weise wurden Ssl1 Varianten mit einer Erhöhung des Redoxpotentials von 16 bis 81 mV erzeugt. Als entscheidende Faktoren wurden der axiale Ligand des Typ 1-Kupferzentrums und die Hydrophobizität in dessen Umgebung identifiziert. Mutationen in unmittelbarer Umgebung des Typ 1-Kupferzentrums störten dabei offensichtlich den Elektronentransport innerhalb der Laccase, was zu geringeren spezifischen Aktivitäten in den entsprechenden Varianten führte. Dagegen waren die Varianten Ssl1 M220L/T222L und Ssl1 ΔC-terminus mit Mutationen abseits des Typ 1-Kupferzentrums in der Lage, die industriellen Farbstoffe Alizarinrot S und Indigokarmin schneller umzusetzen als das Wildtypenzym.

Strategien zur Erhöhung der Kupferbeladung heterolog exprimierter bakterieller Laccasen wurde am Beispiel der Modelllaccase CotA aus *Bacillus licheniformis* untersucht. Dabei zeigte sich, dass eine Erhöhung der intrazellulären Kupferkonzentration durch Ausschalten des aeroben Kupferdetoxifikationsmechanismus in *Escherichia coli* nicht ausreicht, um den Kupfergehalt von CotA zu steigern. Dagegen führte die Coexpression des zuvor durch Genomscreening identifizierten Kupferchaperons CopZ zu einer Erhöhung des Kupfergehalts um 20 %. Gleichzeitig stieg die spezifische Aktivität von CotA um 26 %. Dadurch war zwar noch keine vollständige Kupferbeladung von CotA erreicht, aber das entwickelte System ist einfach im Gebrauch und verlangt keinen großen experimentellen Zusatzaufwand. Daher kann es in Standardexpressionen eingesetzt werden und dabei die Ausbeuten aktiver CotA-Laccase erhöhen.

# 2 Abstract

Laccases (EC 1.10.3.2) belong to the blue multicopper oxidases. They couple the oxidation of four substrate molecules by abstraction of one electron with the reduction of molecular oxygen by four electrons. Laccases accept a wide range of various phenolic and non-phenolic aromatic compounds as substrates and produce water as sole by-product. This makes laccases suitable enzymes for a variety of 'green' oxidation processes. At the moment, exclusively laccases from fungi are used in industrial processes, although laccases from bacteria provide several benefits. Often these are active in neutral to alkaline conditions, they are stable at elevated temperatures and tolerate the presence of common laccase inhibitors. Moreover, they can be produced in prokaryotic expression hosts. This allows simple genetic manipulation and high production yields. The industrial utilization of bacterial laccases is hindered by the low number of characterized enzymes. Further, since the activity depends on the redox potential difference between laccase and substrate, the substrate range and the specific activity of bacterial laccases are limited. Additionally, copper depletion is frequently reported for heterologously expressed bacterial laccases which results in partially inactive enzyme. In the presented work, the usability of bacterial laccases should be improved to facilitate utilization of these versatile enzymes in industrial processes.

The number of available enzymes was increased by cloning and characterization of the laccase Ssl1 from *Streptomyces sviceus*. Ssl1 is a so-called small laccase with a two-domain architecture. It possesses alkaline activity optima with phenolic substrates. The demonstrated optima at pH 9 with guaiacol and 2,6-dimethoxyphenol belong to the highest reported pH optima of laccases. Besides the activity, the stability of Ssl1 at alkaline pH was remarkably high. After seven days at pH 11 a residual activity of 75 % could be observed. The addition of organic solvents and detergents did hardly affect the stability of Ssl1. Although the activity of Ssl1 decreased, even with addition of 40 % water miscible organic solvent at least 20 %

activity was detected. With detergents at least 60 % remaining activity was observed. Addition of 10 mM of the common laccase inhibitor sodium azide did not alter the activity of Ssl1. Moreover, the thermostability of Ssl1 is noteworthy. Thus, the half-time of residual Ssl1 activity at 60 °C was 88 min.

Ssl1 displays a redox potential of 375 mV and, therefore, belongs to low redox potential laccases. To increase its redox potential, first Ssl1 was crystallized and its structure was solved by X-ray crystallography. This enabled rational selection and substitution of amino acids that might influence the redox potential of Ssl1. Thereby created variants showed redox potential increases of 16 to 81 mV. The nature of the axial ligand of the type-1 copper ion and the hydrophobicity in its environment were identified as key determinants of the redox potential of Ssl1. Obviously, mutations close to the type-1 center perturbed the intermolecular electron transfer and resulted in lower specific activities of corresponding variants. The variants Ssl1 M220L/T222L and Ssl1 ΔC-terminus with mutations at larger distance to the type-1 center were able to convert the industrial dyes Alizarin Red S and indigo carmine more efficiently than wildtype enzyme.

Strategies for increasing the copper content of heterologously expressed bacterial laccases were tested with the model laccase CotA from *Bacillus licheniformis*. It was demonstrated that an increased intracellular copper concentration by knock-out of the aerobic copper detoxification mechanism in *Escherichia coli* was insufficient to increase the copper content of CotA. In contrast, coexpression of the copper chaperone CopZ that was identified by genome screening increased the copper content by 20 %. At the same time the specific activity of CotA increased by 26 %. Although a full copper complement could not be achieved, the established system is simple to use and does hardly require additional experimental efforts. As a consequence it can be used in standard expressions in order to maximize the yield of active CotA laccase.

# 3 Introduction

## 3.1 Multicopper oxidases

Multicopper oxidases (MCO) are a superfamily of oxidoreductases that oxidize four substrate molecules by a one-electron abstraction coupled to the four-electron reduction of molecular oxygen to water. They contain at least four characteristic and eponymous copper ions that form the catalytic center of the enzyme. Depending on spectroscopic features in the oxidized state, each copper center is classified as type 1 (T1), type 2 (T2) or type 3 (T3) [1]. T1 copper is coordinated to the enzyme via two histidine residues and one cysteine residue. The resulting cysteine-sulfur to copper bond forms a charge-transfer complex with a strong absorption around 600 nm which causes an intense blue color. This has led to the denotation of MCOs as 'blue' oxidases [2]. The MCO superfamily consists of the enzyme families ceruloplasmin or ferroxidase (EC 1.16.3.1), ascorbate oxidase (EC 1.10.3.3), nitrite reductase (EC 1.7.2.1), and laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) which are involved in diverse physiological processes.

## 3.2 Laccases

Laccases accept a wide range of different electron rich substrates. Among them are diphenols, aromatic amines, phenylpropanoids and metal ions. The substrate spectrum of laccases widely overlaps with the substrate spectrum of tyrosinases (monophenol monooxygenase). Both enzymes can be distinguished by oxidation of the substrates tyrosine and syringaldazine. Tyrosine is oxidized by tyrosinases but not by laccases and syringaldazine is converted by laccases but not by tyrosinases [3].

Due to their high industrial potential laccases have drawn more and more attention by scientists and researchers in recent years. Thus, the number of publications about laccases has constantly risen from 25 per year in the end of the 1980s to more than 600 publications in 2012 (see Fig. 3.1). Most of the research efforts have been concentrated on fungal laccases, although laccases are widely distributed in all domains of life and were identified also in plants, insects, bacteria and even archaea [4–6].

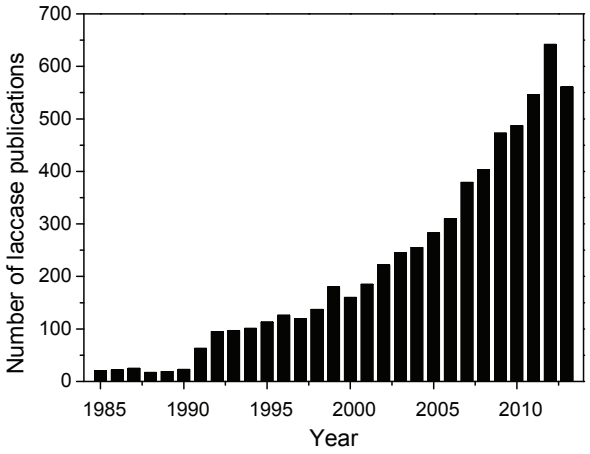


**Figure 3.1:** Annual number of publications on laccases. Numbers are based on data from the ISI Web of Knowledge (http://www.webofknowledge.com, December 2013).

### 3.2.1 Source and function of laccases

#### Plant laccases

The first laccase was discovered already in 1883 [7] and, thus, is one of the first enzymes ever described. The enzyme was found in sap from the Japanese lacquer tree *Rhus vernicifera* and was therefore named laccase. The sap of the lacquer tree contains derivatives of catechol, the so-called urushiols. *R. vernicifera* laccase oxidizes urushiols to radicals which triggers the polymerization of natural lacquer [8]. This reaction has been used for several thousand years in the production of traditional decorative lacquer ware in East Asia (urushi art) and is therefore one of the first examples of enzyme technology in human history.

Although the laccase from *R. vernicifera* is well characterized, the number of reports on plant laccases is limited [9]. Detection of laccase was reported for mango, mung bean, peach, pine, prune, tobacco, ryegrass, corn, and sycamore, but the corresponding enzymes were characterized only partially [10, 11]. Plant laccases together with peroxidases are supposed to oxidize monolignols in plant cell walls. The resulting phenolic radicals then undergo radical-radical coupling which leads to the formation of lignin [12]. Consequently, the disruption of laccase genes lead to reduced lignin contents, as was shown for *Arabidopsis thaliana* [13]. Further, plant laccases may participate in iron metabolism, wound healing, and protection from

herbivores and microorganisms [9, 11, 14]. All known plant laccases are glycoproteins with glycocontents of up to 45 % [11] and their expression was exclusively reported in plants.

### Fungal laccases

Laccases from fungi are by far the best examined group of laccases and nearly each examined fungus contains at least one of them [9]. Their biological function is manifold and their involvement was shown in the development of fruiting bodies [15], rhizomorph production [16], spore pigmentation [17, 18], resistance to environmental stress [18], and pathogenicity [18, 19]. In white-rot fungi, like *Trametes* or *Pleurotus* species, laccases are a part of a secreted multi-enzyme composition which is able to attack and break down the lignin barrier of dead wood. The remaining cellulose and hemicellulose fibers are degraded by cellulases and serve as substrate for the fungus. Enzymes involved in the lignin degradation beside laccases are lignin peroxidases, manganese peroxidases, and peroxide producing oxidases that supply hydrogen peroxide for peroxide dependent oxidases [20].

The expression of fungal laccases is often induced by the presence of phenolic or related compounds in the cultivation medium [21]. Lignin, the natural substrate of laccases from white-rot fungi, is an unordered polymer of the phenylpropanoid building blocks *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [22]. The presence of phenolic compounds that resemble structural features of the lignin building blocks might mimic the presence of lignin. Thus, the induction of laccase expression by phenolic and related compounds in fungi is in accordance with the natural function in lignin degradation.

Depending on the redox potential of their T1 copper site fungal laccases were classified into three groups: low potential laccases with a potential of about 420 mV, middle potential laccases with 470 to 710 mV, and high potential laccases with about 780 mV [23]. Since a correlation between the redox potential of laccases and their oxidative ability was demonstrated [24], high potential laccases from fungi seem especially promising for technical applications [25].

Similar to laccases from plants, fungal laccases are glycoproteins with carbohydrate contents of 5 to 30 % [26]. Glycosylation is believed to be important for secretion, activation, structure, and stability of fungal laccases [27, 28]. Glycosylation causes problems in heterologous expression of fungal laccases that cannot be over-

come easily [28] and that make expression in prokaryotic systems nearly impossible [27]. To date the laccase from *Cyathus bulleri* is the only fungal laccase that could be expressed heterologously in *E. coli* [29]. Recombinant *C. bulleri* laccase was active but migrated at higher molecular mass on a native acrylamide gel than purified native enzyme. The influence of lacking glycosylation on further characteristics like specific activity, thermostability, or substrate spectrum has not been investigated [29].

### Bacterial laccases

Since 1993 when the first bacterial laccase was detected in *Azospirillum lipoferum* [30] (compared to 1883 for plant and 1896 for fungal laccases) the presence of laccases and related enzymes has been demonstrated in numerous bacteria including *Bacillus* species, *Streptomyces* species, and *E. coli* [31]. Two bioinformatic analysis approaches of available sequence data revealed more than 1200 or 1000 putative bacterial laccase genes, respectively [4, 6]. Despite this considerable number of existing bacterial laccase genes only a few have been characterized in detail so far [32].

The function of bacterial laccases is diverse and could not be dedicated for all described enzymes [32]. CotA from *B. subtilis* is part of the endospore coat [33] and it is involved in melanization of the spore coat. CotA deficient spores are unable to build the brown spore pigment and, as a consequence, fail to build full resistance towards UV radiation and hydrogen peroxide [34, 35]. The well-studied periplasmic CueO laccase participates in copper homeostasis of *E. coli* under aerobic conditions [36]. CueO possesses high cuprous oxidase activity. This has led to the assumption that CueO detoxifies extremely toxic Cu(I) by oxidation to less toxic Cu(II) in vivo [37]. Involvement in copper resistance has been shown for several bacterial laccases [38, 39]. Further suggested functions of bacterial laccases include manganese oxidation [40], detoxification of phenolic compounds [41], and synthesis of antibiotics [42].

### Insect laccases

Laccases or laccase-like enzymes have been detected in numerous insects such as *Drosophila* species [43, 44], the silkmoth *Bombyx mori* [45], the red flour beetle *Tribolium castaneum* [46], and the desert locust *Schistocerca gregaria* [47]. Analysis of existing insect genomes showed that all examined insects contained at least two MCO genes

[48]. Based on the MCOs that were used in this phylogenetic analysis, insect laccases can be grouped into orthologues of MCO1 and of MCO2 from *Anopheles gambiae*.

Laccases from the MCO2 group were found to be associated with the insect cuticle where they are supposed to contribute to cuticle sclerotization by oxidation of diphenols to quinones [49]. Subsequent chemical cross-linking of proteins by the quinones makes cuticle proteins insoluble and promotes hardening and tanning of insect cuticles. Accordingly, it was shown that silencing of the MCO2 type laccase TcLac2 in *T. castaneum* inhibited cuticle tanning and was lethal within several days [46]. Other enzymes that participate in cuticle sclerotization are copper containing tyrosinases and heme containing peroxidases [50].

The function of laccases from the MCO1 group has not been completely elucidated yet. However, their localization and expression profiles led to the suggestion of possible roles in iron metabolism, immunoresponse, e.g. by decreasing the available iron amount for infective microbes or by encapsulation thereof, and detoxification [48]. For MCO1 from *Drosophila melanogaster* a role in iron homeostasis was confirmed recently [51].

Genomes of the mosquitos *A. gambiae* and *A. aegypti* contain three additional MCO genes that share a common ancestor with the MCO2 group and whose function is unknown but seems to be mosquito specific [48].

### Archaeal laccases

Although several archaeal laccase or laccase-like genes were discovered in a computational approach based on genome and metagenome data [4], LccA from *Haloferax volcanii* is the only archaeal laccase that has been described experimentally to date [52]. Similar to plant and fungal laccases LccA is a glycoprotein. It contains a twin arginine motif that facilitates its translocation across the cell membrane. Its biological function is unknown. LccA showed characteristics like moderate thermostability and tolerance towards salts and solvents that indicate its biotechnological potential. The production yield of LccA in a homologous overexpression system was $8\,mg\,mL^{-1}$ and in a heterologous expression system in *E. coli* $1\,mg\,mL^{-1}$ was achieved [53]. So far, these yields are considerably lower compared to other prokaryotic laccases that show similar potentials in terms of activity, stability and tolerance towards solvents and additives but which can be expressed with yields of 50 to $300\,mg\,mL^{-1}$ [54, 55].

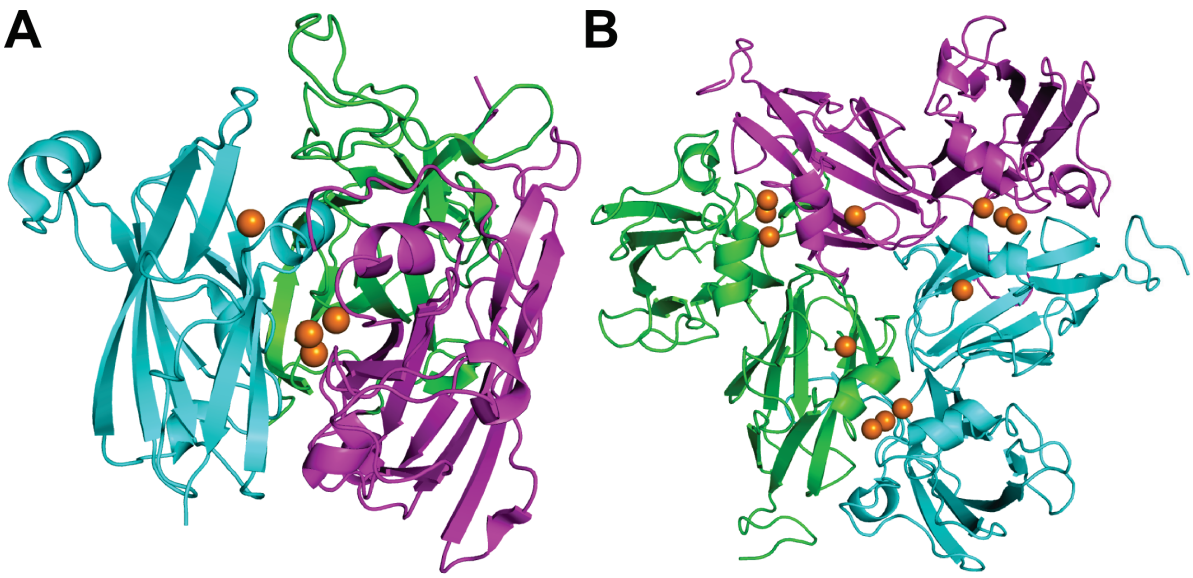


**Figure 3.2:** Architecture of three-domain and two-domain laccases. Copper ions are shown as orange spheres. A: Structure of the three-domain laccase CotA from *B. subtilis* (PDB: 1GSK), domains are colored in magenta (domain 1), green (domain 2), and cyan (domain 3). CotA consists of three cupredoxin-like domains. The trinuclear copper cluster is located at the interface of domains 1 and 3. B: Structure of the two-domain laccase SLAC from *S. coelicolor* (PDB: 3CG8), monomers are colored in magenta, green and cyan. SLAC forms homotrimers and the trinuclear cluster is located at the interface of domains 1 and 2 of neighboring monomers.

### 3.2.2 Structure of laccases

#### Three-domain laccases

The first crystal structure of a copper depleted form of *Coprinus cinereus* laccase was solved in 1998 [56]. Since then the 3D-structure of several fungal and bacterial laccases with a full copper complement have been elucidated [57–59].

The common architecture of laccases consists of three cupredoxin-like domains that contain four copper centers (Fig. 3.2). Based on their spectroscopic features the copper centers are classified in type 1 (T1), type 2 (T2), and type 3 (T3) [1]. Domain 3 contains the so-called T1 or 'blue' copper center. T1 copper ions are coordinated by two histidine residues and one cysteine residue (Fig. 3.3). The strong ligand-to-metal-charge-transfer complex between cysteine-sulfur and copper(II) shows a strong absorption band around 600 nm and was eponymous for the class of 'blue' multicopper oxidases. T1 copper is coordinated in a planar trigonal geometry. In high redox potential laccases from fungi the axial position of T1 copper is occupied by non-coordinating hydrophobic residues such as phenylalanine or leucine. In middle or

low redox potential laccases methionine is located at the axial position of T1 copper. At the interface of domain 1 and 3 and at a distance of approximately 12 Å from the T1 copper, the T2 copper and the binuclear T3 copper center form the trinuclear cluster (TNC) of laccases [10]. Copper ions of the trinuclear cluster are ligated by eight histidine residues, three at each T3 copper ion and two at the T2 copper ion (Fig. 3.3). T2 copper is not detectable via UV-Vis spectroscopy, whereas the T3 copper pair causes a shoulder around 330 nm. In electron paramagnetic resonance spectra the T3 coppers are silent due to antiferromagnetical coupling by a hydroxyl bridge. T1 and T2 copper ions show specific ultrafine splittings which are characteristic for the geometry of their coordination.

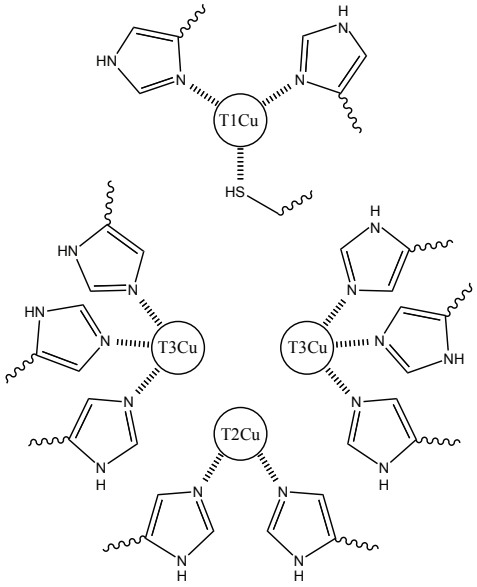


**Figure 3.3:** Schematic represention of laccase copper sites. The copper coordinating residues are highly conserved in laccases. T1 copper is coordinated by two histidines and one cysteine in trigonal planar geometry. Two histidines ligate T2 copper, the T3 copper pair is ligated by six histidine residues. T2 and T3 copper ions together form the trinuclear cluster.

### Two-domain laccases

Recently another class of laccases has been described with SLAC from *Streptomyces coelicolor* as archetype [60]. These so-called small laccases consist of only two domains with domain 2 of common laccases lacking. Domain 2 of three-domain laccases is responsible for connection and positioning of domain 1 and 3 in a way that enables formation of the trinuclear cluster at the interface of domain 1 und 3. As a result of the absence of this domain in small, two-domain laccases, the formation of a trinuclear cluster within single molecules is impossible [61]. Instead, the trinuclear cluster is built at the interface of individual laccase monomers (Fig. 3.2). Therefore two-domain laccases require oligomerization to form intact catalytic sites. Actually, for both two-domain laccases structurally characterized so far, formation of homotrimers has been reported [62, 63].

The discovery of two-domain laccases has promoted a model for evolution of multicopper oxidases [61]. According to this model, evolution of contemporary mul-

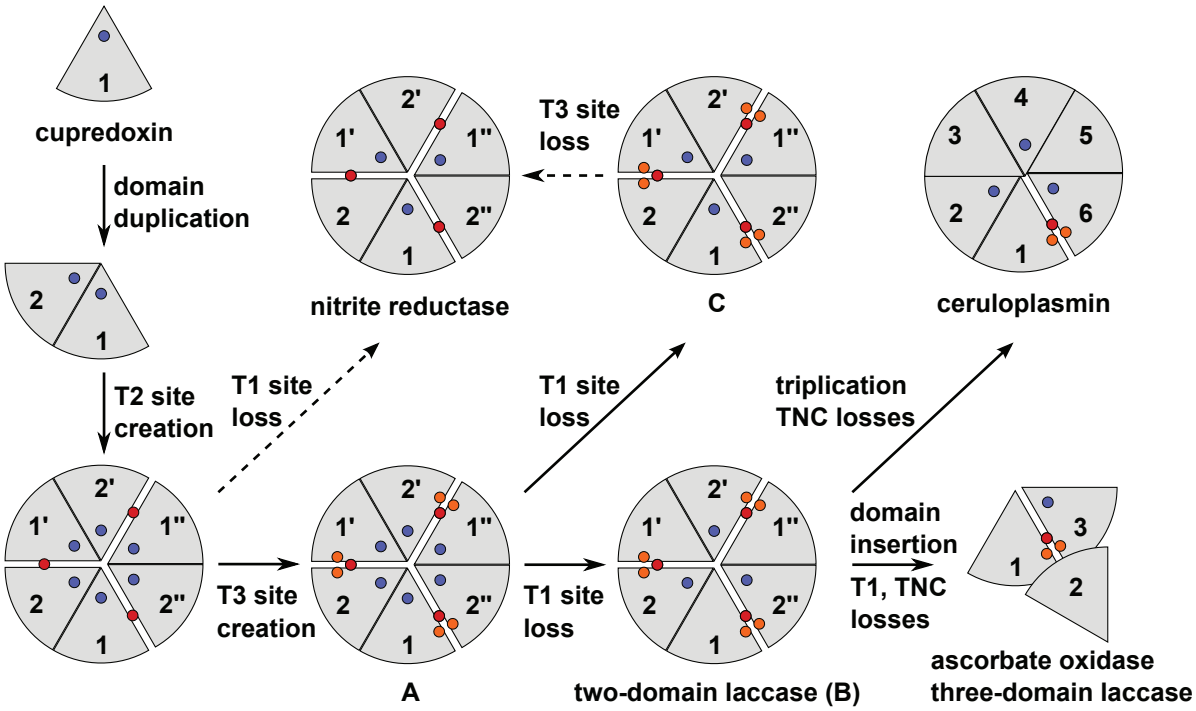


**Figure 3.4:** Model of evolution of multicopper oxidases and nitrite reductase. The common ancestor of multicopper oxidases and nitrite reductase is cupredoxin. Several evolutionary events of domain duplication, creation and loss of specific copper sites, and domain insertion have led to the formation of two-domain laccases, three-domain laccases and ascorbate oxidases, nitrite reductases, and six-domain ceruloplasmins. Intermediates A and C have been identified by genome screening. Dashed arrows represent uncertain events. Adopted from Nakamura et al. [61]. Numbered circle sectors: cupredoxin domains, small circles: T1 copper (blue), T2 copper (red), and T3 copper (orange) centers, TNC: trinuclear cluster.

ticopper oxidases and also nitrite reductases (enzymes with three T1 and T2 copper centers in homotrimers of two-domain monomers) started by a domain duplication of single-domain cupredoxins harboring one T1 copper center (Fig. 3.4). Several further evolutionary events of specific copper site creation, copper site loss, domain insertion, and domain triplication can explain evolution of contemporary three-domain laccases, six-domain multicopper oxidases, and nitrite oxidase. Depending on the course of evolution Nakamura et al. defined A-, B-, and C-type multicopper oxidases [61]. All three consist of two-domain enzymes that form homotrimers with TNCs at the interface of neighboring monomers. A-type MCOs contain two T1 copper centers, one in each domain, B-type MCOs contain one T1 copper center in domain 2, and C-type MCOs contain one T1 copper center in domain 1. Within this model two-domain laccases represent Nakamura B-type intermediates in the evolution of multicopper oxidases. Representatives of hypothetical A- and C-type

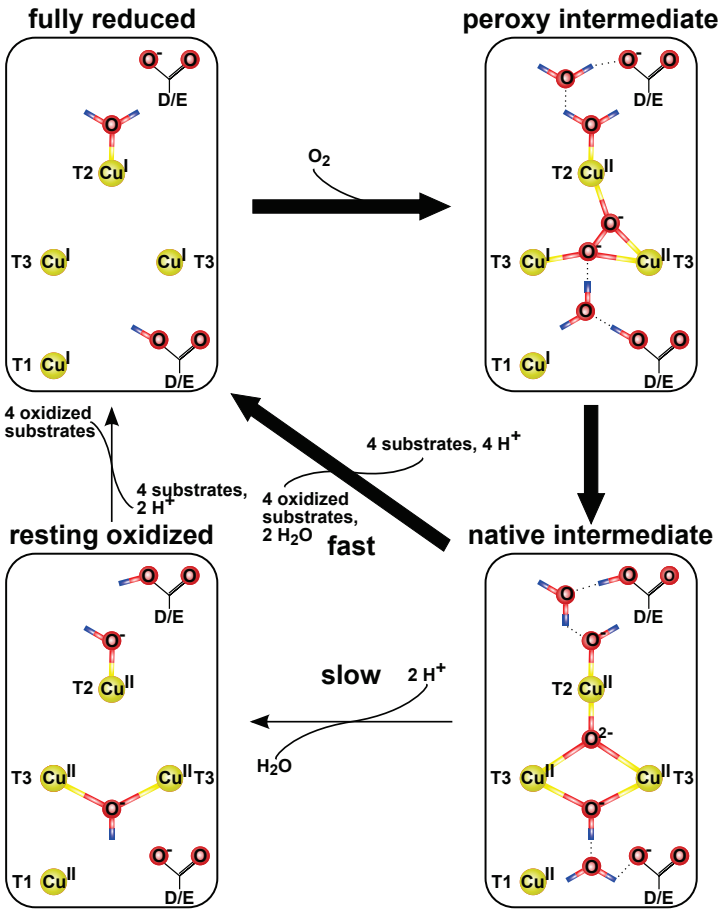


**Figure 3.5:** Reaction mechanism of laccases. Fully reduced laccase binds dioxygen at the trinuclear cluster. Dioxygen is then reduced by two electrons which generates the so-called peroxy intermediate. After further reduction with two electrons the native intermediate is formed. In presence of substrate the laccase copper ions get fully reduced and the reaction cycle is completed. In absence of substrate the native intermediate slowly decays into the resting oxidized form. The acidic residues near the trinuclear cluster are involved in binding of oxygen and peroxide and in the protonation process. Adopted from Solomon et al. [64].

two-domain cupredoxins have been detected as well and support the validity of the proposed evolutionary model [61].

### 3.2.3 Reaction mechanism of laccases

The reaction mechanism of laccases involves the one-electron reduction of four substrate molecules and transfer of the abstracted electrons to molecular oxygen which is reduced to two molecules of water [1]. Substrate molecules are bound near the T1 copper center. One-electron abstraction occurs by an outer-sphere mechanism from substrate to T1 copper. Thereby substrate molecules are converted into free radicals that can undergo further oxidation or radical coupling reactions resulting

in formation of oligomers or polymers. Abstracted electrons are transferred from the T1 center to the trinuclear cluster via a cysteine-histidine pathway that is highly conserved among multicopper oxidases. This so-called superexchange pathway is built by overlapping redox active molecule orbitals of T1 coordinating cysteine, backbone atoms, and T3 copper coordinating histidine residues [65].

In the fully reduced state, when all four catalytic copper ions are in Cu(I) state, a water molecule is coordinated at T2 copper [64]. Oxygen reduction starts with binding and two-electron reduction of dioxygen at the trinuclear cluster which yields the peroxy intermediate (Fig. 3.5). Reduction of the peroxy intermediate with two further electrons leads to formation of the native intermediate in which one $\mu_3$-oxo ligand bridges all copper ions of the trinuclear cluster and one $\mu_2$-oxo ligand bridges both T3 copper ions [66]. With assistance of acidic residues near the trinuclear cluster the oxygen moieties in the native intermediate are protonated yielding two molecules of water [67, 68]. Subsequently, the copper centers are reduced again by four substrate molecules which completes the catalytic cycle of laccases ($k = 350\,s^{-1}$). In absence of substrate the native intermediate undergoes a slow decay ($k = 0.05\,s^{-1}$) to the oxidized resting state in which the T3 copper pair is hydroxyl bridged [64]. The oxidized resting state is the common state of heterologously expressed and isolated laccases.

### 3.2.4 Laccase substrates

With the described reaction mechanism laccases are able to oxidize a wide range of organic substrates (Fig. 3.6). These include phenolic compounds, aromatic amines, polyaromatic hydrocarbons, phenylpropanoids, azo dyes, and indigo dyes. Some of these substrates cannot be oxidized directly by laccase but require the use of redox mediators.

Redox mediators are low molecular weight molecules that are able to shuttle electrons between laccases and target molecules that could not be oxidized otherwise. In a laccase mediator system, laccase oxidizes the mediator and the oxidized mediator in turn oxidizes the final substrate. Such systems can be applied to bulky substrates such as lignin polymers that are not accessible for laccases or to substrates with redox potentials beyond the redox potential of a laccase. By use of redox mediators the substrate range of laccases can be increased [69].

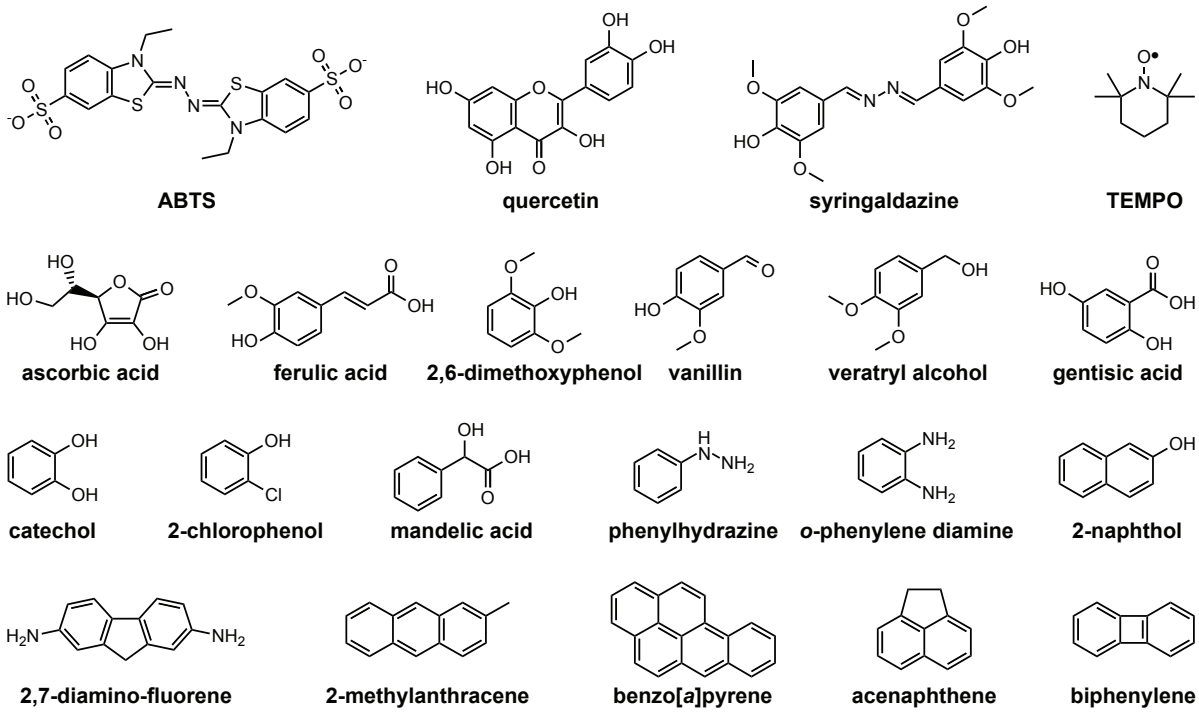


**Figure 3.6:** Selection of organic laccase substrates. ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), TEMPO: (2,2,6,6-tetramethylpiperidine-1-yl)oxyl.

Common artificial mediators are TEMPO (2,2,6,6-tetramethylpiperidine-1-yl)oxyl), HBT (*N*-hydroxybenzotriazole), violuric acid, and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) [70]. Several naturally occurring compounds that mediate laccase activity were detected as well [71–73]. Typical natural laccase mediators are connected to the physiological function e.g. as secondary metabolites or as phenolic degradation products of lignin such as HAA (3-hydroxyanthranilic acid), acetosyringone, vanillin, acetovanillone, *p*-coumaric acid, and syringaldehyde.

Besides organic molecules, some laccases are able to oxidize inorganic ions or metal complexes. Thus, periplasmic CueO from *E. coli* is involved in copper homeostasis and oxidizes Cu(I) to less toxic Cu(II), McoA from *Aquifex aeolicus* oxidizes Cu(I) as well as Fe(II), and *Trametes hirsuta* laccase is able to oxidize Mn(II) [37, 74, 75].

### 3.2.5 Redox potential of laccases

Whether a given laccase is able to oxidize a potential substrate or redox mediator depends largely on the redox potential difference between substrate and T1 copper of the laccase. Moreover, oxidation efficiency depends on this redox potential difference

[24, 76]. The highest redox potentials among laccases of up to 800 mV are observed for laccases from white-rot fungi like *Trametes versicolor*, *T. villosa*, *T. hirsuta*, *T. zonatus*, and *Coriolopsis fulvocinerea* [77]. Plant and bacterial laccases generally fall into the class of low-redox potential laccases with redox potential below 470 mV.

Characterization of point-mutations in the surrounding of T1 centers and theoretical studies by combined quantum mechanics/molecular mechanics and molecular dynamics simulations have revealed further factors that influence the redox potential [78–81]. The five main determinants are a) the hydrophobic effect, b) metal-ligand interactions, c) hydrogen bonding to coordinating sulfur atoms, d) protein constraints, and e) electrostatic interactions [82, 83]. The hydrophobic effect is caused by the relative stabilization of the less charged Cu(I) state. This makes the Cu(I) state energetically more favorable and raises the susceptibility of T1 copper for electrons. In general, a more hydrophobic surrounding of T1 copper is correlated with higher redox potentials [80].

It is well established that the metal-ligand interaction between T1 copper and the amino acid in axial position is one of the key determinants for the redox potential of laccases [84]. In high redox potential fungal laccases non-ligating residues are in axial position while in laccases from plant and bacteria with low redox potential, the axial position is occupied by methionine. In stellacyanine, a one-domain cupredoxin that only harbors a T1 center, replacement of strongly coordinating glutamine to mildly coordinating methionine raised the redox potential by 160 mV. Replacement by non-coordinating leucine raised the potential by 320 mV [85]. For a subset of laccases with different redox potentials, TvL from *T. versicolor*, CotA from *B. subtilis*, CueO from *E. coli*, and SLAC from *S. coelicolor*, the effect of alternated axial ligands could be estimated by a combined approach of quantum mechanics/molecular mechanics and molecular dynamics simulations and matched experimental data [81].

Additional hydrogen bonding of backbone atoms to T1 copper coordinating cysteine have been shown to increase the redox potential in variants of the one-domain cupredoxins pseudazurin from *Alcaligenes faecalis* and amicyanin from *Paracoccus denitrificans* [86, 87]. On the contrary, removal of a corresponding hydrogen bond in *Pseudomonas aeruginosa* azurin, another one-domain cupredoxin, led to a decreased redox potential [88]. Additional hydrogen bonds to copper coordinating cysteine can allow T1 copper to adopt its preferred geometry after reduction to the Cu(I) state [87]. Or they can increase the electrostatic repulsion of copper ion with

N-H dipoles which destabilizes the Cu(II) state [83].

Rigidity of the protein fold determines the position and orientation of T1 copper coordinating amino acids. Although the complex geometry in T1 sites is close to the optimal geometry of free Cu(II) with the given ligands, protein constraints adjust the length of the T1 copper to cysteine-sulfur bond which leads to redox potential modulations [89].

Finally, simple electrostatic repulsion between T1 copper and charged amino acids (or dipoles) destabilize the Cu(II) state in favor of the Cu(I) state. For example in azurin from *P. aeruginosa* replacement of methionine by positively charged lysine raised the redox potential by 60 mV [90]. As a consequence the redox potential of T1 copper is modulated by the orientation of pre-organized dipoles either to higher or to lower values [91].

Based on this knowledge, an increase of redox potential by site-directed mutagenesis has been achieved for several laccases [78, 79]. However, resulting variants showed decreased turnover numbers. Due to functional relevance and wide conserved regions it was stated that only few residues in laccases can be mutated without impairing activity [92].

### 3.2.6 Applications of laccases

Laccases are able to perform the oxidation of a broad range of different substrates. Thereby the only required cofactor is oxygen which can be easily added by aeration and the only by-product of the reaction is non-toxic water. This makes laccases attractive candidates for applications in different industrial areas [93, 94]. Consequently, many laccase related processes have been granted a patent [95].

Traditional delignification processes in the pulp and paper industry require large amounts of chemical oxidants. In order to establish safer and environmentally friendly processes the use of bleaching enzymes is in discussion. Among the used enzymes, laccases are the most promising for industrial delignification [96] and might help to reduce the amount of chemical waste that is produced in traditional delignification processes. A pulping process with laccase from *Trametes versicolor* in combination with chemical mediators was found to efficiently catalyze the delignification of different kinds of pulp and was filed as patent (Lignozym-process) [97].

In the textile industry several processes involving laccases have been commercialized, e.g. as Denilite, Novoprime Base 268 (both Novozymes), PrimaGreen EcoFade LT100, and Ecostone LC10 (both Genenchor). They are used to improve the whiteness in the bleaching of cotton and for denim finishing in the production of jeans and other denim dyed fabrics with a faded look [98]. In these processes undesired dyes are degraded by laccases or by laccase mediator systems. Degradation of undesired molecules by laccases can be utilized as well in the treatment of waste waters and the bioremediation of contaminated soils [99–101].

In cosmetic compositions laccases were described as mild and practicable alternatives in hair colorization, skin lightening, and oral hygiene [98].

There are numerous potential applications of laccases in the food industry [102]. Stabilization of wine and beer can be achieved by removal of polyphenols and oxygen upon laccase addition. By the same means, the clarification of fruit juices is facilitated and use of laccases in combination with ultrafiltration leads to clear and stable fruit juices [103]. A commercialized laccase preparation, Suberase (Novozymes), is used for treatment of cork stoppers. 2,4,6-trichloroanisole is the main component of corks taint in wine and concentrations as low as 10 ppt can be perceived as off-flavor while amounts of 100 ppt and higher completely destroy the quality of wine [104]. Oxidation and polymerization of trichloroanisole by Suberase prevents leaching of the substance from cork stoppers and minimizes spoilage of wine by cork taint.

Laccases are able to modify polymers. When added to flour or gluten dough, laccases alter the dough texture and, thus, can be used to tailor the structure of dough and bread [105]. Further, grafting of natural and artificial polymers has been demonstrated with laccase based reaction systems [106, 107]. Grafting by laccases has been used to improve the mechanical robustness, to introduce antioxidative and antimicrobial properties, and to increase the hydrophobicity of polymers [108].

In organic synthesis laccases were used for *in-situ* generation of quinones for Diels-Alder reactions yielding 1,4-naphthoquinones [109], for oxidative condensation of an indamine dye [110], for regioselective oxidation of hydroxy groups of natural glycosides and sugar derivatives to the corresponding carboxylic acids [111, 112], and for the synthesis of novel antibiotics [113, 114].

Laccases can be immobilized on electrodes and mediate direct electron transfer facilitating their integration on bioelectrochemical devices [115]. This can be utilized in biosensors e.g. for determination of polyphenols in wine [116]. In cooperation

with glucose oxidases, laccases can be used in compartment-less glucose-oxygen dependent biofuel cells. These cells could be used to power implanted sensors and transmitters for medical surveillance and research without external power supply [117].

### 3.2.7 Limitations of current laccase applications

Existing applications focus exclusively on fungal laccases because of the large number of characterized fungal enzymes, their high redox potential, and their catalytic efficiency towards a vast variety of substrates. However, this constitutes some limitations to industrial laccase biocatalysis.

Fungal laccases are heavily glycosylated enzymes and, thus, cannot be expressed in prokaryotic hosts. To date, expression in *E. coli* has been described for a single fungal laccase [29]. This makes eukaryotic expression systems mandatory. Cultivation and genetic manipulation of eukaryotic systems is more complicated which makes production and optimization of fungal laccases more time-consuming.

Moreover, fungal laccases are active in acidic reaction conditions [28] what restricts possible reaction conditions. Particularly in applications at neutral or alkaline pH values, e.g. in treatment of certain waste waters, in bleaching of paper pulp, in washing powder, and in implanted biofuel cells, the usability of fungal laccases is limited. Researchers have tried to broaden the pH-activity profile of fungal laccases and were able to generate *Myceliophthora thermophila* laccase by directed evolution with a pH-activity profile broadened by one pH unit in alkaline direction [118]. The created variant retained 80 % of its maximal activity towards 2,6-DMP at pH 7, whereas wildtype enzyme demonstrated 80 % activity at pH 6. However, activity optima in alkaline conditions could not be achieved.

Another obstacle in biocatalysis with fungal laccases is their susceptibility to several substances. It has been demonstrated that halides, metal ions, detergents, and fatty acids inhibit fungal laccases even in low concentrations [115].

By nature, bacterial laccases possess a wider pH range and activity optima at pH 9 have been reported [54, 60]. Laccases from *Bacilli* demonstrated activity at neutral to alkaline pH [119–121]. Bacterial laccases generally exhibit higher thermal stability and often they are more tolerant towards organic solvents, high salt concentrations, and common laccase inhibitors compared to fungal laccases. Despite

$$\begin{aligned} Cu^{1+} + O_2 &\longrightarrow Cu^{2+} + O_2^{-\bullet} \\ 2\,O_2^{-\bullet} + 2\,H^+ &\longrightarrow H_2O_2 + O_2 \\ Cu^{1+} + H_2O_2 &\longrightarrow Cu^{2+} + OH^\bullet + OH^- \end{aligned}$$

**Figure 3.7:** Fenton-like reactions of copper ions can create reactive oxygen species like superoxide, peroxide, and hydroxide radical.

these advantages for practical applications, the utilization of bacterial laccases is negligible due to their low redox potential and limited range of substrates [32]. Furthermore, although bacterial laccases can be expressed in standard prokaryotic expression systems like *E. coli*, their heterologous expression often results in enzymes that do not contain a full complement of copper ions. For example, copper depletion was reported for CotA from *B. subtilis*, CotA from *B. licheniformis*, Ssl1 from *S. sviceus*, McoA from *M. mediterranea*, and LccA from *H. volcanii* [53, 54, 58, 74, 120, 122]. As a consequence, activity yields of bacterial laccases in heterologous expressions are unsatisfactory.

## 3.3 Copper homeostasis systems

### 3.3.1 Copper toxicity

Copper ions are highly cytotoxic. In the past, this was attributed to Fenton-type reactions in which copper ions shuttle between oxidized and reduced state in the presence of water (Fig 3.7). This generates reactive oxygen species like superoxide, hydrogen peroxide, and hydroxy radicals which could damage cellular macromolecules (DNA, RNA, proteins) [123]. More recently, it was shown that copper ions are able to displace other metal cofactors, e.g. in iron-sulfur clusters, resulting in physiological loss of function of these enzymes [124]. Correspondingly, an investigation of regulation targets under copper excess in *B. subtilis* revealed upregulation of genes from the iron-sulfur biogenesis cluster and genes coding for iron-sulfur cluster dependent proteins [125]. A global oxidative stress response was not triggered by copper excess. This indicates that reactive oxygen radicals are not the primary reason of copper toxicity in *B. subtilis*.

Nonetheless, copper redox chemistry is not only cytotoxic but is widely utilized

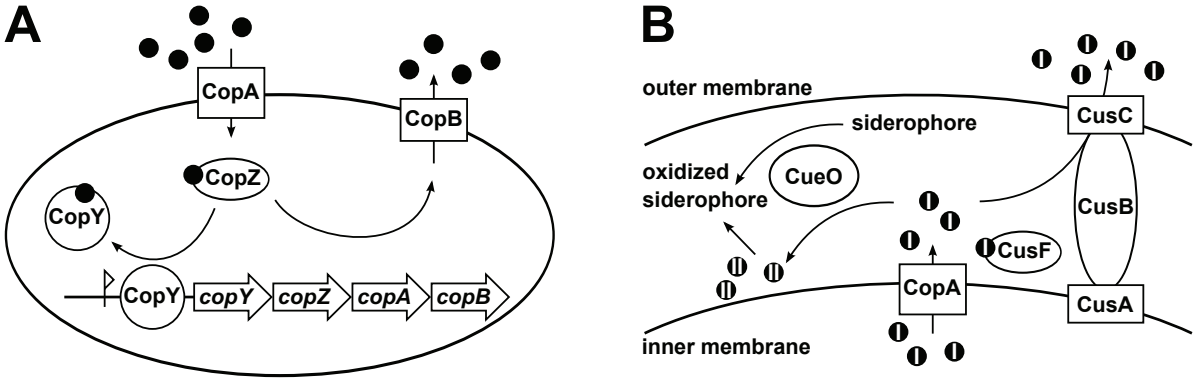


**Figure 3.8:** Prokaryotic copper homeostasis systems. Copper ions are shown as filled circle. A: In *Enterococcus hirae* copper ions enter the cell via the uptake transporter CopA. Intracellular copper is bound the the copper chaperone CopZ which delivers copper to the ATP dependent export pump CopB or to the transcriptional repressor CopY. In copper bound form CopY is released from the promoter region and transcription of the *cop* operon is derepressed. B: In *E. coli* excess Cu(I) is exported from the cytosol to the periplasmic space by the ATP dependent CopA translocase. Under aerobic conditions Cu(I) gets oxidized to less toxic Cu(II) by the periplasmic multicopper oxidase CueO. Additionally CueO oxidizes siderophores to Cu(II) sequestering products. Under anaerobic conditions the proton dependent *cus* system extrudes Cu(I) from the periplasm out of the cell. The *cus* efflux pump is composed of the outer membrane protein CusC, the cytoplasmic membrane protein CusA, and the periplasmic fusion protein CusB. CusF serves for instant copper sequestration until the export pump complex is established.

for redox reactions in cell metabolism and copper containing metalloenzymes are involved in important cellular processes making copper an indispensable micronutrient. Thus, copper containing enzymes participate in mitochondrial respiration (cytochrome *c* oxidase), iron transport and homeostasis (ceruloplasmin, hephaestin), oxidative stress protection (copper,zinc superoxide dismutase), blood clotting (factor VIII), and pigmentation (tyrosinase, laccase) [126, 127].

To handle the toxic copper cargo, organisms have evolved delicately balanced homeostasis systems. These systems include copper export pumps for excess copper ions, copper chaperones and metallothioneines for copper sequestration, multicopper oxidases for copper oxidation from highly toxic Cu(I) to less toxic Cu(II), copper import pumps in case of copper limiting conditions, and copper sensing systems for regulation of copper homeostasis systems.

### 3.3.2 Copper homeostasis in prokaryotes

Copper homeostasis in *Enterococcus hirae* is the best understood prokaryotic copper homeostasis system [128]. It is encoded in the copper resistance operon (*cop*)

containing four genes (Fig. 3.8). Copper ions enter the cell by the copper import pump CopA and are bound intracellularly by the copper chaperone CopZ. CopZ delivers copper ions to the copper efflux pump CopB for exporting excess copper. The second target of CopZ for copper ion delivery is the transcriptional repressor CopY. Two copper ions replace one zinc ion in CopY upon which CopY is released from the promoter region and the genes of the *cop* operon, *copY*, *copZ*, *copA*, and *copB*, are induced [128, 129].

A similar system with a copper inducible transcription repressor (CopR), a cytoplasmic copper chaperone (CopZ), and two copper exporting ATPases (CopA, CopB) exists in *Lactococcus lactis*. A copper import pump for maintenance of intracellular copper concentration under copper starvation conditions was not detected [130].

In *Corynebacterium glutamicum* apart from the intracellular copper sensing regulator CsoR, an extra two-component signal transduction system was found to control transcription of the *cop* operon [131]. At high extracellular copper concentrations the transmembrane sensor kinase CopS undergoes autophosphorylation with subsequent phosphotransfer to the intracellular response regulator CopR. In phosphorylated form CopR stimulates transcription of copper homeostasis related genes including the copper exporter CopB and the multicopper oxidase CopO [131].

Beside the system in *E. hirae*, copper trafficking and homeostasis in *E. coli* is best understood among bacteria [126]. *E. coli* contains two genomic copper detoxification systems, the oxygen dependent *cue* (Cu efflux) system and the proton gradient driven *cus* (Cu sensing) system (Fig. 3.8). The housekeeping copper homeostasis system consists of the copper export ATPase CopA and of the periplasmic multicopper oxidase CueO [132]. CueO and CopA transcription are under control of the cytoplasmic copper sensor CueR. Excess intracellular Cu(I) is translocated by CopA from the cytosol to the periplasmic space where CueO oxidizes Cu(I) to less toxic Cu(II) [37]. Moreover, CueO oxidizes the copper reductant enterobactin and its precursor 2,3-dihydroxybenzoic acid which are secreted by *E. coli* as iron siderophores [133]. The product of this reaction, 2-carboxymuconate, does not reduce Cu(II) and is able to sequester Cu(II). This additionally contributes to copper detoxification. Since the reaction cycle of multicopper oxidases involves oxygen as final electron acceptor, CueO is only functional in aerobic conditions. Copper detoxification under anaerobic conditions is provided by the oxygen independent *cus* system [132]. In presence of oxygen the *cus* system is functional as well and contributes to copper tolerance of

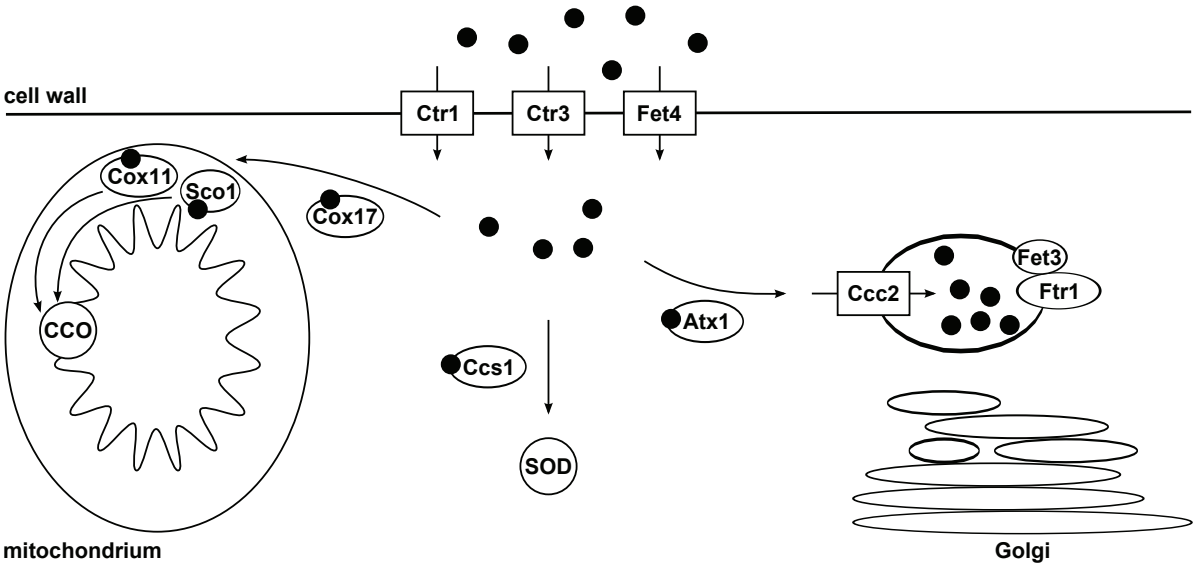


**Figure 3.9:** Copper homeostasis in *Saccharomyces cerevisiae*. High and low affinity importers, Ctr1, Ctr3, Fet4, import copper ions to the cytosol where they are sequestered by metallothioneines, chelating agents, and the copper chaperones Cox 17, Ccs1, and Atx1. Cox 17 transfers copper ions to the mitochondrial copper chaperones Sco1 and Cox11 which assemble specifically the $Cu_A$ and $Cu_B$ center of cytochrome *c* oxidase (CCO). Ccs1 delivers copper ions to the Zn,Cu-superoxide dismutase (SOD) which acts as superoxide scavenger in the cytoplasm. Atx1 donates copper to Ccc2 for copper loading of the Golgi and secretory vesicles. Copper in the Golgi is required for insertion into the multicopper oxidase Fet3. Fet3 and Ftr1 build a copper dependent iron oxidase-permease complex that is required for high affinity iron uptake at the cell surface.

*E. coli* but is not essential. The *cus* operon is regulated by a two-component regulatory system with CusS as sensor kinase at the cytoplasmic membrane and with CusR as response regulator that induces genes of the *cus* operon at high copper concentrations [134]. The products of *cusCFBA* form a copper-extruding complex that spans the periplasmic space with CusA as cytoplasmic membrane transporter, CusC as outer membrane protein, and CusB as periplasmic fusion protein. CusF may function as periplasmic copper chaperone for instant sequestration of periplasmic copper and protection until the efflux pump is established [135]. Since *copA* deleted strains showed lower copper tolerance under anaerobic conditions, it was suggested that the *cus* system exports copper ions mainly from the periplasmic space but not from the cytoplasm [126]. According to this model export of cytoplasmic copper ions under anaerobic conditions is performed by CopA.

### 3.3.3 Copper homeostasis in eukaryotes

The complex compartmentalization of eukaryotic cells with copper dependent enzymes in differing cell compartments, requires more complex copper homeostasis and copper distribution pathways compared to prokaryotic systems. The baker's yeast *Saccharomyces cerevisiae* has proven to be an invaluable tool for discovery and investigation of copper trafficking pathways in eukaryotic cells [136]. Copper enters *S. cerevisiae* cells either by high affinity importers like Ctr1 and Ctr3 (copper transporter 1 and 3) or by low affinity importers like Fet4 (Fe transporter 4) (Fig. 3.9) [137]. Intracellular copper is bound by metallothioneines or by the copper chaperones Atx1 (anti-oxidant 1), Ccs1 (copper chaperone for superoxide dismutase 1), and Cox17 (cytochrome *c* oxidase subunit 17). The copper chaperones mark entry points to three copper delivery pathways within *S. cerevisiae* [138]. Ccs1 transfers copper to cytosolic Cu,Zn-superoxide dismutase (SOD) which acts as superoxide scavenger in oxidative defense. Ccs1 deficient yeast cells produce SOD that is inactive due to copper ion depletion [139]. The target of Atx1 is the secretory pathway [137]. Atx1 delivers copper to the copper transporter Ccc2 for loading into the Golgi and secretory vesicles. Copper ions from the Golgi are utilized for incorporation to the multicopper oxidase Fet3 which builds together with Ftr1 (Fe reductase 1) an iron oxidase-permease complex at the cell surface [136]. The third intracellular copper chaperone, Cox17, transfers copper from the cytoplasm to copper chaperones of the inner membrane space of mitochondria [140]. The recipients are Sco1 (synthesis of cytochrome *c* oxidase 1) and Cox11 which forward copper ions specifically to the $Cu_A$ and the $Cu_B$ center of cytochrome *c* oxidase, the terminal enzyme in the mitochondrial oxidative electron transport chain [141].

## 3.4 Aim of the work

Current technical applications of laccases focus exclusively on fungal enzymes. This implies limitations to laccase applications, since fungal laccases are restricted to acidic reaction conditions, require eukaryotic expression systems, and are prone to inhibition by many substances (see 3.2.7). By nature, bacterial laccases show alkaline activity, can be expressed in prokaryotic hosts, and possess intrinsic robustness in terms of elevated temperature, presence of organic solvent, inhibitors, detergents,

and inorganic salts [31]. Their utilization could circumvent the limitations of fungal laccases. However, applications of bacterial laccases is hindered by several obstacles and to date there is no commercialized bacterial laccase preparation available. In this work, new bacterial laccases should be identified and the disadvantages of bacterial laccases should be addressed in order to make their application more feasible.

### 3.4.1 Cloning and characterization of novel bacterial laccases

As mentioned above, fungal laccases are long known and a large number has been characterized, whereas the number of described bacterial laccases is still low which is in strong contrast to their widespread occurrence in nature. Thus, more than 1000 gene sequences of bacterial laccases and laccase-like enzymes have been detected by classification of available sequence data [4, 6]. The low number of available bacterial enzymes reduces the sequence space for detection of suitable enzymes for a given target reaction.

To increase the number of available bacterial laccases, suitable bacterial laccase genes were selected, cloned in expression vectors, and the corresponding enzymes were produced in *E. coli*. The expression was optimized in order to obtain sufficient amounts of enzyme for in-depth characterization.

### 3.4.2 Tuning the redox potential of a bacterial laccase

The initial step of substrate oxidation by laccases involves one electron abstraction and transfer from substrate to the copper ion of the laccase T1 center. The major factor that determines the velocity of this reaction, and whether the electron abstraction occurs at all, is the redox potential difference between substrate and the T1 copper ion [24, 76, 79]. Bacterial laccases belong to the class of low redox potential laccases with potentials below 470 mV. This limits their substrate range and their suitability for industrial applications [31].

For creation of an enzyme with increased substrate range and higher oxidation activity the redox potential of a bacterial laccase should be increased by site-specific mutagenesis. Structural information is a prerequisite for rational site-specific mutagenesis. Therefore the previously characterized small bacterial laccase Ssl1 from *S. sviceus* [54] was crystallized and its structure was solved by molecular replacement.

Obtained structural information was used for selection of promising positions and appropriate amino acid substitutions for an increased redox potential of Ssl1.

### 3.4.3 Improvement of copper content of a bacterial laccase

Copper depletion upon heterologous expression of bacterial laccases in *E. coli* has been observed frequently [54, 58, 74, 120] which causes suboptimal volumetric activity yields and thereby limits the applicability of bacterial laccases. Several strategies can be pursued to increase copper loading.

The addition of copper salts to the expression medium can increase the specific activity of heterologously expressed bacterial laccases but does not lead to full copper loading [120]. Expression under microaerobic conditions (expression without aeration) was reported for CotA from *B. subtilis* [142]. Although this resulted in fully copper-loaded enzyme, microaerobic expression inevitably leads to reduced growth rates which reduces its practicability for large scale production of laccases. Further, depleted copper centers can be reconstituted after expression and purification by incubation or dialysis at high copper concentrations [143, 144]. However, reconstitution of copper centers in copper depleted bacterial laccases does not result in fully active enzyme [142]. Obviously, formation of natural copper cluster geometry requires copper incorporation immediately during the folding process. Therefore, copper absence during protein folding causes perturbations at the copper clusters which then result in enzyme with reduced activity even with subsequently reconstituted copper centers.

For CotA from *B. licheniformis* microaerobic expression conditions and reconstitution of copper centers after expression and purification did not result in fully copper loaded enzyme (K. Koschorreck, personal communication). For increasing the copper content of CotA two further strategies were applied. Knock-down of a host copper detoxification system should increase the available intracellular copper concentration and coexpression of a copper chaperone should facilitate copper ion incorporation into CotA from *B. licheniformis*.

# 4 Manuscripts

## 4.1 Manuscript 1

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| Title: | Characterization of the alkaline laccase Ssl1 from *Streptomyces sviceus* with unusual properties discovered by genome mining |
| Authors: | Matthias Gunne, Vlada B. Urlacher |
| Published in: | PLoS ONE 7(12): e52360 |
| doi: | 10.1371/journal.pone.0052360 |
| Contribution: | Sequence analysis and cloning of *ssl1*<br>Expression optimization and purification of Ssl1<br>Characterization of Ssl1 (activity-pH profiles, enzyme kinetics, solvent tolerance, thermostability, UV-Vis spectrum)<br>Writing of the manuscript |

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# Characterization of the Alkaline Laccase Ssl1 from *Streptomyces sviceus* with Unusual Properties Discovered by Genome Mining

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## Abstract

Fungal laccases are well investigated enzymes with high potential in diverse applications like bleaching of waste waters and textiles, cellulose delignification, and organic synthesis. However, they are limited to acidic reaction conditions and require eukaryotic expression systems. This raises a demand for novel laccases without these constraints. We have taken advantage of the laccase engineering database LccED derived from genome mining to identify and clone the laccase Ssl1 from *Streptomyces sviceus* which can circumvent the limitations of fungal laccases. Ssl1 belongs to the family of small laccases that contains only few characterized enzymes. After removal of the twin-arginine signal peptide Ssl1 was readily expressed in *E. coli*. Ssl1 is a small laccase with 32.5 kDa, consists of only two cupredoxin-like domains, and forms trimers in solution. Ssl1 oxidizes 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and phenolic substrates like 2,6-dimethoxy phenol, guaiacol, and syringaldazine. The $k_{cat}$ value for ABTS oxidation was at least 20 times higher than for other substrates. The optimal pH for oxidation reactions is substrate dependent: for phenolic substrates the highest activities were detected at alkaline conditions (pH 9.0 for 2,6-dimethoxy phenol and guaiacol and pH 8.0 for syringaldazine), while the highest reaction rates with ABTS were observed at pH 4.0. Though originating from a mesophilic organism, Ssl demonstrates remarkable stability at elevated temperatures ($T_{1/2,60^{\circ}C}$ = 88 min) and in a wide pH range (pH 5.0 to 11.0). Notably, the enzyme retained 80% residual activity after 5 days of incubation at pH 11. Detergents and organic co-solvents do not affect Ssl1 stability. The described robustness makes Ssl1 a potential candidate for industrial applications, preferably in processes that require alkaline reaction conditions.

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## Introduction

Although significant progress has been achieved in enzyme engineering, the discovery and characterization of novel enzymes from diverse (micro)organisms still plays an essential role for the development of biocatalytic processes. Especially in the case of laccases it has been demonstrated that very few positions can be mutated without loss of activity [1]. This is due to highly conserved functionally essential regions of these enzymes. Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase) belong to the enzyme family of multicopper oxidases. They catalyze the one-electron oxidation of substrate molecules and transfer thereby abstracted electrons to molecular oxygen which is reduced to water. The electrons are channeled through highly conserved copper binding residues from the substrate oxidizing T1 copper site to the T2/T3 trinuclear copper cluster where oxygen is bound and reduced to water by four electrons [2]. Typical substrates are mono-, di- and polyphenols, hydroxylated aryls, aromatic or aliphatic amines and metal ions. Most laccases show low substrate specificity and accept a large variety of different substrates. It was proposed that the redox potential at the T1 site is the key factor determining whether a substrate can be oxidized by laccase [3].

The most exhaustively investigated laccases originate from white-rot fungi such as *Trametes* sp. or *Pleurotus* sp. Many of those fungal laccases exhibit high redox potentials and therefore possess high activities towards their substrates. However, owing to pH preference and stability [4], their use is restricted to acidic reaction conditions and mesophilic temperatures. Moreover, fungal laccases are highly glycosylated enzymes and cannot be produced with bacterial expression systems. Recent approaches based on metagenomic libraries [5] or available and fast growing sequence data [6] demonstrate the wide distribution of laccases or laccase-like enzymes in bacteria. Sirim et al. classified more than 2200 laccases and related enzymes from available genome sequences and structural data and assigned more than 1000 potential bacterial laccases into 5 different superfamilies [7].

The physiological functions of most characterized bacterial laccases remain unknown. The few described functions include spore pigmentation as found for the laccase CotA from *B. subtilis* [8] and copper homeostasis as suggested for CueO from *E. coli* [9,10] and CopO from *Corynebacterium glutamicum* [11].

By now, the characteristics and biotechnological potential of these enzymes are poorly investigated and still few reports on bacterial laccases have been published. Nevertheless, these reports

demonstrate the thermal robustness and more alkaline activity profiles of bacterial laccases compared to fungal enzymes. Exemplarily, the laccase from *Thermus thermophilus* shows extreme stability at high temperatures with a half-life of thermal inactivation at 80°C of more than 14 h [12], and laccases from *Bacillus halodurans* and *Streptomyces coelicolor* exhibit maximum activities towards syringaldazine or 2,6-dimethoxyphenol at pH values of 7.5 or 9.4 [13,14]. This kind of bacterial alkaline laccase may circumvent the limitations of fungal laccases and extend the range of feasible reaction conditions in industrial applications of laccase towards higher pH values, elevated reaction temperatures and prolonged production processes owing to more robust biocatalysts.

Similar to other multicopper oxidases, laccases commonly consist of three cupredoxin-like domains with the T1 copper coordinated by two histidines and a cysteine residue in domain 3 and the trinuclear T2/T3 cluster at the interface of domain 1 and 3 coordinated by eight histidines [15]. In 2002, a novel type of laccase was described which showed low sequence similarity to known eukaryotic and bacterial laccases and a smaller molecular size [16,17] due to lack of the second domain present in most laccases [14].

Here, we describe the cloning, expression and characterization of the small two-domain Ssl1 laccase from *Streptomyces sviceus*. Ssl1 demonstrated moderate thermostability, alkaline pH-activity profile, and stability in a wide pH range up to pH 11 and in presence of organic solvents. Thereby its catalytic properties were distinct from other laccases.

## Materials and Methods

### Materials and Strains

All reagents were of analytical grade or higher and purchased from commercial sources. Enzymes for molecular cloning, nucleotide ladders and protein ladders were obtained by Fermentas (St. Leon-Rot, Germany). Molecular cloning and plasmid propagation were carried out in *E. coli* DH5α (Novagen, Darmstadt, Germany). *E. coli* BL21(DE3), *E. coli* BL21(DE3) pLys, *E. coli* Rosetta(DE3) (all from Novagen) and *E. coli* BL21-CodonPlus (DE3)-RP (Stratagene, Waldbronn, Germany) served as expression hosts. Genomic DNA of *Streptomyces sviceus* (DMS 924) was purchased from the DSMZ (Braunschweig, Germany).

### Cloning of *ssl1*

The *ssl1* gene (SSEG_02446) was amplified by PCR with the primers CTTgctagcATGCATCATCATCATCATCATGCCCCGGGCGGCGAG and GGCaagcttTCAGTGGTGGTGTTCGGCCCGC (Eurofins MWG Operon, Ebersberg, Germany) using genomic DNA of *S. sviceus* as template. NheI and HindIII endonuclease recognition sites are shown in lowercase, the sequence of the hexahistidine tag is underlined. The genomic sequence of *ss1l* was truncated at the 5′ end in order to remove a natural signal sequence of the twin arginine translocation pathway. The PCR product was purified and cloned into the pET22H plasmid [18] using the *Nhe*I and *Hind*III restriction endonucleases. The sequence of the *ssl1* insert in the resulting pET22-ssl1 plasmid was verified by sequencing (Eurofins MWG Operon).

### Expression Optimization and Purification of Ssl1

*E. coli* expression strains were transformed with pET22ssl1 and grown in 200 mL medium containing ampicillin (100 μg $ml^{-1}$) and, when required, chloramphenicol (34 μg $ml^{-1}$). Cultures were grown at 30°C or 37°C and 140 rpm. Expression conditions were optimized with regard to *E. coli* expression strain (BL21(DE3), BL21(DE3) pLys, Rosetta(DE3), BL21-CodonPlus (DE3)-RP), medium (LB, TB, M9, 2xYT), induction $OD_{600}$ (0.5, 1, 1.5, 2), inducer concentration (10 μM, 40 μM, 200 μM, 1 mM), expression temperature (20°C, 25°C, 30°C, 37°C) and expression duration (3 to 24 h).

Highest volumetric activities were obtained in *E. coli* BL21-CodonPlus (DE3)-RP grown at 30°C and 140 rpm in TB medium, when expression was induced at an $OD_{600}$ of 1.0 with 40 μM IPTG. 2 mM copper(II) sulfate were added to the culture medium upon induction, and the expression was carried out for 8 h at 25°C and 140 rpm.

After expression, cultures were harvested by centrifugation at 11000 g at 4°C for 20 min. The cell pellets were resuspended in potassium phosphate buffer (50 mM, pH 7.5) containing 0.1 mM PMSF and 0.3 mM copper(II) sulfate. Cell disruption was done by sonication with a Branson sonifier SLPe (3 cycles: energy pulse mode for 90 s at 50% amplitude with 10 J, 2 s off time) with at least 1 min on ice between the cycles. Cell debris was removed by centrifugation at 31000 g for 30 min at 4°C. The soluble fraction was incubated for 20 min at 65°C and precipitate was removed by centrifugation at 48000 g for 30 min at 4°C. The resulting supernatant was filtered through a cellulose acetate membrane with 0.45 μm pores. For purification of Ssl1 by immobilized metal affinity chromatography (IMAC), the filtrate was loaded onto a Talon (BD Biosciences, Heidelberg, Germany) packed and preequilibrated gravity flow column (7 mL bed volume). The column was washed with 5 column volumes (CV) equilibration buffer (50 mM potassium phosphate, pH 7.5, 500 mM sodium chloride) and,thereafter, with 5 CV washing buffer (50 mM potassium phosphate, pH 7.5, 500 mM sodium chloride, 5 mM imidazole). Ssl1 was eluted in 1 CV elution buffer (50 mM potassium phosphate, pH 7.5, 500 mM sodium chloride, 100 mM imidazole). The eluate was concentrated with Vivaspin 15 columns (10 kDa cut-off, Sartorius, Göttingen, Germany) and imidazole and sodium chloride were removed by use of PD miditrap G-25 desalting columns (GE Healthcare, München, Germany). Purified Ssl1 was stored at -20°C without loss of activity until use. The purity of Ssl1 was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel.

### Spectroscopy and Light Scattering

The absorption spectrum of Ssl1 was determined in the range of 300 to 700 nm with a Lamdba 35 spectrophotometer (Perkin Elmer, Rodgau, Germany). The solution contained 125 μM Ssl1 in 50 mM MOPS buffer at pH 7.5. Ssl1 concentrations were determined by the Bradford method with BSA as standard, calculation of molar concentrations of Ssl1 were based on the computationally determined and experimentally verified molar mass of 32.5 kDa.

The copper content of Ssl1 was determined by atom absorption spectroscopy with an AAnalyst 100 (Perkin Elmer, Rodgau, Germany) at 324.8 nm with a slit width of 0.7 nm.

The molar mass of Ssl1 in native form was determined by multi-angle static light scattering. Therefore, Ssl1 was applied to an equilibrated Superdex 200 10/300 GL size exclusion chromatography (SEC) column mounted on an ÄKTA purifier FPLC system (GE Healthcare, München, Germany). The SEC was conducted at 0.5 mL $min^{-1}$ flow with 50 mM potassium phosphate buffer at pH 7.5. The injection volume was 100 μL with a Ssl1 concentration of 7.5 mg $mL^{-1}$. Refraction index and scattering intensity were measured on-line by an Optilab rEX differential refractometer and a miniDAWN TREOS triple-angle light scattering detector (both Wyatt Technology

Europe, Dernbach, Germany). Data were integrated and analyzed by the Astra software package.

### Activity Assays

The activity of Ssl1 towards the substrates 2,6-dimethoxyphenol (2,6-DMP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), guaiacol, and syringaldazine (SGZ) was tested in 50 mM McIlvaine's buffer (pH 3–6), 50 mM potassium phosphate buffer (pH 7–8), 50 mM glycine-sodium hydroxide buffer (pH 9–10) and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-sodium hydroxide buffer (pH 11) at room temperature. The oxidation of 2,6-DMP was followed spectrophotometrically at 468 nm ($\varepsilon = 49.6\ mM^{-1}\ cm^{-1}$), the reaction mixture contained 0.5 mM 2,6-DMP. The oxidation of ABTS (0.5 mM) was followed at 420 nm ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$), oxidation of guaiacol (2 mM) to the dimeric product tetraguaiacol was followed at 470 nm ($\varepsilon = 26\ mM^{-1}\ cm^{-1}$) and oxidation of SGZ (50 μM, 10% dimethyl sulfoxide) was followed at 525 nm ($\varepsilon = 65\ mM^{-1}\ cm^{-1}$). Ssl1 activity towards tyrosine was tested at pH 4.0, 7.0 and 9.0. The reaction contained 0.1 mM tyrosine and 2 μM Ssl1, oxidation was monitored at 280 nm ($\varepsilon = 4.4\ mM^{-1}\ cm^{-1}$). Activity assays were performed in triplicate. For pH activity profiles, activities at optimal pH were set as 100%.

For testing thermal stability, Ssl1 (75 μM in 50 mM glycine-sodium hydroxide buffer with pH 9.0) was incubated at 50°C, 60°C, 70°C and 80°C for 0 to 240 min. After incubation, samples were immediately chilled on ice. Residual activities were measured with 2,6-dimethoxyphenol as described above and the half-time of activity loss was determined by non-linear regression to the equation of exponential decay ($a(t) = a_0 \exp(-t \ln 2/T_{1/2})$) by OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA). Data were collected as triplicate.

For testing the stability at different pH values, Ssl1 (75 μM) was incubated at pH 3, 4, 5, 6 (in 50 mM McIlvaine's buffer), 7, 8 (in 50 mM potassium phosphate buffer), 9, 10 (in 50 mM glycine-sodium hydroxide buffer) and 11 (in 50 mM CAPS-sodium hydroxide buffer) at room temperature and residual activities were determined with 2,6-dimethoxyphenol.

Relative activities of Ssl1 in presence of organic solvents were determined after 10 min incubation in glycine-sodium hydroxide buffer (50 mM, pH 9.0) containing solvent in indicated concentrations at ambient temperature. In case of water immiscible solvents (isooctane and *n*-hexane), samples were agitated with 600 rpm in a thermomixer for the same period. Activities were measured with 2,6-dimethoxyphenol as described above. Stability towards organic solvents was accessed by incubation with solvent for 20 h at ambient temperature and subsequent activity measurement with 2,6-dimethoxyphenol.

Kinetic constants of Ssl1 were determined with the substrates 2,6-DMP, ABTS and SGZ. Reactions contained 3.5 μM Ssl1 and 0 to 6 mM 2,6-DMP in 50 mM glycine-NaOH buffer (pH 9.0), 2.3 μM Ssl1 and 0 to 10 mM ABTS in 50 mM McIlvaine buffer (pH 4.0), or 22 nM Ssl1, 0 to 40 μM SGZ and 2% dimethyl sulfoxide in 50 mM potassium phosphate buffer (pH 8.0). The reactions were conducted at room temperature and followed spectrophotometrically at 468 nm (2,6-DMP, $\varepsilon = 49.6\ mM^{-1}\ cm^{-1}$), 420 nm (ABTS, $\varepsilon = 36\ mM^{-1}\ cm^{-1}$) and 525 nm (SGZ, $\varepsilon = 65\ mM^{-1}\ cm^{-1}$). Initial rates were determined in triplicate and resulting data were fitted by non-linear regression to the hyperbolic equation ($v = v_{max}\,[S]/(K_m + [S])$) in OriginPro 8.5.

## Results and Discussion

### Sequence Analysis and Cloning of ssl1 from *Streptomyces sviceus*

*S. sviceus* is a mesophilic soil bacterium best known for its ability to produce the glutamine antagonist acividin (or U-42,126) [19]. According to the Laccase Engineering Database [7] *S. sviceus* contains two laccases of different type, one belonging to SUBfamiliy K of SLAC homologues from *S. coelicolor* and the second belonging to SUBfamily J with homologues of CueO from *E. coli*. Here we describe the cloning, expression and characterization of the laccase Ssl1 (*S. sviceus* laccase 1) from SUBfamiliy K. To this subfamily belong also the previously described EpoA laccase from *Streptomyces griseus*, SLAC laccase from *S. coelicolor*, and SilA laccase from *S. ipomoea*. An alignment of the Ssl1 amino acid sequence with these two-domain laccases (Fig. 1) demonstrates the high sequence similarity within this family. All copper coordinating residues (one cysteine and ten histidins) in these laccases are strictly conserved and conservation among neighboring amino acids is high. Sequence variations are mainly located at both termini, including the tat signal, and in some regions that were found to form loops in the crystal structure of SLAC.

As indicated by a Conserved Domain Search [20] Ssl1 seems to possess an architecture of two cupredoxin-like domains missing domain 2 of large laccases. Analysis of the amino acid sequence by SignalP 4.0 server [21] revealed a potential signal sequence spanning the first 39 amino acids and belonging to the twin-arginine translocation (tat) pathway. In contrast to the sec pathway, translocation via the tat pathway allows secretion of fully folded and cofactor-bound enzymes. A bioinformatics analysis of known laccases showed that 76% of bacterial laccases contain a secretion signal [6]. Cloning and expression of *ssl1* containing the tat signal did not result in active enzyme. Therefore, *ssl1* was amplified without the signal sequence. For simple purification of Ssl1 by immobilized metal affinity chromatography (IMAC) a 6-hexahistidine tag was introduced. The resulting 882 bp PCR fragment was cloned into pET22H [18] to give pET22-ssl1.

### Recombinant Expression and Purification of Ssl1

Highest volumetric activities of Ssl1 were obtained with *E. coli* BL21-CodonPlus (DE3)-RP as expression host (800–1000 $U\ L^{-1}$). This expression strain contains a plasmid encoding rare tRNAs for arginine and proline that are frequently found in genes of GC-rich genomes such as in *Streptomyces* strains. In case of Ssl1 there are three prolines (P72, P206 and P248) and four arginines (R46, R54, R68 and R200) that are encoded by the CCC or AGG codon which are transcribed by two of the CodonPlus-RP tRNAs. Since *ssl1* contains further codons that are rarely used in *E. coli*, codon optimization of the *ssl1* nucleotide sequence can be expected to increase expression efficiency and volumetric activities.

For optimal Ssl1 expression, cultures were grown in TB medium at 30°C until $OD_{600}$ reached 1.0 when expression was induced by only 40 μM IPTG with 2 mM copper sulfate as supplement. After 16 h at 25°C the cells were harvested and lysed. The soluble fraction was incubated for 20 min at 65°C which lead to precipitation of most *E. coli* proteins while the activity of Ssl1 remained stable. Precipitated proteins were removed and the lysate was applied to a single IMAC purification step which resulted in homogeneously pure Ssl1 as judged by SDS-PAGE (Fig. 2). Typical expressions yielded 40 to 50 mg Ssl1 per liter of culture with specific activity of $21.7 \pm 0.25\ U\ mg^{-1}$ (with ABTS as substrate).

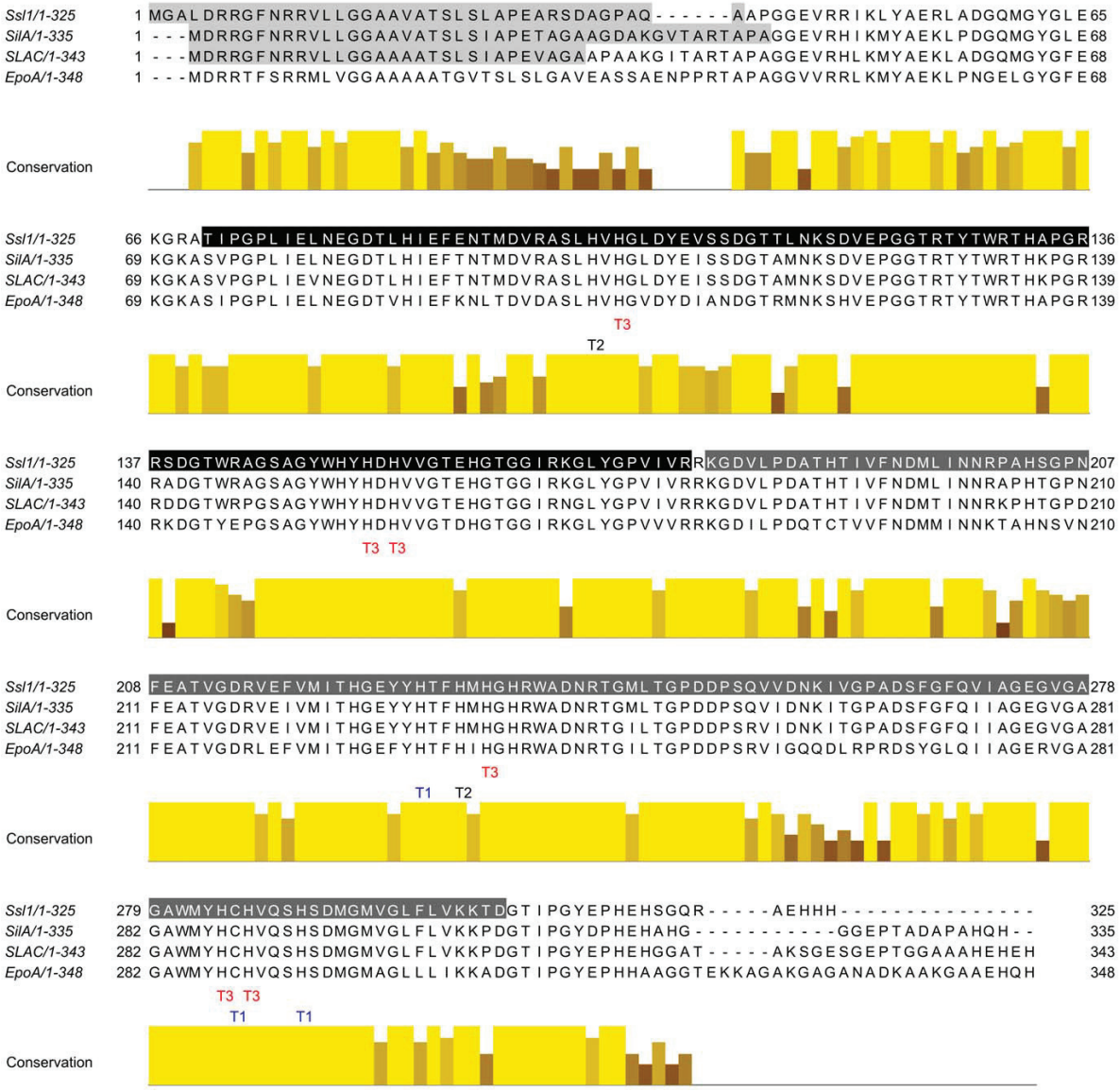


**Figure 1. Multiple sequence alignment of homologue small laccases.** Ssl1 from *Streptomyces sviceus*, SilA from *S. ipomoea*, EpoA from *S. griseus* and SLAC from *S. coelicolor*. The copper binding residues are conserved in all 4 laccases as indicated. All four laccases consist of 2 domains (indicated in Ssl1, black: domain 1, dark grey: domain 2). Signal sequences of the twin-arginine pathway are indicated in light grey. Sequence identity between the four laccases is high and variations are mainly located at the termini.
doi:10.1371/journal.pone.0052360.g001

As determined by atomic absorption spectroscopy Ssl1 contained 2.5 copper ions per molecule. According to the four canonical copper binding sites of laccase, we expected four copper equivalents instead of 2.5. The used expression and purification strategy resulted in partially copper depleted enzyme. Partial copper depletion of bacterial laccases when heterologously expressed in *E. coli* was observed repeatedly [22–24]. Since all four canonical copper ions are required for activity copper depletion during expression and purification limits the obtainable yield of active laccase. This might be due to the high level of protein expressed under the control of the strong T7-promoter, which is not completely loaded with copper ion upon expression.

In SDS-PAGE Ssl1 migrated at 33 kDa corresponding to the theoretically determined molecular weight of 32.5 kDa. When incubated in loading buffer containing SDS but without reducing agent, Ssl1 migrated at approximately 100 kDa (not shown). Accordingly, in multiangle static light scattering experiments the molecular weight of active Ssl1 was determined to be 98.3 kDa. This molecular weight corresponds to a homotrimeric oligomerization state of active Ssl1. Other two-domain laccases were found to form either homotrimers (EpoA, [16]) or homodimers (SilA,

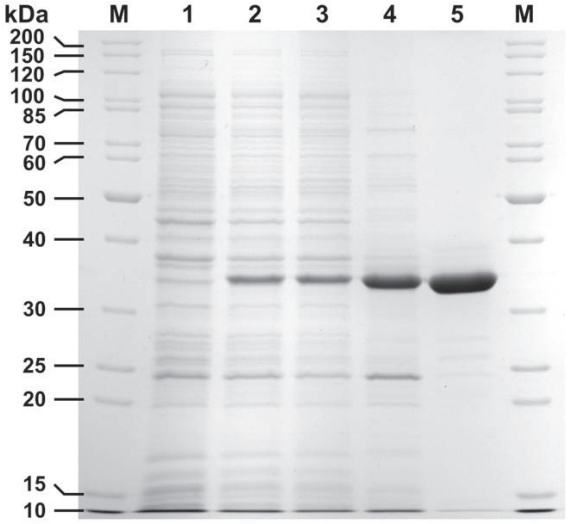


**Figure 2. SDS-PAGE analysis of recombinant expression and purification process of Ssl1.** Ssl1 migrates at 33 kDa. It was expressed in *E coli*-CodonPlus (DE3)-RP and purified to homogeneity by heat precipitation and immobilized metal affinity chromatography (IMAC). M: molecular size marker, lane 1: cell extract before induction, lane 2: cell extract after expression, lane 3: soluble fraction from cell disruption, lane 4: soluble fraction from heat precipitation, lane 5: IMAC eluate.
doi:10.1371/journal.pone.0052360.g002

[25]), for SLAC both forms were observed [14,26]. The need for oligomerization of two-domain laccases was attributed to the fact that the trinuclear cluster is located at the interface of domain 1 and 2 of neighboring laccase molecules, thereby forming catalytic entities of electronically connected T1 and T2/T3 copper centers as seen in the SLAC crystal structure [26]. In three-domain laccases, domain 2 connects the domains 1 und 3 in a fashion that enables the formation of the trinuclear center at the interface of domain 1 and 3 of a single enzyme molecule. Owing to the lack of domain 2, in two-domain laccases this is not possible. Thus, formation of the trinuclear cluster is achieved by assembly into an appropriate quarternary structure.

## UV-vis Spectrum of Ssl1

Purified Ssl1 solutions are characterized by a deep blue color. Accordingly, the absorption spectrum of Ssl1 in the range of 300 to 700 nm (Fig. 3) showed the typical laccase features, with a maximum at 592 nm corresponding to the T1 copper center and a shoulder at approximately 330 nm reflecting the binuclear T3 copper center. The maximum at 592 nm can be utilized for quantification of Ssl1 ($\varepsilon = 2.796 \pm 0.191$ mM$^{-1}$ cm$^{-1}$).

## Biochemical Properties of Ssl1

Ssl1 was able to oxidize syringaldazine but did not accept tyrosine as substrate. In combination with the typical UV-vis spectrum and conservation of the canonical copper binding residues, Ssl1 could be classified as laccase, not as tyrosinase. Ssl1 oxidized a range of other typical laccase substrates like 2,6-DMP and guaiacol. ABTS was also oxidized by this enzyme. Thereby the activity-pH dependence was bell-shaped for all substrates (Fig. 4). Maximal oxidation activity towards ABTS was reached at pH 4.0 as observed for most laccases with this substrate, whereas Ssl1 activity peaked at alkaline pH values with each of the phenolic

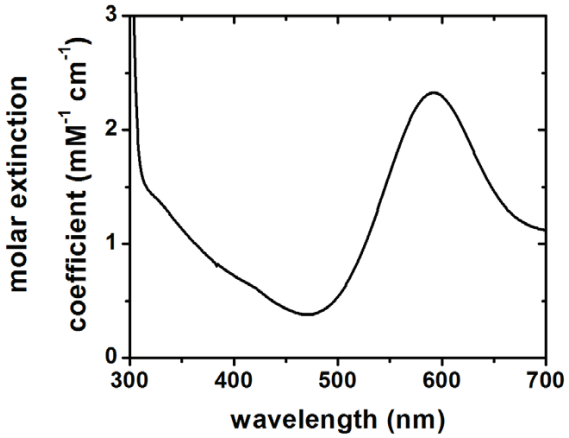


**Figure 3. UV/vis spectrum of Ssl1 in potassium phosphate buffer.** Ssl1 shows a laccase-typical absorption spectrum. The maximum at 592 nm ($\varepsilon_{Ssl1,\ 592\ nm} = 2.796 \pm 0.191$ mM$^{-1}$ cm$^{-1}$) corresponds to type I or blue copper and the shoulder around 330 nm is characteristic for type 3 copper centers.
doi:10.1371/journal.pone.0052360.g003

substrates 2,6-DMP, guaiacol (both pH 9.0) and syringaldazine (pH 8.0).

The bell-shaped activity profiles with phenolic substrates can be ascribed mainly to two antagonistic effects: (a) the redox potential of phenolic substrates decreases with increasing pH, which results in a larger redox potential difference of substrate and T1 copper and thus in higher activity; (b) hydroxide ions bind at the trinuclear cluster and inhibit the oxygen reduction which reduces activities at high pH [27,28]. The observed optimum at pH 9 is one of the most alkaline activity optima of laccases reported so far. Generally, fungal laccases are active under acidic conditions [4] and only few bacterial laccases show activity in alkaline milieu [29]. For certain industrial processes like addition of laccase to washing powder, decolourization of waste waters, or treatment of Kraft pulps, where alkaline reaction milieus prevail, alkaline activity would be preferable.

Ssl1 reactions followed Michaelis-Menten kinetics and analysis of kinetic parameters showed higher catalytic efficiency for ABTS (20.6 s$^{-1}$ mM$^{-1}$) than for syringaldazine (3.66 s$^{-1}$ mM$^{-1}$) and 2,6-DMP (0.361 s$^{-1}$ mM$^{-1}$). Observed $K_m$ values were in the micromolar range, turn over numbers ($k_{cat}$) strongly depended on the substrate and ranged from 443 min$^{-1}$ for ABTS to 19.3 min$^{-1}$ and 3.47 min$^{-1}$ for 2,6-DMP and syringaldazine, respectively (Table 1).

Despite high similarity to other two-domain laccases (Ssl1 shares 84% identity with SLAC, 88% with SilA, and 74% with EpoA), the catalytic features of these four two-domain laccases differ substantially. It is notable that EpoA could not oxidize 2,6-DMP, guaiacol and syringaldazine. SLAC and SilA accept these substrates but show catalytic constants that differ from the constants measured for Ssl1. For 2,6-DMP oxidation by Ssl1 the $K_m$ was lower by a factor of 5 and $k_{cat}$ by a factor of 14 compared to SilA and SLAC. Further, SilA and SLAC show shifted pH optima (see Table 1). In a future comparative study, ideally on structural level, the minor sequence variations in these four enzymes would help to understand the role that the differing residues play in substrate binding and oxidation.

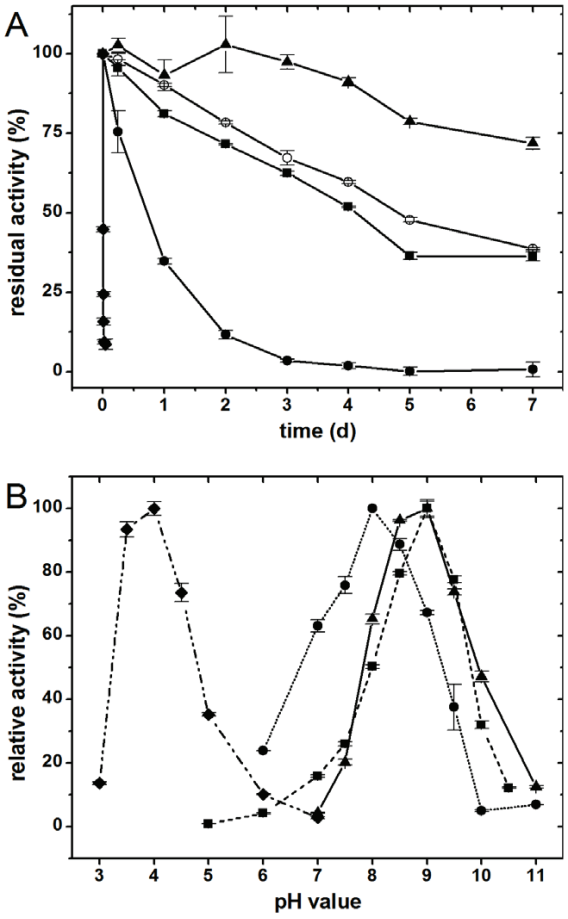


**Figure 4. pH optima and pH stability of Ssl1.** A: Stability of Ssl1 in buffers with different pH values was tested as residual oxidation activity towards 2,6-dimethoxy phenol. Ssl1 was inactivated within 30 min at pH 3 (values shown as diamonds), and within 3 days at pH 4 (circles). Stability at pH 5 (open circles) and pH 7 (squares) was similar with a half-time around 4 to 5 days. At pH 11 (triangles) Ssl1 was most stable with more than 70% residual activity after 7 days. B: Relative activities of Ssl1 at different pH values towards the substrates ABTS (values shown as diamonds), syringaldazine (circles), 2,6-dimethoxy phenol (triangles) and guaiacol (squares). All activities were normalized to the values at optimum pH with the respective substrate. Optimal pH values are 4 for ABTS, 8 for syringaldazine, and 9 for 2,6-dimethoxy phenol and guaiacol.
doi:10.1371/journal.pone.0052360.g004

## Stability of Ssl1

Ssl1 showed remarkable stability with regard to pH, temperature and presence of organic solvents. In a broad pH range from 5 to 10, Ssl1 retained 40 to 60% residual activity after 5 days of incubation (Fig. 4). At more acidic conditions it was less stable, at pH 4 Ssl1 lost 65% activity within 1 day and at pH 3 Ssl1 was almost completely inactivated within 30 min. Interestingly, the highest stability was observed at pH 11 with approximately 80% residual activity after 5 days incubation. Stability at high pH values can be explained by the fact that inhibition of the trinuclear cluster by hydroxide ions reduces auto-oxidation of laccase and thereby stabilizes the enzyme [30].

Ssl1 also showed moderate stability at elevated temperatures, the half-times of residual activities were 226±12 min at 50°C, 88±9 min at 60°C, 29±4 min at 70°C and 10±0.4 min at 80°C. This thermal robustness was utilized in the purification process by heat precipitation of most *E. coli* host proteins, while Ssl1 activity remained unaltered. Thermal stability is not only beneficial for this simple and efficient purification step, but is also an ideal prerequisite for directed evolution experiments since stable enzymes can tolerate more destabilizing mutations and thereby allow screening within a larger mutational space as was shown e.g. for P450 monooxygenases [31]. Moreover, thermal stability is usually considered as beneficial for industrial processes since it is often connected to operational stability of the enzyme which allows higher reaction temperature, longer process duration, and in general a more flexible process management.

Further, stability of Ssl1 in the presence of several organic solvents was examined. Presence of a second phase of the water immiscible organic solvents *n*-hexane and isooctane did not alter either activity or stability of the enzyme (Fig. 5). With 40% water miscible solvents like DMSO, methanol, ethanol, 2-propanol, acetonitrile or acetone in the reaction system, the activity dropped to 20 to 40%. However, the stability of Ssl1 remained unchanged with most solvents and about 75% residual activity were detected after 20 h. Ssl1 was destabilized by acetonitrile (53% residual activity), whereas DMSO acted as stabilizer and adaption over 20 h even increased measured activities to 131%. Addition of 50 mM sodium dodecyl sulfate or 1% Triton-X-100 lead to a reduction of activity to 62 and 79% but showed no effect on Ssl1 stability. Addition of 10 mM sodium azide, a well-known laccase inhibitor [32], led to a slight decrease of activity by 5%, whereas many laccases are completely inhibited by concentrations in the micromolar range [33,34]. The relative stability of Ssl1 with organic co-solvents and other chemicals allows use of the enzyme in a wide variety of reaction compositions. This is particularly useful since many described laccase substrates, like polyaromatic hydrocarbons or phenylpropanoids, are poorly soluble in water and use of an additional organic phase as substrate reservoir in the

**Table 1.**

| laccase | ABTS | | | 2,6-dimethoxy phenol | | | syringaldazine | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH optimum | $K_m$ ($mM^{-1}$) | $k_{cat}$ ($min^{-1}$) | pH optimum | $K_m$ ($mM^{-1}$) | $k_{cat}$ ($min^{-1}$) | pH optimum | $K_m$ ($\mu M^{-1}$) | $k_{cat}$ ($min^{-1}$) |
| Ssl1 | 4.0 | 0.36 | 443 | 9.0 | 0.89 | 19.3 | 8.0 | 15.8 | 3.47 |
| EpoA[16] | n.a. | n.a. | n.a. | not oxidized | | | not oxidized | | |
| SilA[25] | 5.0 | 0.40 | 599 | 8.0 | 4.27 | 252 | 8 | n.a. | n.a. |
| SLAC[14,35] | 4.0 | n.a. | n.a. | 9.4 | ~4 | ~270 | n.a. | n.a. | n.a. |

n.a.: not available.
doi:10.1371/journal.pone.0052360.t001

reaction could facilitate the conversion of higher amounts of substrate. Further the robustness of Ssl1 activity enables its use in processes where reaction compositions cannot be entirely controlled. E.g. waste waters will usually contain a diverse mixture of all kinds of chemical compounds that might interfere with enzyme activity. Since Ssl1 tolerated all studied additives to a certain degree, its use in such undefined reaction compositions is feasible. Since Ssl1 showed both, thermal stability and stability in presence of additives, we conclude that it possesses the operational stability required for biocatalytic processes.

## Conclusions

Ssl1 from *S. sviceus* is a small two-domain laccase with unusual properties. It can be easily expressed in *E. coli* and combines stability in a wide pH range, at elevated temperatures and in presence of organic solvents with an alkaline activity profile. This makes Ssl1 a suitable candidate for industrial biocatalysis, especially in processes that cannot be accessed by other laccases due to the requirement of high pH values or organic co-solvents.

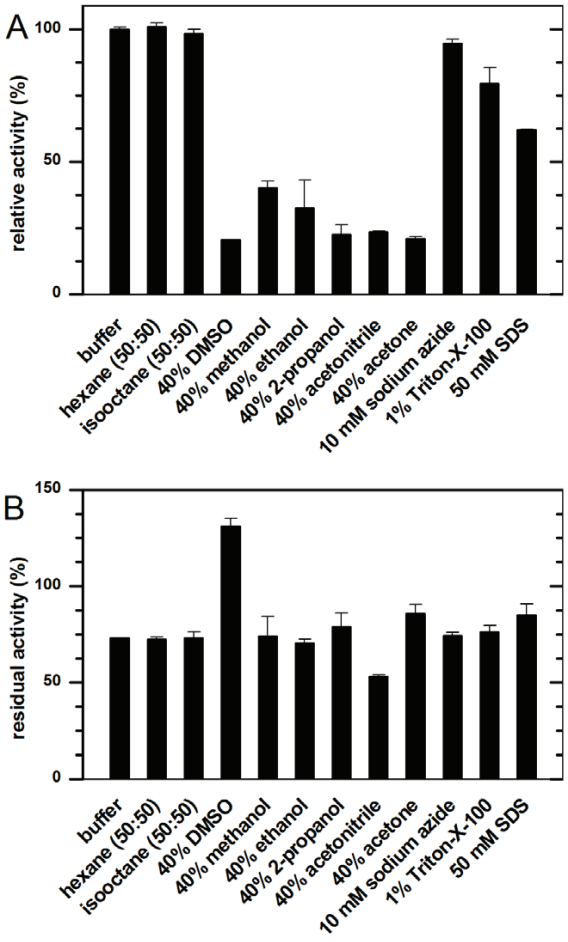


**Figure 5. Effect of detergents, salts and organic co-solvents on Ssl1.** A: Relative activity of Ssl1 in presence of organic solvents, salts and detergents towards 2,6-dimethoxyphenol oxidation. Water immiscible solvents did not affect Ssl1 activity, with water miscible co-solvents the activity dropped to 20–40%. Ssl1 tolerated the addition of 10 mM sodium azide, with the detergents SDS and Triton-X-100 the activity was reduced to 60 or 80%. B: Residual activity of Ssl1 after 20 h incubation with organic solvents, salts and deteregents. DMSO stabilized Ssl1 whereas acetonitrile lead to a reduction in residual activity. All other studied additives did not lead to major changes of Ssl1 stability.
doi:10.1371/journal.pone.0052360.g005

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## Author Contributions

Conceived and designed the experiments: VBU MG. Performed the experiments: MG. Analyzed the data: VBU MG. Contributed reagents/materials/analysis tools: VBU. Wrote the paper: VBU MG.

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## 4.2 Manuscript 2

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|---|---|
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the FEBS Journal

# Structural and redox properties of the small laccase Ssl1 from *Streptomyces sviceus*

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**Keywords**
bacterial laccase; multicopper oxidase; trinuclear cluster; two-domain laccase; type 1 copper center

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Laccases (EC 1.10.3.2) are members of the multicopper oxidase family. They oxidize diverse electron-rich substrates through electron abstraction by the type 1 copper ion in the enzyme active site. Abstracted electrons are transferred to the trinuclear copper cluster, where molecular oxygen serves as final acceptor and is reduced to water. Laccase activity is assumed to depend on the redox potential of its type 1 copper ion. Whereas numerous studies have been undertaken to elucidate the determinants of the redox potential of type 1 copper ions in one-domain cupredoxins and in three-domain laccases, such experimental investigations are lacking for recently described, small, two-domain laccases. In this work, the crystal structure of the small laccase Ssl1 from *Streptomyces sviceus* was solved, and the positions that might influence the redox potential of Ssl1 were depicted. On the basis of this knowledge, several Ssl1 variants were constructed with an increase in redox potential of 16–81 mV, from 375 mV to 391–456 mV. Mutation of residues in close proximity to the type 1 copper center resulted in a predicted increase in the redox potential of the copper center; however, there was a reduced specific activity for the oxidation of 2,6-dimethoxyphenol, which has a relatively low redox potential. Mutations more distant to the type 1 copper also led to an increased redox potential of the copper center, and resulted in variants able to oxidize the high redox potential substrates 1,2-dihydroxyanthraquinone-3-sulfonic acid (Alizarin Red S) and indigo carmine more efficiently than wild-type Ssl1.

**Database**
The atomic coordinates of the structure of Ssl1 laccase from *Streptomyces sviceus* and structure factors have been deposited in the RCSB Protein Data Bank (4M3H)

**Structured digital abstract**
• Ssl1 and Ssl1 bind by x-ray crystallography (View interaction)

## Introduction

Laccases (EC 1.10.3.2) are oxidoreductases that are capable of oxidizing diverse electron-rich substrates, such as phenols, aryl diamines, benzenethiols, hydroxyindoles, and inorganic metal ions [1]. They are involved in diverse biological processes, e.g. the lignification of plant cell walls, heavy metal homeostasis, spore morphology, and lignin degradation [2]. Laccases belong to the group of blue multicopper oxidases, and

**Abbreviations**
2,6-DMP, 2,6-dimethoxyphenol; CV, column volume; ESRF, European Synchrotron Radiation Facility; PDB, Protein Data Bank; T1Cu, type 1 copper; T2Cu, type 2 copper; T3Cu, type 3 copper; TNC, trinuclear cluster.

contain four canonical copper ions that are organized in three copper sites. The copper ions are coordinated by one cysteine and 10 histidines. The ligand-to-metal charge-transfer complex of cysteine sulfur with copper (II) at the type 1 copper (T1Cu) site is responsible for the absorbance around 600 nm and the deep blue color of laccases [3]. In course of the reaction, four substrate molecules are oxidized by one-electron abstraction at the T1Cu site. Electrons are then shuttled via a cysteine–histidine bridge to the trinuclear cluster (TNC), where one type 2 copper (T2Cu) site and one type 3 copper (T3Cu) site with a pair of copper ions are involved in the four-electron reduction of molecular oxygen to water.

Laccases occur in fungi, plants, bacteria, archaea, and insects [4,5], and differ greatly in the redox potential of T1Cu. For laccases from plants and bacteria, this is usually ~ 0.4 V, whereas for high-potential fungal laccases, it can be up to 0.8 V [6]. The catalytic activity of laccases is believed to be associated with the redox potential difference between the substrate and T1Cu [7,8]. For example, the degradation efficiency of azo dyes with a laccase–mediator system, *Trametes villosa* laccase–1-hydroxybenzotriazole, can be predicted by the redox potential difference between the T1Cu and the dyes [9]. Consequently, in most cases, high-potential fungal laccases possess higher specific activities and accept more substrates than low-potential laccases from plants and bacteria [10]. Apart from the redox potential difference between laccase and substrate, other factors seem to play a role in substrate oxidation by laccases [11]. For instance, reshaping of the substrate-binding site in SLAC created space for substrate molecules to access T1Cu, and increased its catalytic efficiency [12].

Laccases usually consist of three cupredoxin-like domains, with the T1Cu site in domain 3 and the TNC at the interface of domains 1 and 3 [13]. Domain 2 is involved in formation of the substrate-binding pocket [14], and assists in the correct positioning of domains 1 and 3, which facilitates formation of the TNC. A few years ago, a novel laccase family of small, two-domain laccases was discovered [15]. In two-domain laccases, domain 2 of their three-domain counterparts is lacking. This leads to a substantially different architecture, as shown for SLAC from *Streptomyces coelicolor* [16]. In order to build a functional TNC, small laccases oligomerize as homotrimers [15,17,18] with the TNC located at the interface of adjacent monomers [16].

The structural basis of differences in T1Cu redox potentials among laccases has been investigated in several studies with three-domain laccases [19–21]. However, experimental investigations of determinants of the redox potential of small two-domain laccases are still lacking. The available data are restricted to a theoretical study in which the redox potential of the small laccase SLAC from *S. coelicolor* was modeled with a combination of first-principles density functional theory and empirical molecular dynamics simulations [22].

In this study, we aimed at understanding the molecular basis of T1Cu redox potentials in small, two-domain laccases. Ssl1 from *Streptomyces sviceus*, which has been recently cloned and characterized by our group, was chosen as a model laccase [17]. The solved 3D structure of Ssl1 in combination with site-directed mutagenesis provided insights into determinants of the redox potential of small laccases.

## Results

### Global structure of Ssl1

In order to reveal factors contributing to the T1Cu redox potential of the small bacterial laccase Ssl1 from *S. sviceus*, the enzyme was crystallized, and its structure was solved by molecular replacement with SLAC from *S. coelicolor* as the starting model [Protein Data Bank (PDB) ID 3CG8]. Ssl1 formed rod-shaped, blue crystals within 7–14 days. Collected X-ray data allowed solution of the crystal structure of Ssl1 with a resolution of 2.2 Å (PDB ID: 4M3H). Crystals of Ssl1 have a good stereochemistry, as shown in Table 1. The asymmetric unit contains a homotrimer. The trimeric organization is in accordance with previous multiangle scattering light experiments with soluble Ssl1 [17]. Residues 1–41 (for numbering, see Fig. 1) from the N-terminus and residues 312–328 of the C-terminus could not be determined in the electron density. At the N-terminus, the unresolved part accounts for the hexahistidine tag, two residues of the enzyme core, and the twin-arginine translocation signal that was removed during the cloning process. Each Ssl1 molecule consists of two cupredoxin-like domains that comprise a β-sandwich fold with two aligned β-sheets in each domain. Domain 2 of each chain is oriented towards domain 1 of the neighboring chain (Fig. 2).

As in other two-domain multicopper oxidases [16,23], the TNC is positioned at the interface of domain 2 and domain 1 of neighboring Ssl1 molecules. As a consequence, oligomerization is mandatory for formation of the TNC and a prerequisite for enzymatic activity. The T1Cu ion is located ~ 4 Å beneath the surface of domain 2, and, together with the TNC,

**Table 1.** Summary of crystallographic data collection and atomic model refinement statistics for Ssl1 from *S. sviceus*. Data in parentheses represent values for the highest-resolution shell.

| | |
|---|---|
| Crystal parameters at 100 K (ESRF, ID23-1) | |
| Space group | $P2_12_12_1$ |
| Unit cell parameters | |
| *a*, *b*, *c* (Å) | 51.54, 103.82, 162.26 |
| Data collection and processing | |
| Wavelength (Å) | 0.9144 |
| Resolution (Å) | 47.97–2.2 (2.3–2.2) |
| Mean redundancy | 4.4 (4.1) |
| Unique reflections | 49 366 |
| Completeness (%) | 99.47 (98.7) |
| $I/\sigma$ | 17.3 (3.3) |
| $R_{sym}$ (%) | 6.4 (42.5) |
| $R_{meas}$ (%) | 6.9 (41.7) |
| CC(1/2) (%) | 99.9 (93.6) |
| Refinement statistics | |
| $R_{work}$ (%) | 20.6 |
| $R_{free}$ (%) | 26.8 |
| rmsd from ideal | |
| Bond lengths (Å) | 0.020 |
| Bond angles (°) | 1.837 |
| Average *B*-factors (Å$^2$) | 29.9 |
| Ramachandran plot | |
| Most favored (%) | 97.0 |
| Allowed (%) | 2.7 |
| Generously allowed (%) | 0.2 |
| Disallowed (%) | – |
| Model content | |
| Monomers/ASU | 3 |
| Protein residues | 43–311, 43–311, 42–311 |
| No. of atoms | 6419 |
| Ligand | Cu, O |
| No. of water molecules | 129 |

domain 2 and domain 1 of neighboring molecules, forms catalytic units facilitating intramolecular electron transfer from T1Cu to T2Cu/T3Cu, spanning a distance of ~ 13 Å. Thus, considering that one T1Cu is located in domain 2 and that the TNC is built at monomer interfaces within a homotrimer, Ssl1 belongs to the family of Nakamura-type B two-domain multicopper oxidases [24].

The trimeric organization of Ssl1 results in the formation of a cavity along the central axis of the homotrimer (Fig. 3). The cavity is covered with hydrophilic residues (Asp239, Asp249, Asp250, Ser252, Gln253, Lys258, and Asp264). Two hydrophilic residues, Ser265 and Asp256, connect the central cavity with the TNC and coordinate a water molecule. As well as this connection to the central cavity, the structure of Ssl1 contains two branched channels that lead from the TNC to the trimer surface.

## Copper coordination sites

The Ssl1 homotrimer contains the four canonical copper coordination sites of multicopper oxidases in triplicate. T1Cu is bound by two histidines (with $N\delta_{his}$ atoms) and one cysteine in trigonal planar geometry (Fig. 2B). The axial position is occupied by methionine (Met295 at a distance of 3.4 Å from T1Cu), as found in all described bacterial laccases. The axial position is known to be one of the key determinants of the redox potential of bacterial laccases [25].

The trinuclear copper cluster with a T2Cu site and a binuclear T3Cu site is located at a distance of ~ 13 Å and is connected to the T1Cu site with the canonical cysteine–histidine bridge of multicopper oxidases. Both copper ions of the T3Cu site are coordinated by three histidines, and the T2Cu ion is coordinated by two histidines, all by Nε atoms (Fig. 2B). The distance between the T3Cu pair is ~ 5 Å. Two of the three trinuclear sites are fully occupied by three copper ions, and one oxygen molecule is bound within the cluster. In the third trinuclear site of the homotrimer, T2Cu is depleted, and no oxygen is bound at the TNC. A tyrosine is located at a distance of ~ 7 Å from the TNC (Fig. 2B). In SLAC from *S. coelicolor*, an analogous tyrosine (at a shorter distance of 5 Å) was shown to

*MGALDRRGFN RRVLLGGAAV ATSLSLAPEA RSDAGPAQA*A PGGEVRRIKL YAERLADGQM 60
GYGLEKGRAT IPGPLIELNE GDTLHIEFEN TMDVRASLHV HGLDYEVSSD GTTLNKSDVE 120
PGGTRTYTWR THAPGRRSDG TWRAGSAGYW HYHDHVVGTE HGTGGIRKGL YGPVIVRRKG 180
DVLPDATHTI VFNDMLINNR PAHSGPNFEA TVGDRVEFVM ITHGEYYHTF HMHGHRWADN 240
RTGMLTGPDD PSQVVDNKIV GPADSFGFQV IAGEGVGAGA WMYHCHVQSH SDMGMVGLFL 300
VKKTDGTIPG YEPHEHSGQR AEHHHSTP 328

**Fig. 1.** Sequence of wild-type Ssl1 with the numbering as used in the text. The signal sequence (italic) was replaced by a $His_6$ tag (MHHHHHH); mutated positions are indicated by underlines.

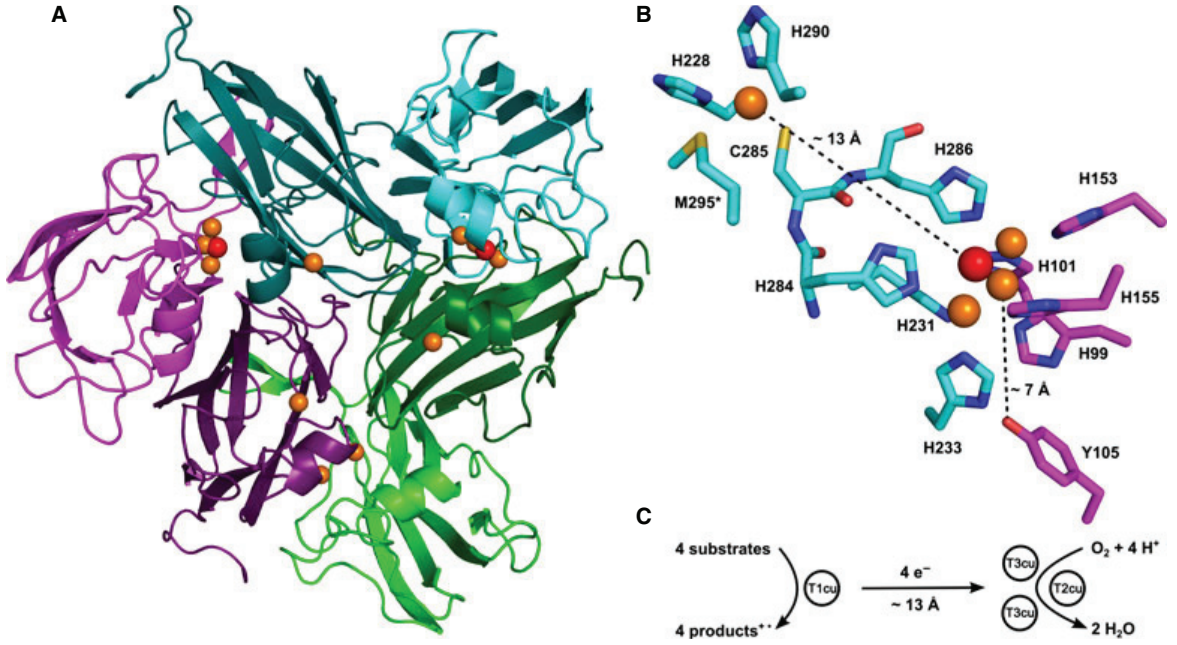


**Fig. 2.** (A) Cartoon representation of the homotrimeric structure of Ssl1. Ssl1 monomers are indicated by different colors (green, blue, and magenta), and domain 2 of each monomer is colored in darker shade. Copper ions (orange spheres) of the TNC are located at the interface of neighboring Ssl1 monomers. Intact TNCs contain oxygen of a water or hydroxyl moiety (red spheres). (B) Detail of Ssl1 copper sites. Copper ions (orange spheres) are coordinated by the canonical histidines and cysteines. The axial position of T1Cu, which is a major determinant of the redox potential of laccases, is occupied by methionine (M295*; substituted in this study). Intramolecular electron transfer occurs via a cysteine–histidine bridge spanning ~ 13 Å from T1Cu to the TNC. Copper ions of the TCN are coordinated by eight histidines from neighboring monomers (shown in cyan and magenta), and bind oxygen (red sphere). A tyrosine residue is located at a distance of ~ 7 Å from the TNC. (C) Scheme of the electron transfer path in laccases. Four substrate molecules are oxidized by electron abstraction at T1Cu to the corresponding radicals. The electrons are transferred to the TNC with a T3Cu pair and a T2Cu, where molecular oxygen is reduced by four electrons to yield two molecules of water.

participate in oxygen reduction at the trinuclear site by providing one electron under substrate-limiting conditions [26].

### Putative substrate-binding site

Apart from the coordinating axial methionine ligand at the T1Cu site, two additional methionines (Met195 and Met293) are located near the T1Cu and close to the enzyme surface that participate in forming two opposing ridges of the putative substrate-binding site (Fig. 4A). These methionines are not present in large, three-domain laccases. Met293 is widely exposed to the solvent. Other residues involved in shaping the putative substrate binding site are Ser289, the T1Cu-coordinating residues Met295 and His290, and, at the opposite site of the substrate-binding cleft, Leu196 and Ala202. In this way, a bilateral substrate-binding pocket is formed by hydrophilic residues (Ser289 and His290) on one side and by hydrophobic residues (Leu196 and Ala202) on the other side.

### Environment of the T1Cu site

The interior of the β-sandwich of domain 2 is in proximity to the T1Cu and contains mainly hydrophobic residues such as phenylalanine and tryptophan, as would be expected for the core region of a soluble protein (Fig. 4B). An exception is Thr222 at a distance of 8 Å from T1Cu. The hydroxide group of Thr222 points towards the sulfur atom of the neighboring Met220. The presence of Thr222 might reduce the hydrophobic character of the environment of the T1Cu, and might induce a dipole momentum in the core region.

### Positions contributing to redox potential

The structural data were used to identify amino acid positions that might contribute to the redox potential of Ssl1. Corresponding residues were selected for substitution to test whether the assumed effects are valid.

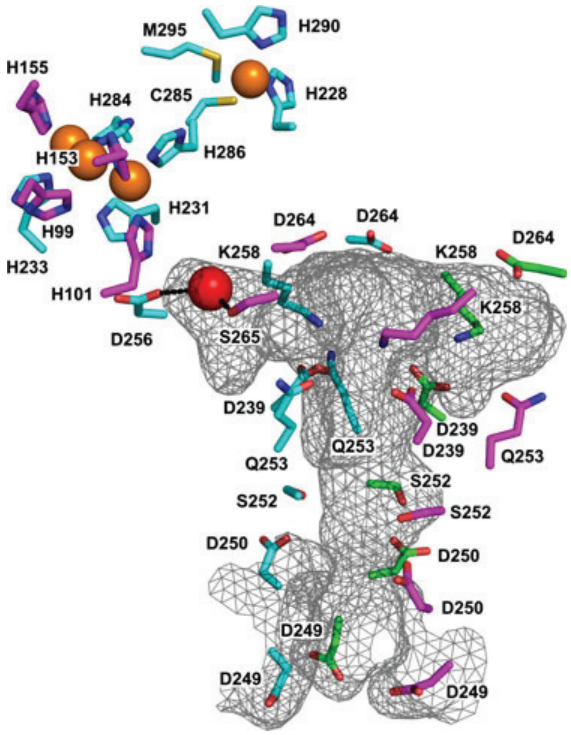


**Fig. 3.** Central cavity of the Ssl1 trimer (shown as mesh). Residues from the same monomer are colored the same (green, magenta, and blue). Copper ions are depicted as orange spheres; for clarity, only one TNC is shown. The cavity is covered by hydrophilic residues. Glu256 and Ser265 bind water (red sphere) near the TNC.

The major impact of the residue in the axial position of T1Cu has been well investigated in large monomeric laccases. In fungal laccases, which contain hydrophobic residues such as phenylalanine or leucine, at that position, redox potentials of up to 0.8 V have been observed [27]. Bacterial laccases with methionine in the axial position usually have lower potentials of approximately 0.4–0.5 mV [20,28,29]. The obvious assumption that a more hydrophobic axial residue than methionine would raise the redox potential of Ssl1 was tested by substitution with a noncoordinating leucine in the M295L Ssl1variant.

Another factor that is thought to influence the redox potential of T1Cu is the desolvation (or hydrophobic) effect [25]. A more hydrophobic environment of copper ions leads to relative destabilization of the oxidized $Cu^{2+}$ state over the reduced $Cu^{1+}$ state, and thus results in a higher redox potential of the corresponding copper center [30]. The hydrophobic core of domain 2 could participate in the creation of a hydrophobic environment of T1Cu, although the hydrophobicity is

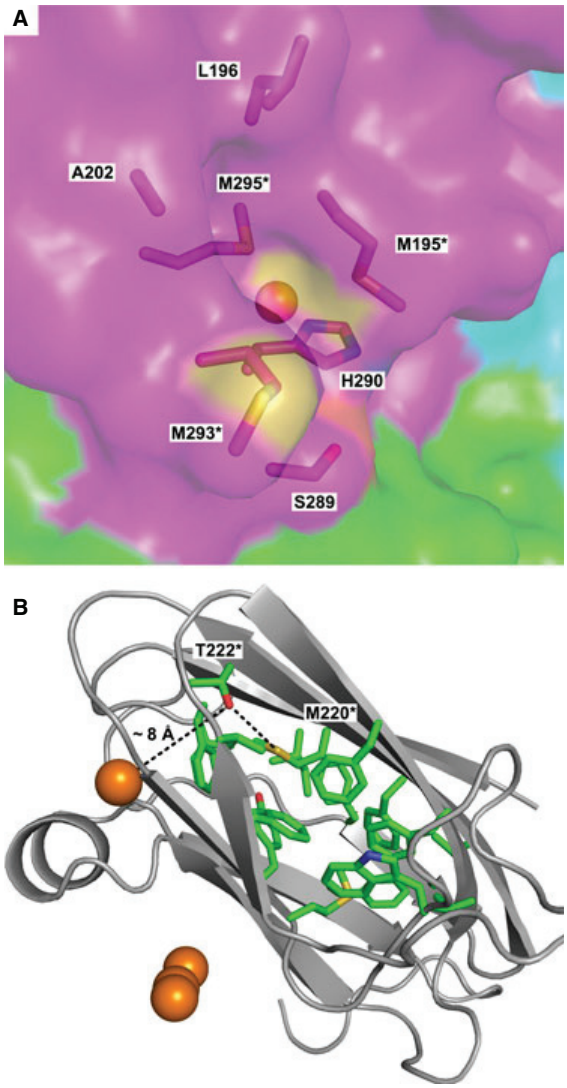


**Fig. 4.** (A) Surface representation of the putative substrate-binding site of Ssl1 (T1Cu is depicted as a sphere; residues that were replaced are indicated by asterisks). (B) Hydrophobic core near the T1Cu site. The interior of the β-sandwich in proximity to T1Cu (upper orange sphere) contains mostly hydrophobic residues, with the exception of Thr222.

limited by the above-mentioned residues Thr222 and Met220. The more hydrophobic leucine was introduced at both positions in the M220L and M220L Ssl1 variants. A higher redox potential in these variants would indicate that the hydrophobic core has an influence on the redox potential by increasing the hydrophobicity of the T1Cu environment.

We also tested the possible contribution of both methionines of the putative substrate-binding site (Fig. 4A) to the redox potential. Replacement of the

two methionines with leucine in the M195L and M293L Ssl1 variants should also produce a more hydrophobic environment, and, as a consequence, should increase the redox potential of T1Cu.

The C-terminus of Ssl1 could not be resolved in the structure, so its potential influence on the redox potential could not be rationalized. To test for a potential contribution of the C-terminus to the redox potential, we created the variant Ssl1 ΔC-terminus, which has a truncation of the 17 unresolved residues.

### Redox potential of Ssl1 variants

The redox potential of T1Cu in wild-type Ssl1 and the variants was determined by spectrophotometric redox titration with the potassium hexacyanoferrate(II/III) redox couple. Wild-type Ssl1 showed a midpoint potential of 375 ± 8 mV, which is in the typical range of bacterial laccases (Table 2). All constructed variants showed higher redox potentials than wild-type Ssl1. The largest increase, of 81 ± 10 mV (from 375 to 456 mV), was observed with substitution of the axial methionine of the T1Cu in Ssl1 M295L. In this variant, T1Cu is coordinated by only three ligands. Obviously, this leads to a relative destabilization of the oxidized state, as observed previously in other multicopper oxidases [20], and confirms the major role of the axial ligand of T1Cu in the redox potential of laccases. The replacement of methionines in the putative substrate-binding side with leucines in Ssl1 M195L and Ssl1 M293L increased the redox potential from 375 ± 8 mV to 403 ± 10 mV and 398 ± 11 mV, respectively. Higher hydrophobicity in the core of domain 2 in the M220L and M220L/T222L variants increased the redox potential to 391 ± 1 mV and 408 ± 3 mV, respectively. Deletion of the Ssl1 C-terminus resulted in an increase in the redox potential to 403 ± 11 mV.

**Table 2.** Redox potentials of wild-type Ssl1 and variants, and their catalytic constants for 2,6-DMP.

| Variant | Redox potential (mV) | $K_m$ (mM) | $k_{cat}$ ($min^{-1}$) |
|---|---|---|---|
| Wild-type Ssl1 | 375 ± 8 | 0.89[a] | 19.3[a] |
| Ssl1 ΔC-terminus[b] | 403 ± 11 | 0.95 ± 0.06 | 4.8 ± 0.2 |
| Ssl1 M195L | 404 ± 10 | 1.27 ± 0.08 | 4.2 ± 0.2 |
| Ssl1 M220L | 391 ± 1 | 0.74 ± 0.02 | 1.9 ± 0.1 |
| Ssl1 M220L/T222L | 408 ± 3 | 0.85 ± 0.01 | 3.8 ± 0.1 |
| Ssl1 M293L | 398 ± 11 | 1.23 ± 0.04 | 3.2 ± 0.3 |
| Ssl1 M295L | 456 ± 6 | 0.71 ± 0.01 | 1.4 ± 0.1 |

[a] Kinetic data of wild-type Ssl1 are from [17].
[b] The 17 C-terminal residues (312–328) were deleted in the Ssl1 ΔC-terminus.

### Activity of the Ssl1 variants

The oxidation activity of Ssl1 variants was tested with the common laccase substrate 2,6-dimethoxyphenol (2,6-DMP). All Ssl1 variants were active, but, despite their higher redox potentials, $k_{cat}$ values in this reaction decreased by factors of 4–14 as compared with the wild-type enzyme (Table 2). Actually, Ssl1 M295L, the variant with the largest redox potential increase (81 mV), showed the lowest rate constant, with a decline from 19.3 $min^{-1}$ in the wild-type to 1.4 $min^{-1}$. Substitutions in the putative substrate-binding site resulted in decreases to 3.2 $min^{-1}$ for the widely exposed Met293 and to 4.2 $min^{-1}$ for the less exposed Met195. Deletion of the C-terminus reduced the rate constant to 4.8 $min^{-1}$, and substitutions in the hydrophobic core led to rate constants of 1.9 $min^{-1}$ in Ssl1 M220L and 3.8 $min^{-1}$ in Ssl1 M220L/T222L. Obviously, there was no simple correlation between Ssl1 redox potential and the rate constant with 2,6-DMP.

The $K_m$ values for 2,6-DMP did not change substantially with substitutions in the core region and with deletion of the C-terminus of Ssl1 (Table 2). However, replacement of methionines in the putative substrate-binding site with leucines resulted in 40% higher $K_m$ values: 1.27 and 1.23 mM in Ssl1 M195L and M293L, as compared with 0.89 mM in wild-type Ssl1. This may reflect a lower affinity of the enzyme for the substrate in a more hydrophobic active site.

As compared with other laccase substrates, 2,6-DMP has a relatively low redox potential ($E'_0$ = 0.58 mV for the redox couple 2,6-dimethoxyphenoxyl radical/2,6-DMP [31]). For all six Ssl1 variants, the oxidative activities against 1,2-dihydroxyanthraquinone-3-sulfonic acid (also known as Alizarin Red S), which has a higher redox potential ($E_0$ = 0.79 mV for the redox couple oxidation product/quinone [32]) and against the indigo dye indigo carmine were tested. Wild-type Ssl1 showed conversion rates of 31% with indigo carmine (after 24 h) and 45% with Alizarin Red S (after 4 h) (Fig. 5). The variants Ssl1 ΔC-terminus and Ssl1 M220L/T222L showed higher conversion rates of 49% and 57% with indigo carmine and of 56% and 63% with Alizarin Red S, respectively. These are variants with substitutions located rather distant to the T1Cu. Substitutions in close proximity to the T1Cu, as in Ssl1 M195L, M293L, and M295L, did not prove to be beneficial for the conversion of either dye.

## Discussion

In the current study, we sought to elucidate factors that contribute to the redox potential of the small, two-domain laccase Ssl1 from *S. sviceus*. Therefore,

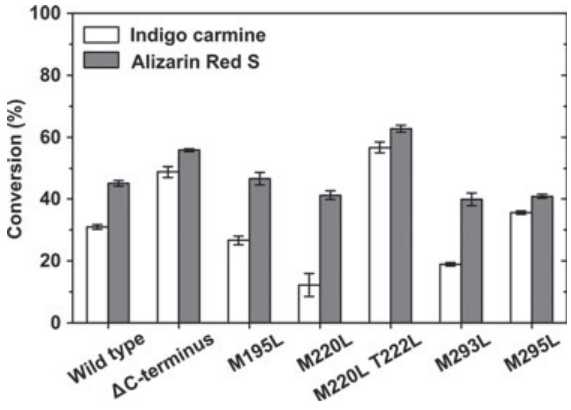


**Fig. 5.** Conversion of indigo carmine (white columns) within 24 h and of Alizarin Red S (gray columns) within 4 h by wild-type Ssl1 and variants.

the structure of Ssl1 was solved, positions that might have an effect on the redox potential were identified, and residues at the corresponding positions were substituted.

Similarly to other two-domain multicopper oxidases described up to now [16,18,23,33], Ssl1 was found to form homotrimers. The resulting central cavity is covered with hydrophilic residues. Two of them coordinate a water molecule in close proximity to the TNC. This indicates that the central cavity might serve as reservoir for oxygen supply and water removal after reduction thereof. It is noteworthy that both water-coordinating residues, Asp256 and Ser265, originate from neighboring Ssl1 molecules, which underlines the close and complex functional connection within the Ssl1 homotrimer. Oxygen and water might also be supplied by the two solvent channels that are formed at the interface of two neighboring monomers and that connect the TNC with the enzyme surface. The same solvent channel arrangement has been reported for the two-domain multicopper oxidase from *Nitrosomonas europaea* [33], but not for SLAC, which contains exclusively the channel leading from the TNC to the central cavity [16].

At the N-terminus, the hexahistidine tag and the two subsequent residues Ala40 and Gly41 were unresolved. The tag was introduced at the position where the native Ssl1 sequence contains a signal sequence of the twin-arginine translocation pathway. The small residues Ala40 and Gly41 might act as a flexible linker between signal sequence and enzyme. The consequent flexibility could have prevented the formation of a defined crystal structure, frustrating the structural characterization of the N-terminal residues.

A major impact of the axial ligand on the redox potential of T1Cu has been described frequently for large laccases [19,25,34]. Studies on one-domain cupredoxins that contain a single T1Cu site, such as azurin, stellacyanine, and plastocyanine, have revealed further factors that might influence the redox potential of T1Cu-containing enzymes. Among them are the degree of exposure of T1Cu to solvent, the rigidity of the copper coordination geometry provided by the protein backbone, hydrogen bridges with the conserved cysteines of T1Cu sites, and intraprotein electrostatic interactions [25,34,35]. The major impact of the axial ligand on the redox potential of T1Cu could be confirmed for Ssl1. The replacement of the axial methionine ligand with leucine in Ssl1 M295L resulted in a redox potential increase of $81 \pm 10$ mV, which was the highest increase observed in this study. A redox potential increase was also observed in variants with more hydrophobic environments of T1Cu (as compared with the wild-type). The replacement of methionines with more hydrophobic leucines in the putative substrate-binding site in Ssl1 M195L and M293L, as well as the introduction of leucine instead of threonine and methionine in Ssl1 M220L and M220L/T222L, resulted in redox potential increases of ~ 20 mV. In conclusion, the creation of a more hydrophobic environment of T1Cu seems to be a successful strategy to increase the redox potential of Ssl1.

Truncation at the C-terminus by 17 residues increased the redox potential of T1Cu by ~ 20 mV. Although this part of the molecule was not resolved in the crystal structure, the fact that it was capable of generating such an effect suggests that it could be located near T1Cu. Taking into account the positions of resolved residues from the C-terminus, location of the unresolved part of the C-terminus close to T1Cu is plausible (Fig. 6). However, the C-terminus seems not to be involved in substrate binding, as C-terminal deletion did not alter the $K_m$ for 2,6-DMP. In crystals of SLAC from *S. coelicolor* soaked with potassium hexacyanoferrate(II), iron ions were bound at the interface of neighboring trimers, which is not far from the last observed residues of the C-terminus [16]. The redox titration experiments with Ssl1 were performed with hexacyanoferrate, and thus an effect of this on the redox potential (via interaction with the C-terminus) cannot be completely excluded.

Replacement of Met195 and Met293 with leucines in the putative substrate-binding site resulted in an increased $K_m$. This indicates that both residues may be involved in substrate binding. This is supported by recent work with the homologous SLAC laccase from *S. coelicolor*. In SLAC, replacement of the corresponding

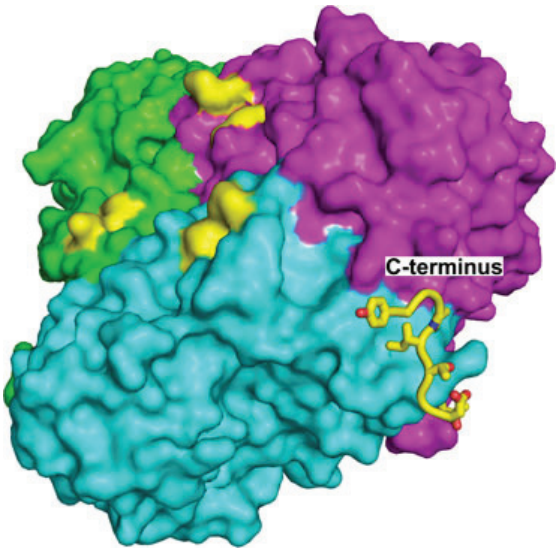


**Fig. 6.** Positions of resolved residues at the C-terminus. Residues 304–311 are represented as yellow sticks. Putative substrate-binding sites are depicted in yellow.

methionines with smaller residues (glycine or alanine) led to higher rate constants with 2,6-DMP, presumably because of improved access of substrate to the T1Cu [12]. Therefore, in both laccases, Ssl1 and SLAC, methionines in the substrate-binding site seem to contribute considerably to substrate binding. Both methionines and adjacent residues might be used as 'hot spots' in saturation mutagenesis for the creation of Ssl1 variants tailored for optimized oxidation of unnatural substrates by reshaping the substrate-binding pocket.

The activity of laccase has been connected to the difference between the redox potentials of T1Cu and a substrate [8,10]. As the created Ssl1 variants possess higher redox potentials than the wild-type, we expected higher activities as well. However, the variants showed reduced rate constants in the oxidation of 2,6-DMP. With its relatively low redox potential for a laccase substrate, 2,6-DMP is relatively easy to oxidize, which is one of the reasons for its extensive use in enzymatic oxidation assays. Obviously, for such substrates with relatively low redox potentials, the redox potential difference between substrate and T1Cu is not rate-limiting. Moreover, substitutions near the catalytic T1Cu might trigger subtle changes in the intramolecular electron transfer pathway from T1Cu to the TNC. This might, in turn, reduce the electron transfer rate and, simultaneously, the overall oxidation rate of 2,6-DMP. Variants with substitutions close to the T1Cu, i.e. Ssl1 M195L and M293L, showed lower conversion rates for the high redox potential substrate Alizarin Red S and for indigo carmine. This might also be attributable to disturbances in the intramolecular electron transfer. The variants Ssl1 ΔC-terminus and M220L/T222L, with substitutions that are more distant from the T1Cu, showed higher conversion rates with both dyes. More distant substitutions from the T1Cu might cause less severe disturbances, and the beneficial effect of an increased redox potential might predominate.

In conclusion, the redox potential difference between the T1Cu and the substrate can actually be limiting for high redox potential substrates such as anthraquinone and indigo dyes. In that case, an increase in laccase redox potential leads to increased conversion rates, unless the intramolecular electron transfer is hampered substantially. Thus, side effects of mutations on intramolecular electron transfer need to be considered as well. However, apart from the conserved cysteine–histidine bridge, little is known about residues that participate in or support the electron transfer between T1Cu and the the TNC. The developed crystallization conditions for Ssl1 could be used to investigate mutations at positions surrounding the electron transfer pathway. A deeper knowledge of the molecular mechanisms that influence intramolecular electron transfer would contribute to the understanding of the complex catalytic mechanism not only of small laccases but also of multicopper oxidases in general.

## Experimental procedures

### Materials and strains

All reagents were of analytical grade or higher purity, and were purchased from commercial sources. Enzymes for molecular cloning, nucleotide ladders and protein ladders were purchased from Fermentas (St Leon-Rot, Germany). For molecular cloning and plasmid propagation *Escherichia coli* DH5α (Novagen, Darmstadt, Germany) was used as the host, and *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene, Waldbronn, Germany) was used as the expression host.

### Cloning of *ssl1* variants

Variants of the *ssl1* gene (SSEG_02446) were generated by use of the QuikChange protocol (Stratagene). The plasmid pET22ssl1 was used as the template [17]. It contains *ssl1* with an N-terminal hexahistidine tag sequence and without the N-terminal signal peptide sequence. Mutations were introduced with primers harboring the desired codon exchanges (indicated by underlining). Numbering of amino acid positions follows the Ssl1 sequence containing the N-terminal

signal sequence of the twin-arginine translocation pathway (according to EDY5586; Fig. 1). For creation of the variants, the following primer sets were used: M195L, ATC GTC TTC AAC GAC CTG CTC ATC AAC AAC AGG and CCT GTT GTT GAT GAG CAG GTC GTT GAA GAC GAT; M220L, GTC GAG TTC GTG CTG ATC ACG CAC GGC and GCC GTG CGT GAT CAG CAC GAA CTC GAC; M293L, GC CAC TCC GAC CTG GGG ATG GTG GG and CC CAC CAT CCC CAG GTC GGA GTG GC; and M295L, TCC GAC ATG GGG CTG GTG GGC CTG TTC and GAA CAG GCC CAC CAG CCC CAT GTC GGA. Deletion of the 17 C-terminal residues was achieved by introduction of a stop codon with the primers CCG GGG TAC GAG TAG CAC GAG CAC AGC and GCT GTG CTC GTG CTA CTC GTA CCC CGG. The M220L/T222L variant was created with the primer set GTC GAG TTC GTG CTG ATC CTG CAC GGC GAG TAC and GTA CTC GCC GTG CAG GAT CAG CAC GAA CTC GAC, with the template pET22ssl1M220L which was generated previously. The identity of created sequences was confirmed by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

## Expression and purification of Ssl1 variants

Ssl1 and variants thereof were expressed and purified as described previously for the wild-type enzyme [17]. In brief, *E. coli* BL21-CodonPlus(DE3)-RP was transformed with pET22ssl1 (or the corresponding variants) and grown in 200 mL of TB medium (24 $g \cdot L^{-1}$ yeast extract, 12 $g \cdot L^{-1}$ tryptone, 4 $mL \cdot L^{-1}$ glycerol, 17 mm monopotassium phosphate, 72 mM dipotassium phosphate) containing ampicillin (100 $\mu g \cdot mL^{-1}$) and chloramphenicol (34 $\mu g \cdot mL^{-1}$) at 30 °C with stirring at 140 r.p.m. to a $D_{600\ nm}$ of 1.0. Then, expression was induced with 40 μM isopropyl thio-β-D-galactoside supplemented with 2 mM copper(II) sulfate. After 8 h at 25 °C and 140 r.p.m., cells were harvested by centrifugation at 11 000 ***g*** and 4 °C for 20 min. Supernatants were discarded, and cells were resuspended in chilled potassium phosphate buffer (50 mM, pH 7.5) containing 0.3 mM copper(II) sulfate and 100 μM phenylmethanesulfonyl fluoride. Cell disruption was carried out with a Branson SLPe sonifier (three cycles of 90 s in energy pulse mode with 50% amplitude, 10 J, 2 s of off-time, and at least 1 min of chilling on ice between cycles). Cell lysates were cleared by centrifugation at 31 000 ***g*** and 4 °C for 30 min.

Supernatants were subjected to a heat precipitation step (20 min at 65 °C), and precipitated endogenous host proteins were removed by centrifugation at 48 000 ***g*** and 4 °C for 30 min. Ssl1 variants were further purified by immobilized metal affinity chromatography on a Talon column (BD Biosciences, Heidelberg, Germany) with a column volume (CV) of 7 mL. The immobilized metal affinity chromatography column was equilibrated with three CVs of binding buffer (50 mM potassium phosphate, pH 7.5, 500 mM sodium chloride), and loaded with supernatant from the heat precipitation step. The column was washed with five CVs of binding buffer and with five CVs of washing buffer (binding buffer with 5 mM imidazole). Elution was carried out with one CV of elution buffer (binding buffer containing 100 mM imidazole). Eluted fractions containing Ssl1 were concentrated through Vivaspin Turbo 15 columns (molecular mass cut-off of 10 kDa; Sartorius, Göttingen, Germany). For crystallization, Ssl1 solutions were adjusted to a concentration of 10 $mg \cdot mL^{-1}$ with elution buffer. For biochemical and electrochemical characterizations, elution buffer was replaced with potassium phosphate buffer (50 mM, pH 7.5) by use of PD miditrap G-25 columns (GE Healthcare, München, Germany).

## Crystallization

Ssl1 crystals were grown with the sitting-drop vapor diffusion technique. In initial crystallization studies, the pH of the reservoir solution, the ammonium sulfate concentration and the homogeneous protein concentration were optimized. In a second screening with Additive Screens I, II, and III (Hampton Research, Aliso Viejo, CA, USA), an additive for improved crystal quality was identified. Crystals used for data collection were obtained when 1.5 μL of Ssl1 solution (10 $mg \cdot mL^{-1}$ in 50 mM potassium phosphate, pH 7.5, 500 mM sodium chloride, 100 mM imidazole) and 1.5 μL of reservoir solution [50 mM Hepes, pH 7.0, 1–1.3 M ammonium sulfate, 5–20 mM hexamminecobalt(III) chloride] were mixed and equilibrated against 400 μL of reservoir solution at 21 °C. Ssl1 crystals grew within 7–14 days, were rod-shaped with an average size of 120 × 20 × 20 $\mu m^3$, and had a blue appearance. Crystals were cryoprotected by slowly and carefully adding 1 μL of glycerol (100%) to the crystallization drop before freezing the crystals in liquid nitrogen.

## Data collection, structure solution, and refinement

A complete dataset was collected from a single crystal at ID23-1, European Synchrotron Radiation Facility (ESFR, Grenoble, France). Data were processed with XDS [36]. We solved the structure of Ssl1 laccase by the method of molecular replacement with PHASER from the CCP4 package [37], with PDB ID 3CG8 as template. Water molecules were fitted with ARP/WARP [38]. For refinement, we performed iterative cycles of manual modulation with COOT [39], and then molecular refinement with REFMAC [37]. The final models have good stereochemistry as determined with REFMAC and with the validation server of the PDB. Crystal structures were visualized with PYMOL 0.99rc6 (Schrödinger, Mannheim, Germany).

### Redox titrations

Redox titrations were performed under a nitrogen atmosphere. The oxidation state of T1Cu of Ssl1 variants was followed by fading of the blue absorption band at ~ 592 nm upon reduction. Titration solutions contained 50 μM Ssl1 variant, 10 mM Hepes (pH 7.0), and 700 μM to 6.5 mM potassium hexacyanoferrate(III). T1Cu was gradually reduced by the addition of solution containing 100 mM potassium hexacyanidoferrate(II) and 50 μM Ssl1 variant. After each addition, the absorption spectrum from 400 to 700 nm was measured with a Tidas photodiode array spectrometer (J&M Analytik AG, Essingen, Germany). Redox potentials in the cuvette were calculated according to the Nernst equation [$E'_0$ = 0.433 V for the redox couple hexacyanoferrate(III)/hexacyanidoferrate(II)] [40]. Absorption at ~ 592 nm was drawn as a function of the corresponding redox potential, and fitted to Nernst curves with ORIGINPRO 9.0G (Additive, Friedrichsdorf, Germany). The resulting fitting parameters were used to calculate the fraction of oxidized Ssl1 variant.

### Determination of kinetic constants

Reaction mixtures contained 3–9 μM Ssl1 variant, 50 mM glycine/sodium hydroxide buffer (pH 9.0), and 25 μM to 8 mM 2,6-DMP. Reactions were performed at room temperature, and were followed at 468 nm ($\varepsilon$ = 49.6 $mM^{-1} \cdot cm^{-1}$) [41,42] on a SpectraMax Plus384 microplate reader (Molecular Devices, Biberach an der Riss, Germany). Reaction rates were calculated according to the Lambert–Beer law [$v = A/(\varepsilon c)$]. Initial reaction rates were determined in triplicate, and were fitted by nonlinear regression to the hyperbolic equation $v = v_{max}[S]/(K_m + [S])$ in ORIGINPRO 9.0G.

### Conversion of indigo carmine and Alizarin Red S

Decolorization assays were carried out with 10 μM Ssl1 variant in 50 mM potassium phosphate buffer (pH 7.5). Reaction mixtures contained 50 μM indigo carmine or 100 μM Alizarin Red S, and were incubated at 30 °C for 24 h (indigo carmine) or for 4 h (Alizarin Red S). Fading of absorption was recorded at 608 nm for indigo carmine and at 513 nm for Alizarin Red S, and conversions were calculated on the basis of reduction of the absorption, with the starting absorption of both dyes defined as 100%.

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## Author contributions

M. Gunne: conception and design of the study, acquisition, analysis and interpretation of data, and writing of the manuscript. A. Höppner: design of the study, acquisition, analysis and interpretation of data, and writing of the manuscript. P.-L. Hagedoorn: design of the study, analysis and interpretation of data, and revision of the manuscript. V. B. Urlacher: conception of the study, interpretation of data, and writing and revision of the manuscript.

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## 4.3 Manuscript 3

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|---|---|
| Title: | Enhancement of copper content and specific activity of CotA laccase from *Bacillus licheniformis* by coexpression with CopZ copper chaperone in *E. coli* |
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# Enhancement of copper content and specific activity of CotA laccase from *Bacillus licheniformis* by coexpression with CopZ copper chaperone in *E. coli*

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ABSTRACT

Copper depletion of bacterial laccases obtained by heterologous expression in *Escherichia coli* is a common problem in production of these versatile biocatalysts. We demonstrate that coexpression of small soluble copper chaperones can mitigate this problem. The laccase CotA and the copper chaperone CopZ both from *Bacillus licheniformis* were used as model system. The use of the *E. coli* BL21(DE3) strain expressing CopZ and CotA simultaneously from two plasmids resulted in an 20% increase in copper occupancy and in 26% higher specific activity. We conclude that not only intracellular copper ion concentration, but also presence of an appropriate copper chaperone influences copper ion insertion into CotA laccase. Moreover, *E. coli* BL21(DE3) seems to lack such a copper chaperone which can be partially complemented by heterologous expression thereof. The presented system is simple and can routinely be used for improved heterologous production of bacterial laccase in *E. coli*.



Laccases are industrially relevant multicopper oxidases that are used in bleaching of cellulose or textiles, for organic synthesis, in fuel cells and biosensors, and in bioremediation of waste waters (Rodriguez-Couto and Toca-Herrera, 2006). They catalyze the oxidation of phenolic substrates coupled to the reduction of molecular oxygen to water. The catalytic cycle of laccases involves four copper ions that are bound to the enzyme by 8 highly conserved histidines and one cysteine (Fig. 1) (Messerschmidt and Huber, 1990). Bacterial laccases are important alternatives to their well-studied and broadly used fungal relatives. They show high thermal stability and are active under neutral or alkaline conditions while fungal laccases are active in acidic milieu (Baldrian, 2006). Moreover, bacterial laccases are routinely expressed in prokaryotic hosts what is not possible for fungal laccases because of lacking post-translational glycosylation. This facilitates rapid genetic manipulation and selection in directed evolution processes. However, for heterologously produced bacterial laccases an incomplete copper content has been reported upon expression in *Escherichia coli* as it has been demonstrated for CotA from *Bacillus subtilis* (Bento et al., 2005; Durao et al., 2008; Martins et al., 2002), McoA from *Aquifex aeolicus* (Durao et al., 2008), CueO from *E. coli* (Galli et al., 2004), CotA from *B. licheniformis* (Koschorreck et al., 2008), and Ssl1 from *Streptomyces sviceus* (Gunne and Urlacher, 2012).

To face this problem different strategies have been described. Addition of copper ions to culture medium can increase specific activity of laccases but only to a limited extent (Koschorreck et al., 2008). In some cases, reconstitution of copper sites after production of copper depleted apoenzyme increased copper content and/or specific activity (Galli et al., 2004; Miyazaki, 2005). For CotA from *B. subtilis* expression under microaerobic conditions – that is cultivation without agitation and thus with lower oxygen concentration – lead to fully copper-loaded enzyme (Durao et al., 2008). The same study revealed that reconstitution of apoenzyme did not result in fully active laccase and that presence of copper is required for correct folding especially of the trinuclear copper cluster. Thus, copper incorporation during biosynthesis is favorable over incorporation into apoenzyme after expression. For CotA from *B. licheniformis* both strategies, reconstitution with external copper (Koschorreck et al., 2008) and expression in microaerobic conditions, failed to increase the copper content of recombinant laccase. Therefore, we searched for alternatives to produce recombinant CotA with increased copper content and thus higher specific activity.

Copper ions are both highly toxic and indispensable for living organisms. For a long time their toxicity was ascribed to the formation of highly active hydroxide and superoxide through Fenton-type reactions catalyzed by copper ions and the subsequent damage of cellular macromolecules. Recent reports suggest that the toxicity of copper ions is rather due to the displacement of iron ions from iron–sulfur-clusters in Fe–S-enzymes (Macomber and Imlay, 2009). To avoid these harmful processes living organisms contain

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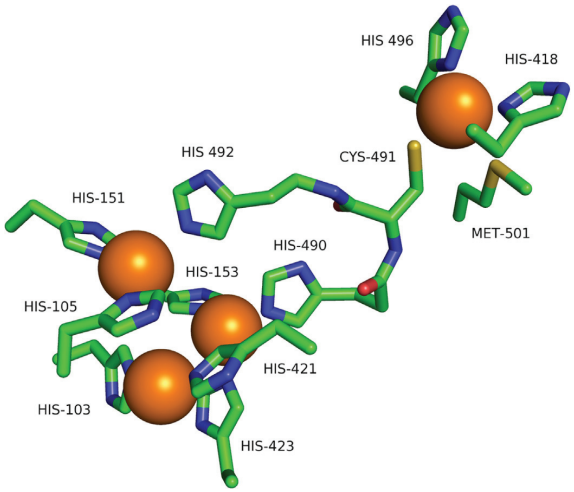


**Fig. 1.** Homology model of copper sites in CotA from *B. licheniformis* (based on PDB 1GSK). Four copper ions (spheres) are located in two sites. At the T1 site substrate molecules are oxidized. The abstracted electrons are transferred to the trinuclear cluster where oxygen is reduced to water.

well-balanced copper homeostasis systems that consist of copper exporters, copper oxidases, metallothioneines, and copper binding chaperones. This limits the number of available copper ions for heterologously expressed enzymes and might be the reason for the observed copper depletion of heterologously expressed bacterial laccases.

To increase the amount of available intracellular copper we created an *E. coli* BL21(DE3) knock-out strain that is deficient in the CueO laccase. CueO functions as copper oxidase in the oxygen dependent copper homeostasis system of *E. coli* (Outten et al., 2001) and deletion of *cueO* lead to intracellular accumulation of copper ions in *E. coli* K12 (Tree et al., 2005). *CueO* knock-out in *E. coli* BL21(DE3) was confirmed functionally (Fig. 2A) and by sequencing. CotA from *B. licheniformis* was expressed in the wild-type and in the CueO deficient strain following the protocol described previously (Koschorreck et al., 2008). The copper content of both enzymes was measured by atomic absorption spectroscopy and specific activity was determined by oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) according to (Koschorreck et al., 2008). We expected that the increased intracellular copper concentration would result in CotA with a higher copper content and thus with higher specific activity. However, the copper content of CotA from the CueO deficient strain was not higher or even decreased by 10–12% and specific activity did not change substantially (Fig. 2, Tab. S2). We concluded that a higher intracellular copper concentration is not sufficient to produce fully copper-loaded CotA. Since intracellular copper ions do not occur as free ions (Rae et al., 1999) but bound to metallothioneines or copper chaperones that deliver copper ions to their target enzymes, we assumed that such a copper chaperone for copper delivery to CotA from *B. licheniformis* is missing in *E. coli* cells. Therefore we aimed at coexpression of an appropriate copper chaperone from *B. licheniformis* simultaneously with CotA to assist in copper incorporation.

In order to find candidate copper chaperones we screened the genome of *B. licheniformis* with sequences of 31 known copper containing proteins (Tab. S1) by applying the tBLASTn algorithm. Thereby 3 putative copper binding proteins were detected, CopZ, YcnJ, and YpmQ (Table 1). YpmQ and YcnJ contain putative transmembrane domains as indicated by the TMHMM prediction tool (http://www.cbs.dtu.dk/services/TMHMM/) and by comparison with characterized homologous proteins (Chillappagari et al., 2009; Mattatall et al., 2000). This might be the reason that YcnJ could not be expressed in *E. coli*. YpmQ could be expressed once the N-terminal membrane-anchor was removed but did not show beneficial effects on copper loading and specific activity of CotA. Therefore we focused on coexpression of CopZ as cytosolic soluble copper chaperone.

The *copZ* gene was inserted into pCOLADuet-1 (Novagen, Germany) that is compatible to pET22cotA and expression was conducted under standard conditions. Coexpression of CotA and CopZ increased the CotA copper content by up to 20% (Fig. 2B). This indicates that CopZ is capable of supporting the copper incorporation into CotA. As a result the specific activity rose by 26% (Fig. 2C). To reduce the number of required plasmids and antibiotics in the expression system, we constructed the bicistronic plasmid pET22copZcotA containing laccase and copper chaperone gene under control of one T7 promoter with two individual ribosome binding sites. Coexpression with the bicistronic construct increased the specific activity of CotA by 16% although the total copper content did not alter. When the bicistronic construct was used for expression in the CueO deficient strain, the copper occupancy of CotA increased by 14% and the specific activity rose by 10% compared to expression with standard construct. This demonstrates again that coexpression of CopZ can increase copper content and specific activity of CotA, although the best results were achieved with expression from two individual plasmids.

Thereby it is counterintuitive that the copper occupancy of CotA is not directly linked to specific activity. Thus specific activity rose by 26% when copper occupancy rose by 20% with two plasmids, but it rose only by 10% when the copper occupancy rose by 14% when the bicistronic construct was used in the CueO deficient strain. According to existing models of the laccase reaction mechanism (Mot and Silaghi-Dumitrescu, 2012) all four copper ions are required for oxidative activity. In reality 5 different states can occur, laccase without copper, with one, two, or three copper centers occupied (all inactive), and fully occupied active laccase. For simplification we can consider 3 different species with copper contents of 0%, 50%, and 100%. Considering an average occupancy of 50% two extreme distributions could occur, (a) all laccase molecules contain two copper ions and are inactive, (b) 50% of laccase molecules contain no copper and 50% contain all four copper ions. In that case 50% of the laccase molecules would be active whereas in case (a) no activity would be observed although in both cases the average copper occupancy is identical. We assume that total copper content is not the key determinant for specific activity, but the fraction of fully copper-loaded CotA. Unfortunately it is experimentally difficult to access distinct occupancies of the three copper clusters and a bundle of different techniques would be necessary like electron spin resonance for T1 and T2 copper, UV-Vis spectroscopy for T1 and T3 copper, resonance Raman spectroscopy for detection of peroxo-bridged T3 coppers, or even X-ray analysis of the enzyme.

The incomplete copper loading after coexpression poses the question why CopZ was not able to raise the copper content to the possible maximum of four copper ions per CotA molecule. Some copper chaperones deliver copper to specific copper centers, i.e. Cox11 in *Rhodobacter sphaeroides* delivers copper ions to the $Cu_B$ but not to the $Cu_A$ center of cytochrome *c* oxidase (Hiser et al., 2000), and YpmQ (or BsSco) from *B. subtilis* was proposed to be involved specifically in the assembly of $Cu_A$ of cytochrome *c* oxidase (Mattatall et al., 2000). As well as Cox11 and BsSco, CopZ might assist in copper insertion into specific copper centers. In that case, non-target copper centers in CotA would remain depleted which would limit the achievable copper content of CotA.

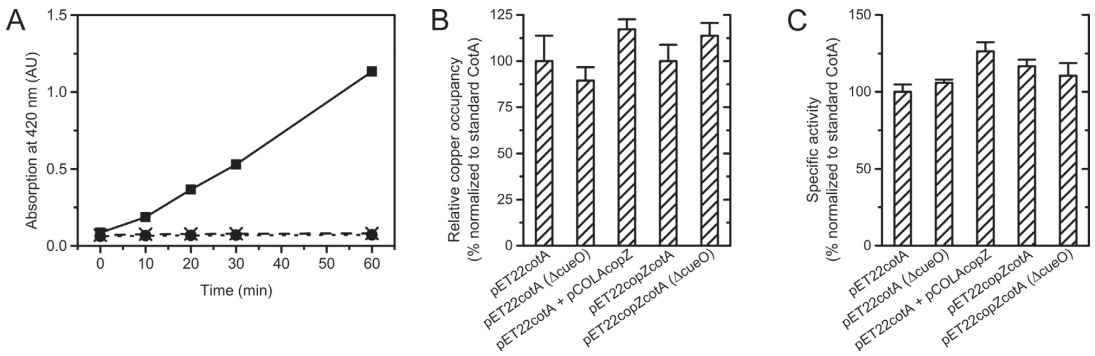


**Fig. 2.** (A) Functional confirmation of *cueO* knock-out in *E. coli* BL21(DE3). The knock-out was introduced by use of TargeTron (Sigma–Aldrich, Germany). Cleared lysate of wild type *E.* coli BL21(DE3) (squares) oxidized 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as followed at 420 nm. Oxidation activity was not detectable in cleared lysates after knock-out of the periplasmic laccase CueO (circles: clone 1; crosses: clone 2). Sequencing confirmed replacement of the *cueO* locus by the kanamycin resistance gene. (B) Relative copper occupancy of CotA. CotA was expressed with standard conditions (pET22cotA), in the *cueO* knock-out strain, or with CopZ coexpressed as indicated. C. Specific activity of CotA expressed from different constructs as indicated in comparison to CotA expressed with standard conditions (pET22cotA).

**Table 1**
Putative copper binding proteins in the genome of *Bacillus licheniformis* DSM13 (GenBank accession number CP000002) detected by tBLASTn searches with known copper binding proteins.

| Putative copper binding protein in *B. licheniformis* | Similarity | Query protein | Function of query protein |
|---|---|---|---|
| CopZ (YP_080648) | 60% | CopZ from *Enterococcus hirae* | Cytosolic copper chaperone for delivery of copper ions to the export pump CopB and to the transcriptional activator of the copper homeostasis system CopY (Solioz et al., 2010) |
| YcnJ (YP_077717) N-terminus | 48% | PcoC from *E. coli* | Periplasmatic copper chaperone for copper delivery to the copper uptake transporter PcoD (Rensing and Grass, 2003) |
| YcnJ (YP_077717) C-terminus | 40% | PcoD from *E. coli* | Copper uptake transporter of the inner membrane (Rensing and Grass, 2003) |
| YpmQ (YP_079474) | 44%/51% | Sco1/Sco2 from *S. cerevisiae* | Copper chaperones anchored in the mitochondrial inner membrane; Sco1 is involved in copper insertion to cytochrome c oxidase (Cobine et al., 2006) |

A simpler explanation would be that the intracellular concentration of CopZ is not sufficient to promote full copper loading of CotA. The ratio of CopZ to CotA cannot be controlled with the used expression construct. Distinct promoters for *copZ* and *cotA* under control of different inducers or use of multiple *copZ* copies would permit to vary the ratio of CopZ to CotA. Such expression systems would enable to determine whether CopZ concentration is really a limiting factor. At the same time these systems could be utilized to raise the limit of obtainable copper occupancies of CotA.

Conclusively we demonstrated that coexpression of CopZ from *B. licheniformis* increases the copper content and the specific activity of CotA laccase from *B. licheniformis*. This proofs the concept that coexpression of copper chaperones can be beneficial for heterologous expression of bacterial laccase in *E. coli*. This approach is simple to perform, does not increase experimental efforts substantially, and can thus be used as standard procedure in the heterologous expression of CotA. Modified expression constructs might further improve efficiency of the method. Whether the described system is universal and works for other bacterial laccases as well remains the subject of further studies.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.06.011.

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## Supplementary

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>cueO
CTGCAACTACCTGGGGCTATAACGGCAATCTGCTGGGGCCGGCGGTGAAATTACAGCGCGGCAAAGCGGT
AACGGTTGATATCTACAACCAACTGACGGAAGAGACAACGTTGCACTGGCACGGGCTGGAAGTACCGGGT
GAAGTCGACGGCGGCCCGCAGGGAATTATTCCGCCAGGTGGCAAGCGCTCGGTGACGTTGAACGTTGATC
AACCTGCCGCTACCTGCTGGTTCCATCCGCATCAGCACGGCAAAACCGGGCGACAGGTGGCGATGGGGCT
GGCTGGGCTGGTGGTGATTGAAGATGACGAGATCCTGAAATTAATGCTGCCAAAACAGTGGGGTATCGAT
GATGTTCCGGTGATCGTTCAGGATAAGAAATTTAGCGCCGACGGGCAGATTGATTATCAACTGGATGTGA
TGACCGCCGCCGTGGGCTGGTTTGGCGATACGTTGCTGACCAACGGTGCAATCTACCCGCAACACGCTGC
CCCGCGTGGTTGGCTGCGCCTGCGTTTGCTCAATGGCTGTAATGCCCGTTCGCTCAATTTCGCCACCAGC
GACAATCGCCCGCTGTATGTGATTGCCAGCGACGGTGGTCTGCTACCTGAACCAGTGAAGGTGAGCGAAC
TGCCGGTGCTGATGGGCGAGCGTTTTGAAGTGCTGGTGGAGGTTAACGATAACAAACCCTTTGACCTGGT
GACGCTGCCGGTCAGCCAGATGGGGATGGCGATTGCGCCGTTTGATAAGCCTCATCCGGTAATGCGGATT
CAGCCGATTGCTATTAGTGCCTCCGGTGCTTTGCCAGACACATTAAGTAGCCTGCCTGCGTTACCTTCGC
TGGAAGGGCTGACGGTACGCAAGCTGCAACTCTCTATGGACCCGATGCTCGATATGATGGGGATGCAGAT
GCTAATGGAGAAATATGGCGATCAGGCGATGGCCGGGATGGATCACAGCCAGATGATGGGCCATATGGGG
CACGGCAATATGAATCATATGAACCACGGCGGGAAGTTCGATTTCCACCATGCCAACAAAATCAACGGTC
AGGCGTTTGATATGAACAAGCCGATGTTTGCGGCGGCGAAAGGGCAATACGAACGTTGGGTTATCTCTGG
CGTGGGCGACATGATGCTGCATCCGTTCCATATCCACGGCACGCAGTTCCGTATCTTGTCAGAAAATGGC
AAACCGCCAGCGGCTCATCGCGCGGGCTGGAAAGATAC
```

Fig. S1: Partial sequence of *cueO* in *Escherichia coli* BL21(DE3) (consensus of 4 sequencing reactions). For automated design of retargeting primers in the TargeTron system the sequence of the *cueO* gene of *E. coli* BL21(DE3) was required. Amplification and sequencing primers (Tab. S2) were designed by use of a multiple sequence alignment of *cueO* sequences from *E. coli* strains with known genomes. The genome of *E. coli* BL21(DE3) was not yet published when the CueO deficient strain was created. The determined sequence is identical to the meanwhile published sequence of *cueO* (NC_012971).

Tab. S1: Query proteins in the tBLASTn approach for identification of potential copper chaperones in the genome of *Bacillus licheniformis* DSM13 (GenBank accession number: CP000002.3).

| **Organism** | **Protein** |
|---|---|
| *Enterococcus hirae* | CopA (AAA61835.1), CopB (P05425.2), CopZ (Q47840.1) |
| *Escherichia coli* | CopA (NP_415017.1), CusF (YP_001461735.1), PcoA (YP_001481473.1), PcoC (CAA58527.1), PcoD (CAA58528.1), PcoE (CAA58532.1) |
| *Homo sapiens* | ACDP1 (AAF86357.1), APP (AAB23646.1), MURR1 (NP_689729.1) |
| *Marinomonas mediterranea* | PpoB2 (AAV49997.1) |
| *Saccharomyces cerevisiae* | ATX1 (CAA65485.1), CCS (P40202.1), CCC2 (P38995.1), COX11 (AAB68227.1), COX17 (CAA97453.1), COX19 (Q3E731.1), CRS5 (P41902.1), CTR1 (Q06686.1), CTR2 (P38865.1), CTR3 (Q06686.1), CUP1-1 (NP_011920.1), Fet4p (CAA90837.1), FRE1 (EDN59435.1), SCO1 (CAA84979.1), SCO2 (CAA84966.1), SMF1 (P38925.2) |
| *Streptomyces griseus* | GriE (BAF36647.1), MelC1 (BAA37084.1) |

Tab. S2: Copper occupancy and specific activity of CotA after expression from different constructs. SD: standard deviation from 3 experiments

| **construct** | **copper content** | | **specific activity** | | **copper occupancy** | | **specific activity** | |
|---|---|---|---|---|---|---|---|---|
| | **(mol mol$^{-1}$)** | **SD** | **(U mg$^{-1}$)** | **SD** | **%** | **SD** | **%** | **SD** |
| pET22cotA | 1,6 | 0,2 | 5,9 | 0,3 | 39 | 5 | 100 | 5 |
| pET22cotA in ΔcueO strain | 1,4 | 0,1 | 6,3 | 0,1 | 35 | 3 | 106 | 25 |
| pET22cotA with pCOLAcopZ | 1,8 | 0,1 | 7,5 | 0,4 | 46 | 2 | 126 | 6 |
| pET22copZcotA | 1,6 | 0,1 | 6,9 | 0,3 | 39 | 4 | 117 | 4 |
| pET22copZcotA in ΔcueO strain | 1,8 | 0,1 | 6,6 | 0,5 | 44 | 3 | 111 | 8 |

Tab. S3: Used strains

| **strains** | **source** |
|---|---|
| *Escherichia coli* DH5 | Novagen, Germany |
| *Escherichia coli* BL21(DE3) | Novagen, Germany |
| *Escherichia coli* BL21(DE3) *cueO::kan*$^{R}$ | this study |

Tab. S4: Used plasmids

| **plasmid** | **source** |
|---|---|
| pACD4K-C | Sigma-Aldrich, Germany |
| pET22cotA | Koschorreck et al., 2008, Appl Microbiol Bioechnol 79:217-224 |
| pCOLADuet-1 | Novagen, Germany |
| pCOLAcopZ | this study |
| pET22copZcotA | this study |

Tab. S5: Used oligonucleotides. Endonuclease restriction sites are indicated in lowercase.

| **oligonucleotide** | **sequence** | **function** |
|---|---|---|
| cueO_fw2 | CCC TGA TTT GCT CAC GAC CGA TG | amplification and sequencing of *cueO* |
| cueO_rev2 | CAT GCT CCA GCA GAT GGC AGT G | amplification and sequencing of *cueO* |
| cue216_IBS | AAA AAA GCT TAT AAT TAT CCT TAA GCA GCT AGC GGG TGC GCC CAG ATA GGG TG | intron retargeting for *cueO* knock-out |
| cue216_EBS1d | CAG ATT GTA CAA ATG TGG TGA TAA CAG ATA AGT CTA GCG GCA TAA CTT ACC TTT CTT TGT | intron retargeting for *cueO* knock-out |
| cue216_EBS2 | TGA ACG CAA GTT TCT AAT TTC GAT TCT GCT TCG ATA GAG GAA AGT GTC T | intron retargeting for *cueO* knock-out |
| copZ_Nde_fw | GGG CGA Gca tat gAT GGA ACA AAA AAC AC | cloning of *copZ* in pCOLADuet-1 |
| copZ_Xho_rev | CTA ctc gag TCA GCC TGC AAC ATC ATA C | cloning of *copZ* in pCOLADuet-1 |
| COLA_RBS2_ XbaI_fw | CGG tct aga TAG TTA AGT ATA AGA AGG AG | cloning of bicistronic *copZ-cotA* construct |
| copZ_XbaI_rev | CAG tct aga TCA GCC TGC AAC ATC ATA C | cloning of bicistronic *copZ-cotA* construct |

# 5 Further results and discussion

Bacterial laccases as many other bacterial enzymes are more promising enzymes for technical application compared to their eukaryotic counterparts. Bacterial laccases possess higher stability at elevated temperatures and can easily be engineered by means of laboratory evolution based on construction of large mutant libraries. Despite these advantages no technical application of bacterial laccases has been reported so far. One of the reasons for this might be the limited number of available bacterial laccases. Their use is further hindered by the low redox potentials of the so far characterized enzymes which results in low specific activities and limited substrate range, and by the difficulty to produce fully copper loaded bacterial laccase. Mitigation of these obstacles could help to develop and implement biocatalytic oxidation processes based on bacterial laccases. Therefore, this work focused on lifting the above mentioned restrictions.

## 5.1 Selection of laccase candidates

Since description of the first bacterial laccase from *Azospirillum lipoferum* [30] only few further bacterial enzymes have been described in detail although genome mining approaches have revealed 1000 to 1200 laccase genes in bacteria [4, 6]. That accounts for approximately half of all detected laccase sequences. In the Laccase Engineering Database (LccED), that is based on sequence similarity, eleven superfamilies, from A to K, are classified [6]. Five of the superfamilies contain genes from bacteria (B, H, I, J, and K). At the beginning of this work bacterial laccases from the superfamilies I (CotA from *B. licheniformis* and CotA from *B. subtilis*) and J (laccase from *Thermus thermophilus*) were available in our laboratory. For creation of a more diverse laccase toolbox, putative laccase genes from other superfamilies were selected for cloning, expression, and characterization. A wide sequence space covered in such a toolbox would increase the probability to detect enzymes with

distinct new and interesting properties. Furthermore, it would make these interesting enzymes available for the development of novel biocatalytic reactions and processes. Considering sequence similarity, laccases from superfamily K are most distinct from the remaining superfamilies. Superfamily K contains the so-called small laccases which are comprised of only two cupredoxin-like domains. All other superfamilies contain common three-domain laccases. Therefore a representative of superfamily K, namely Ssl1 from *S. sviceus*, was selected for cloning and characterization. Apart from Ssl1, three two-domain laccase are known. However, only SLAC from *S. coelicolor* has been characterized in detail [60, 62, 145–147].

A second strategy for selection of novel bacterial laccases in order to create a diverse toolbox was based on the phylogenetic relationship of host organisms. Laccase genes in the LccED originate from nine bacterial phyla (out of fourteen based on the phylogenetic tree of Horiike et al. [148], see Fig. 5.1). The available laccases CotA from *B. subtilis* and CotA from *B. licheniformis* originate from the phyla *Firmicutes* and *T. thermophilus* laccase originates from *Deinococcus*. The putative laccases Cal1 from *Chloroflexus aurantiacus* and CgL1 from *Corynebacterium glutamicum* were additionally chosen to cover further bacterial phyla. *C. aurantiacus* is a non-green sulfur bacterium from hot springs [149] and is therefore adapted to thermophilic conditions. Accordingly, enzymes from *C. aurantiacus* might be thermostable which could be beneficial to process robustness of Cal1. With Ssl1 a laccase from the phylum *Actinobacteria* had already been selected. Since Ssl1 demonstrates low sequence similarity to known enzymes, CgL1 laccase from *C. glutamicum* was selected as a second laccase from *Actinobacteria*, nonetheless. The low sequence similarity of Ssl1 and CgL1 is reflected by their classification in two differing LccED families (K and B). Characterization of *C. aurantiacus* Cal1 and of *C. glutamicum* CgL1 were subject of a diploma and a master thesis in our group [150, 151].

With the three novel bacterial laccases from *S. sviceus*, *C. aurantiacus*, and *C. glutamicum* and the already available ones from *B. licheniformis*, *B. subtilis*, and *T. thermophilus*, the laccase toolbox contains enzymes from the LccED families B, J, I, and K. In order to cover all families containing bacterial laccases, a laccase from family H should be cloned and characterized in future.

This first approach to select candidates for a highly diverse laccase toolbox was based on sequence similarity. The second approach was based on the phylogenetic classification of the organisms of origin. Laccases from the phyla *Proteobacteria* (CueO

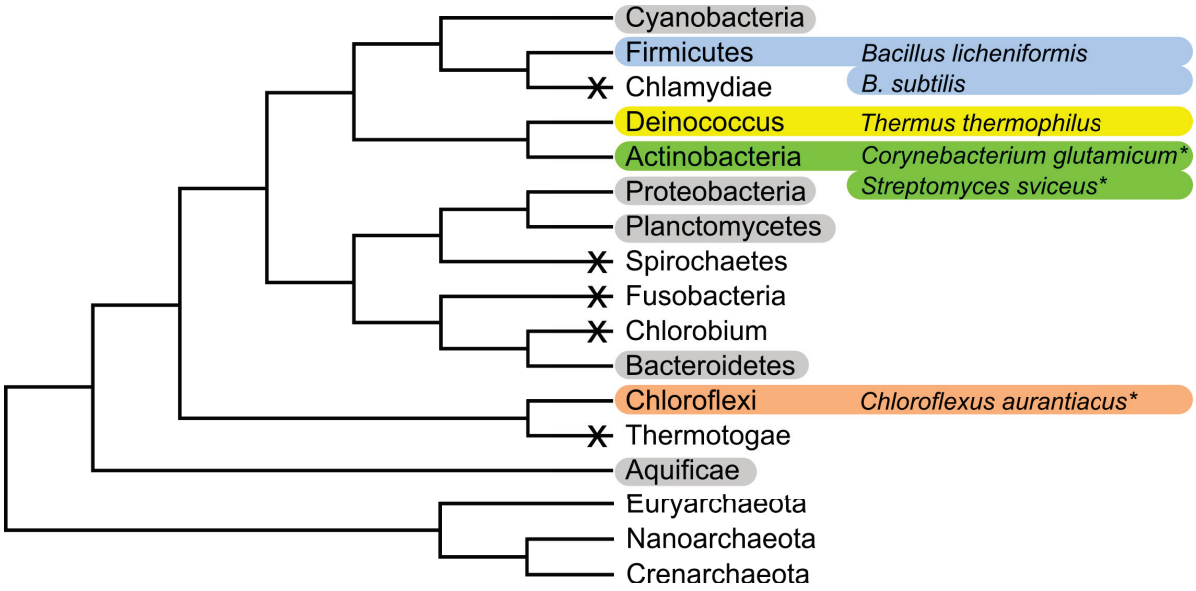


**Figure 5.1:** Distribution of laccase genes in bacteria. The phylogenetic tree with fourteen bacterial phyla with archaea as outgroup was adapted from Horiike et al. [148]. Grey: phyla with detected laccase genes according to the Laccase Engineering Database (LccED) [6], X: phyla without detected laccase genes, specified organisms indicate the origin of laccases that are meanwhile included in the laccase toolbox, *: laccases were not available at the beginning of this work.

from *E. coli* and PpoA from *Marinomonas mediterranea*) [36, 152] and *Aquificae* (McoA from *Aquifex aeolicus*) [74] have already been described in the literature. Laccases from the phyla *Firmicutes*, *Actinobacteria*, and *Chloroflexi* have been characterized in our group [54, 120, 150, 151]. Characterized enzymes from the phyla *Cyanobacteria*, *Planctomycetes*, and *Bacteroides* are still lacking and their cloning and characterization should be performed in future for complete phylogenetic coverage within the bacterial laccase toolbox. Moreover, laccases from *Archaea* might be valuable targets as well since these organisms are adapted to extreme environments and should possess highly stable enzymes. For example, for LccA from *Haloverax volcanii* a remarkably high thermostability has been reported [52].

## 5.2 Sequence analysis and expression of Ssl1

The native gene of Ssl1 contains an N-terminal signal sequence [54] as most of bacterial laccase genes [4] with *Bacillus* laccases as exception. Obviously, the biological functions of most bacterial laccases require activity in the periplasm or outside the cell. For CueO from *E. coli* participation in copper detoxification in the

periplasm has been demonstrated [36, 133]. *Bacillus* laccases seem to be located in the endospore coat where they are responsible for formation of pigments that contribute to radiation resistance of *Bacillus* spores [34, 35]. The endospore coat is formed within the cell. In the final stage of the sporulation process, the mother cell lyses and the endospore is released [33]. As a result *Bacillus* laccases do not need to be translocated across the cytoplasmic membrane and are still able to act outside the cell (or spore).

The physiological function of most bacterial laccases has not been elucidated yet [31] and remains the object of further studies. Suggested functions of bacterial laccases are in copper and iron homeostasis [74], survival and activity of non-motile root bacteria [153], morphogenesis, and degradation of phenolic compounds [154].

Expression of Ssl1 was tested with four constructs: i) *ssl1* without signal sequence and without affinity tag, ii) *ssl1* with signal sequence and with N-terminal $His_6$-tag, iii) *ssl1* without signal sequence and with N-terminal $His_6$-tag, and iv) *ssl1* without signal sequence and with C-terminal $His_6$-tag. Noteworthy amounts were exclusively achieved with an N-terminal $His_6$-tag fused to *ssl1* without signal sequence. The fact that removal of the signal sequence improves expression is not surprising. Incorrect processing of a heterologous signal sequence can lead to degradation of the corresponding protein as suggested in models of the translocation pathway [155]. His-tags, in particular long His-tags, can promote formation of inclusion bodies [156] and often results in reduced solubility of target proteins [157, 158]. Nonetheless, introduction of the N-terminal $His_6$-tag increased the amounts of recombinant Ssl1. The His-tag might mask an aggregation prone site in Ssl1 that is exposed upon removal of the signal sequence, or the His tag might have a stabilizing effect on mRNA of Ssl1. In heterologous expression of a truncated form of murine metalloproteinase-1 in *E. coli*, the introduction of an N-terminal polyhistidine tag resulted in stabilization of the protein within *E. coli* but did not alter mRNA levels [159]. To resolve whether this is the case for N-terminally His-tagged Ssl1 as well, studies of the mRNA level, for instance by quantitative real time PCR, could be performed.

The *ssl1* gene contains several codons that are rarely used by the *E. coli* translation machinery. Therefore, use of *E. coli* CodonPlus(DE3)-RP which carries a plasmid encoding for rare arginine and proline tRNAs, that indeed occur in *ssl1*, resulted in the highest Ssl1 yields of all tested expression hosts. Since further rare codons occur in *ssl1*, codon optimization in a synthetic gene might result in higher

expression yields and would allow to use expression strains without additional plasmid. This would make the corresponding selection marker (chloramphenicol) dispensable. The consequently lower metabolic burden might result in an additionally improved expression yield of Ssl1. Conclusively, expression optimization by codon optimization should be performed before large scale production and industrial use of Ssl1.

Introduction of point mutations for creation of Ssl1 variants with higher redox potentials generally lowered the expression yields. However, all variants could be produced with the established expression system in sufficient amounts for characterization. Expression optimization of Ssl1 variants is currently under investigation and will be part of an upcoming master thesis.

## 5.3 Properties of Ssl1

Ssl1 was able to oxidize common laccase substrates such as ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), SGZ (syringaldazine), guaiacol, and 2,6-DMP (2,6-dimethoxyphenol) (for structures of substrates see Fig. 3.6). As many bacterial laccases Ssl1 was active at neutral to alkaline conditions with optima for phenolic substrates in the pH range between pH 8 (SGZ) and pH 9 (guaiacol, 2,6-DMP). Together with SLAC from *S. coelicolor*, Ssl1 is among the laccases with the highest alkaline activity optima. It has been suggested that the activity at alkaline pH is limited by binding of hydroxide ions at the trinuclear cluster (TNC) [160, 161]. Notably, both Ssl1 and SLAC, are members of the small, two-domain laccases that form homotrimers in order to build the trinuclear cluster at the interface of neighboring laccase monomers. In comparison to large, three-domain laccases, that form the TNC at the interface of domain 1 and 3, this might lead to an altered geometry of the TNC resulting in a lower affinity for hydroxide ions and subsequently in higher activity at alkaline pH. It would be interesting to investigate further small laccases to reveal whether extreme alkaline activity is a general feature of this laccase family.

Applications of fungal laccases are restricted to acidic conditions [28], whereas bacterial laccases, in particular small, two-domain laccases, are active at neutral to alkaline pH. Knowledge of the structural features of small laccases that apparently decrease affinity of the TNC for hydroxide ions might be transferred to fungal

laccases. In such engineered fungal laccases alkaline activity and high redox potential would be combined within one enzyme. In the meantime, laccases with natural alkaline activity, such as Ssl1, can help to extent the feasible pH range in laccase based processes.

Ssl1 demonstrated a half-life of approximately 90 min at 60 °C. Since many industrial processes are carried out at elevated temperatures this is an important feature for application of Ssl1. Furthermore, the relatively high stability makes Ssl1 a good candidate for directed evolution approaches. More stable enzymes can tolerate a higher number of potentially destabilizing mutations and possess enhanced evolvability [162]. Therefore, Ssl1 is suitable for standard directed evolution experiments. Thereby it could be adapted to various reaction conditions and substrates.

The stability and tolerance of Ssl1 in presence of organic solvents, detergents, and laccase inhibitors was tested and demonstrated a high robustness of Ssl1 under the chosen conditions [54]. Within the last years the chemical industry has started to develop more and more sustainable and environmentally friendly processes based on the twelve principles of green chemistry [163]. Novel 'green' processes are often achieved by integration of several catalytic steps within one reaction pot. This involves combined reactions of chemo- and biocatalysts which are often not compatible due to required reaction conditions [164]. The tolerance of Ssl1 towards various organic solvents, detergents, and inhibitors creates a large operational window which might be useful for combination with chemocatalysts. This is applicable for combination of Ssl1 with other enzymes in enzyme cascade reaction as well. Moreover, many laccase substrates are poorly soluble in water [94] and their conversion requires solubilization in organic solvents. For such conversions tolerance towards organic solvents is a requirement that is well met by Ssl1.

## 5.4 Crystal structure of Ssl1

For tuning of the redox potential of Ssl1 structural information was required. Therefore, Ssl1 was crystallized and its three-dimensional structure solved by molecular replacement. Ssl1 crystallized as homotrimer and the particle size measured in multi-angle static light scattering experiments indicated trimeric architecture as well. This is strong evidence that soluble Ssl1 is active as homotrimer as suggested previously for other two-domain laccases and nitrite reductases [62, 154, 165, 166]. Domain 2 in

three-domain laccases is responsible for positioning of domain 1 and 3 in a manner that facilitates formation of the trinuclear cluster. In two-domain laccases domain 2 is missing. As a consequence the TNC cannot be formed between the domains of a monomer. Instead the TNC is located between domain 2 and 1 of adjacent monomers. The interfacial location of the TNC was also observed for Ssl1.

The organization as trimer results in formation of a central cavity that is covered with hydrophilic residues. Two of these residues (D256, S265) coordinate a water molecule near the trinuclear cluster. This indicates that the central cavity is involved in delivery of oxygen to the trinuclear cluster and in elimination of water after reduction and protonation of oxygen. Whether the two residues that coordinate water are important for activity of Ssl1 could be tested by amino acid substitutions, e.g. by replacement with hydrophobic residues.

The copper coordinating residues in laccase are highly conserved and were actually used for creation of signature sequences and extraction of putative laccase genes from public databases [6]. The copper coordination motif of Ssl1 is no exception and contains the common nine amino acids, one cysteine and eight histidine residues. A difference to described copper coordination geometries was detected at the TNC, though. In three-domain multicopper oxidases from fungi and bacteria one of the T3Cu is coordinated by one N$\delta$ and two N$\epsilon$ atoms of three histidine residues while the second T3Cu is coordinated by three N$\epsilon$ ligands as seen, for example, in *Trametes versicolor* laccase, and CotA from *Bacillus subtilis* [58, 167]. For Fet3p from *Saccharomyces cerevisiae*, it was shown that this difference in coordination of both T3Cu results in an asymmetric coordination geometry [168]. The T3Cu of Fet3p with three N$\epsilon$ ligands shows a distorted trigonal pyramidal geometry while T3Cu

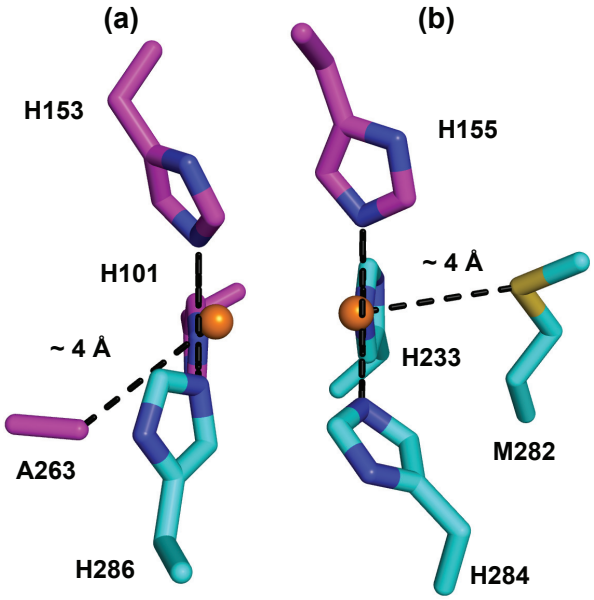


**Figure 5.2:** T3 copper ions (T3Cu, orange spheres) in Ssl1 are coordinated in an asymmetric manner. (a) T3Cu in pyramidal trigonal geometry. The axial position is not occupied by a copper ligand, the closest residue is non-coordinating alanine (A263). (b) T3Cu in planar trigonal geometry. The axial position is occupied by coordinating methionine (M282).

with one N$\delta$ and two N$\epsilon$ ligands shows trigonal planar geometry. This structural asymmetry facilitates binding of dioxygen to the T3Cu with distorted geometry and subsequent formation of the peroxo intermediate by two electron reduction [168]. In Ssl1 both T3Cu have exclusively N$\epsilon$ histidine ligands. Nevertheless, they show structural asymmetry with one T3Cu in trigonal planar geometry and one T3Cu in trigonal pyramidal geometry where the copper ion is located approximately 0.5 Å out of plane (Fig. 5.2). In axial position of the T3Cu with planar geometry methionine 282 is located. The axial position of the T3Cu with distorted geometry is unoccupied with A272 being the closest residue. It can be assumed that methionine in axial position and planar organization leads to relative stabilization of oxidized over reduced state of T3Cu, as frequently reported for T1Cu sites. At the second T3Cu such stabilization does not occur, which makes this copper ion more reactive towards dioxygen binding and reduction. Methionine at the position corresponding to M282 in Ssl1 is highly conserved within small laccases. A272 is frequently replaced in two-domain laccases by the small residue glycine which would leave the axial position unoccupied as in Ssl1. This indicates that creation of an asymmetric trinuclear cluster by placing an axial methionine at one of the T3Cu and leaving the axial position of the second T3Cu unoccupied is conserved within small laccases. Although asymmetry at the trinuclear cluster of small laccases is achieved by a molecular mechanism distinct from three-domain multicopper oxidases, the functional asymmetry that facilitates dioxygen binding and reduction seems to be conserved among multicopper oxidases regardless of their domain organization.

Near the T1 copper center a putative substrate binding cleft is formed by several amino acids. Substitution of two methionines from this site altered the affinity to 2,6-dimethoxyphenol which indicates that M195 and M293 are indeed involved in substrate binding. For confirmation thereof and to reveal further amino acids that are involved in substrate binding, structure solution of Ssl1 in complex with a substrate would be desirable. Simple co-crystallization experiments failed in formation of regular enzyme crystals. Crystallization was performed at room temperature in aerobic conditions and at a pH that allows catalytic activity of Ssl1. Catalytically active Ssl1 might undergo minor structural changes in course of the reaction, e.g. by substrate binding and product release, or by redox state changes and subsequent alterations in electrostatic attraction and repulsion within the enzyme. The minor structural changes of active Ssl1 in the presence of substrate might then

prevent the formation of well-ordered crystals. For crystallization in complex with substrates an inactive form of Ssl1 could be more suitable. This could be achieved by crystallization without oxygen. Without final electron acceptor the reaction would cease when all copper centers are in reduced state. However, working under oxygen-free conditions is uncomfortable and removing oxygen from the crystallization might result in altered crystallization conditions and might in turn require screening and establishment of novel conditions. To avoid this laborious approach, type 2 depleted variants are currently investigated in co-crystallization experiments. In these variants one of the T2 copper coordinating histidines is substituted in order to disrupt copper binding. Resulting T2 depleted variants should possess an intact substrate binding site and an intact T1 copper site. However, the electron transfer pathway from T1 copper ions to the trinuclear cluster would be disrupted rendering the variants inactive. Revelation of substrate binding residues with the T2 depleted variants would facilitate tailoring of the Ssl1 substrate binding site for conversion of substrates of interest. This could be done either in rational approaches by site-directed mutagenesis or by saturation mutagenesis of the relevant amino acids. Variants of saturation mutagenesis could further be collected in a focused library. With such a library available, screening of optimal variants for newly emerging substrates would be possible within very short time.

The crystallization conditions developed for wildtype Ssl1 are suitable for Ssl1 variants as well and crystals of several Ssl1 variants have been obtained. The resulting crystals show sufficient X-ray diffraction to allow the solution of Ssl1 variant structures. Therefore, the developed crystallization conditions are valuable to gain more knowledge on structure-function relationship in Ssl1. Structures of the Ssl1 variants will enable evaluation of the predicted effects of amino acid substitutions on the redox potential. In turn, this knowledge can be used in iterative engineering of Ssl1 in order to obtain variants with even higher redox potentials. Further, the crystallization conditions can be used in co-crystallization experiments with substrates. This would allow to identify residues that are involved in substrate binding and, subsequently, tailor-made optimization of substrate binding residues. Co-crystallization of Ssl1 and inhibitors would allow to identify residues that interact with inhibitor molecules. Replacement of these amino acids would result in laccase variants that are less susceptible to the presence of inhibitors. Laccases are often used for the treatment of waste waters. Typically, these contain not only substrate

molecules but a variety of substances. Some of them might inhibit wildtype laccases and prevent efficient degradation of the contaminating target substance. Laccase variants engineered for lower inhibitor affinity could efficiently oxidize target substances in complex reaction mixtures, such as waste waters, without being inhibited. A combination of amino acid substitutions for redox potential increase, substrate binding optimization, and inhibitor insusceptibility, would result in a more efficient laccase biocatalyst.

## 5.5 Ssl1 variants

The redox potential of Ssl1 wildtype is 375 mV which is in the common range of bacterial laccase. High-redox laccases from fungi reach potentials of up to 800 mV [169] which is of great benefit for their industrial application [170]. The crystal structure allowed selection of residues with impact on the redox potential of the T1 site. Substitution at the corresponding positions resulted in increased redox potentials with an increase of 81 mV to a redox potential of 456 mV in the M295L variant. Residue 295 is located in axial position of T1 copper. The large impact of amino acids in axial position has been demonstrated for a wide array of T1 copper containing proteins such as laccase, ceruloplasmin, azurin, and further one-domain cupredoxins [83]. Amino acids with strong copper coordinating capabilities, i.e. glutamine, result in relative stabilization of the oxidized Cu(II) state and consequently in lower redox potentials [85, 171]. Non-coordinating residues, i.e. hydrophobic amino acids, result in relative stabilization of the Cu(I) state and consequently in higher redox potentials [78, 85]. The same behavior was observed in Ssl1, substitution of weakly copper coordinating methionine with non-coordinating leucine increased the redox potential of T1 copper.

Another important factor that contributes to the redox potential of T1 copper ions is the hydrophobicity in its environment [83]. In Ssl1 two methionine residues are located in the putative substrate binding site (M195 and M293) near the T1 copper center. Moreover, the hydrophobic core of Ssl1 domain 2 in about 8 Å distance of T1 copper contains a threonine and a methionine residue (T222 and M220). By substituting these four residues (M195, M220, T222, and M293) by more hydrophobic leucine, Ssl1 variants were constructed with increases in the redox potential of 16 to 33 mV. This confirms that hydrophobicity in the environment of T1 copper

contributes to the redox potential. This observation can be used to further tune the redox potential of Ssl1 in a rational approach. A combination of the introduced substitutions might have an additive effect and might increase the Ssl1 redox potential further. This hypothesis is currently under investigation. In the presented work leucine was used for hydrophobicity generating substitutions exclusively. The introduction of more hydrophobic residues, for instance phenylalanine or tryptophan, might show elevated redox potential increases and should be tested as well.

All four tested positions were proven to affect the redox potential of Ssl1 and even truncation of the C-terminus had an influence. This demonstrates the high number of residues that play a role in determination of the redox potential. Obviously, the interactions that contribute to the magnitude of the redox potential are numerous and complex. Probing of further positions by amino acid substitutions is therefore likely to reveal more residues that participate in the redox potential of Ssl1.

In contrast to the expectations, variants with increased redox potentials were not able to convert the low-redox potential substrate 2,6-dimethoxyphenol (2,6-DMP) more efficiently. The variants displayed four to fourteen fold reductions in $k_{cat}$ values compared to wildtype Ssl1. A similar behavior has been reported for CotA from *B. subtilis*. Mutation of the axial amino acid in the variants CotA M502L and CotA M502F increased the redox potential by approximately 100 mV but reduced the $k_{\mathrm{cat}}$ values two to tenfold [172]. Apparently, the redox potential difference between the T1 copper ion and 2,6-DMP is not limiting the reaction rate. On the contrary, alterations near the T1 copper center impaired the reaction rate. In general, the closer the substituted amino acid was located to the T1 center the larger was the reduction in $k_{cat}$. Thus, substitution directly at T1 copper in Ssl1 M295L reduced $k_{cat}$ by a factor of fourteen. Substitution in the putative substrate binding site in Ssl1 M293L and Ssl1 M195L reduced $k_{cat}$ by factors of five and six, respectively. Again the substitution closer to T1 copper in M293L compared to M195L resulted in a larger decrease of $k_{cat}$. In conclusion, amino acid substitutions, in particular those close to T1 copper, might perturb the T1 copper center in a manner that impairs the intermolecular electron transfer to the trinuclear cluster.

Additionally to 2,6-DMP, with a relatively low redox potential compared to other laccase substrates, the high-redox potential substrates indigo carmine and

Alizarin Red S were tested with the created Ssl1 variants. The decolorization of such industrial dyes is one of the main applications of laccases, for example in waste water treatment and in bleaching of textiles. Variants with substitutions in larger distance to T1 copper demonstrated higher dye decolorization efficiencies than wildtype Ssl1 and than variants with substitutions close to the T1 site. This demonstrates that the redox potential difference between substrate and T1 copper can be a limiting factor in the oxidation of high-redox potential substrates such as the tested dyes. It also demonstrates that substitutions near the T1 site can impair Ssl1 activity regardless of an increase in the redox potential. For substitutions close to the T1 site the perturbing effect on the intermolecular electron transfer to the trinuclear copper cluster might be larger than for more distant substitutions. For creation of an efficient Ssl1 variant a deeper knowledge of residues that are capable to modulate the electron transfer from T1 copper to the trinuclear cluster would be beneficial. That would allow optimization of the electron transfer pathway or, at least, to select amino acids for substitutions that increase the T1 redox potential without impairing the electron transfer to the trinuclear cluster.

An unexpected change in color from deep blue in wildtype Ssl1 to a black or violet shade was observed with the M295L mutation. This is reflected in the UV-Vis spectrum of Ssl1 M295L. Wildtype Ssl1 shows a strong absorption at 592 nm corresponding to the 'blue' T1 copper ion [54]. In Ssl1 M295L this absorption band was less intense and an additional absorption maximum at 422 nm appeared (Fig. 5.3). The shoulder around 330 nm corresponding to the hydroxide bridged T3 copper pair was unaffected which indicates that mutation at the T1 copper center does not affect the electronic structure or geometry of the trinuclear cluster. The absorption at 592 nm accounts for transition of the $3d_{x^2-y^2}$ orbital of the T1 copper ion to the $\pi$ orbital of coordinating cysteine-sulfur [65]. Absorption bands of blue copper proteins in the $\sim$450 nm region have been assigned to transition of the $3d_{x^2-y^2}$ orbital of the T1

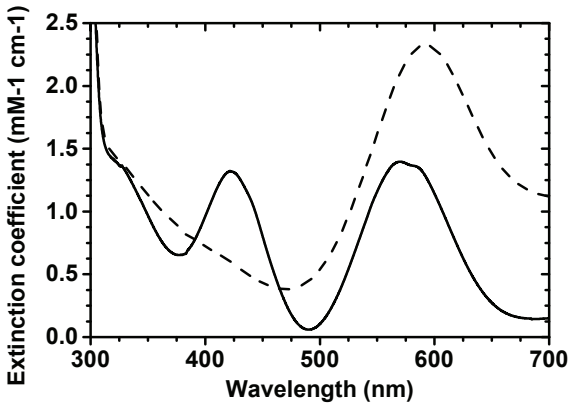


**Figure 5.3:** UV-Vis spectra of Ssl1 wildtype (dashed line) and Ssl1 M295L (solid line). In Ssl1 M295L the maximum at 592 nm showed a reduced intensity, extra maxima occurred at 422 nm and at 570 nm.

copper ion to the pseudo-$\sigma$ orbital of coordinating cysteine-sulfur, for example in nitrite reductase from *Achromobacter cycloclastes* [173]. A redistribution of transitions that result in a decrease of absorption at $\sim 600\,\text{nm}$ and an increase at $\sim 450\,\text{nm}$ can be caused by rotation of the $3d_{x^2-y^2}$ orbital. This can result in increased $\sigma$ overlap and decreased $\pi$ overlap involving cysteine-sulfur p and T1 copper $d_{x^2-y^2}$ orbitals as demonstrated with *R. vernicifera* plastocyanin (mainly absorbing at $\sim 600\,\text{nm}$), cucumber stellacyanine (absorption at $\sim 450\,\text{nm}$ and $\sim 600\,\text{nm}$), and *A. cycloclastes* nitrite reductase (mainly absorbing at $\sim 450\,\text{nm}$) [174]. Substitution of the axial T1 copper ligand methionine in Ssl1 M295L might change the electronic structure of T1 copper in a manner that its $3d_{x^2-y^2}$ orbital rotates for larger overlap with the pseudo-$\sigma$ orbital of cysteine-sulfur. This would increase the $\sigma$ character of the copper to sulfur coordination bond and lead to a decrease in blue absorption. At the same time an increased absorption at $\sim 450\,\text{nm}$ would be expected as observed for Ssl1 M295L. This change in the electronic structure of the T1 copper to sulfur bond should be reflected by a longer coordination bond between T1 copper and cysteine-sulfur [65]. Comparison of the structure of Ssl1 wildtype with a preliminary structure of Ssl1 M295L suggests such a reduction of the copper to sulfur bond from 2.2 Å to 2.1 Å. However, the resolution of the structures (2.2 Å for Ssl1 wildtype) is too low to rely on these bond length values. Solution of structures with higher resolutions will be necessary to confirm this assumption. Verification of the changes in the electronic structure of the T1 copper center by computational methods would be valuable as well.

Electron transfer from T1 copper, where substrate molecules are oxidized, to the trinuclear cluster, where oxygen is reduced to water, occurs via the so-called superexchange pathway [65]. $\pi$ delocalization from T1 copper to cysteine-sulfur extends through backbone atoms and exits at the destination copper with an additional shortcut through a hydrogen bond of a backbone carbonyl [64]. The described rotation of the $3d_{x^2-y^2}$ orbital in Ssl1 M295L might decrease the efficiency of electron transfer from T1 copper to the superexchange pathway which would result in lower electron transfer rates and subsequently in lower specific activities. This assumed perturbation of electron transfer is in accordance to the low activities that were observed for Ssl1 M295L in oxidation of 2,6-dimethoxyphenol, indigo carmine, and Alizarin Red S. Redox potential increases of the T1 copper center will improve the entry of electrons into the superexchange pathway. However, this does not

improve the specific activities when reaction rates are limited by the intermolecular electron transfer. Conclusively, the focus for rational design of Ssl1, and of laccases in general, needs to be extended and should include residues that influence the electron transfer pathway from T1 copper to the trinuclear cluster. Up to now, only residues and corresponding atoms of the protein backbone that contribute directly to the electron transfer by provision of redox active molecular orbitals have been described [64, 65]. Neighboring residues which might change the electronic structure along the superexchange pathway are unknown. Knowledge of such residues might provide a better understanding of the electron transfer process which is a prerequisite for rational optimization of the superexchange pathway. Laccase variants with increased T1 redox potential and, additionally, improved intramolecular electron transfer should be very efficient biooxidative catalysts.

Our experiments showed that substitution of methionines from the putative substrate binding site (M195 and M293) results in variants with an increase in $K_M$ values of approximately 40 %. This indicates that M195 and M293 are indeed involved in shaping the substrate binding site and substitution thereof changes the substrate-enzyme interaction and, consequently, $K_M$. Adjustment of the substrate binding site by substitution of these and neighboring residues could proof valuable for creation of Ssl1 variants tailored for oxidation of specific substrates.

## 5.6 Improvement of the copper content of CotA

Heterologous expression of bacterial laccase often results in partially copper depleted enzyme as was shown for the laccases CotA from *B. subtilis*, CotA from *B. licheniformis*, Ssl1 from *S. sviceus*, and McoA from *A. aeolicus* [54, 58, 74, 120]. CotA from *B. licheniformis* was used as model laccase in heterologous expression with *E. coli* as expression host to test two strategies for overcoming this limitation.

The first strategy involved enhancement of the intracellular copper concentration by knock-down of *E. coli* CueO which participates in aerobic copper detoxification. With an increased intracellular copper content an improved supply of copper during CotA biosynthesis was anticipated. However, the deletion of CueO did not result in an increased copper content of recombinant CotA or in an increased specific activity. Deletion of CueO was reported to lead to copper accumulation in *E. coli* cells [175]. Conclusively, an increased intracellular copper concentration is not sufficient

to improve the copper incorporation of heterologously expressed CotA.

Intracellular copper ions do not occur as free ions but are bound to metallothioneines or copper chaperones to protect the cells from the cytotoxic effects of copper ions. As a result the concentration of free copper ions is extremely low. For *S. cerevisiae* a concentration of less than one free copper ion per cell was detected [176]. Many copper containing enzymes receive copper ions from copper chaperones that deliver copper ions specifically to their target enzymes. Thus, tyrosinase MelC2 from *S. antibioticus* requires the presence of its caddie protein MelC1 for copper dependent activation [177]. Also for *S. cerevisiae* superoxide dismutase the coexpression of its copper chaperone yCCS resulted in recombinant enzyme with high copper contents [178]. The absence of a copper chaperone in the *E. coli* expression strain that is able to deliver copper ions to *B. licheniformis* CotA is a possible reason for the difficulty to produce fully copper loaded CotA even with increased intracellular copper concentrations. Therefore, the second strategy to overcome insufficient copper contents in heterologously expressed CotA involved the identification and coexpression of a suitable copper chaperone.

Sequences of known copper binding proteins were used to screen the genome of *B. licheniformis* for putative copper chaperones. Three candidates were identified, YcnJ, YpmQ, and CopZ. YcnJ was found to be similar to PcoC and to PcoD from *E. coli* in its N- and C-terminus, respectively. PcoC is a small soluble periplasmic copper chaperone that is assumed to bind periplasmic copper ions for delivery to the copper uptake transporter of the inner membrane of *E. coli* [126]. Judged from the similarity to PcoC and PcoD, YcnJ might act as copper transporter in *B. licheniformis* either in uptake or in export of copper ions. The fusion of copper chaperone and copper transporter into a single protein might improve the efficiency of copper transfer between both proteins. It might also be an adjustment to the cellular organization in *B. licheniformis*. In *E. coli* PcoC is located in the periplasm where it is retained by the outer membrane. *B. licheniformis* is a gram-positive bacterium and does not possess an outer membrane. Therefore, the fusion with the transmembrane protein might prevent diffusion and loss of the small soluble copper chaperone. According to sequence analysis YcnJ is a transmembrane protein with nine transmembrane helices. Heterologous expression of transmembrane proteins is often difficult and was not possible for YcnJ in *E. coli* BL21(DE3) with the used expression systems. Therefore YcnJ was not used for coexpression with CotA.

YpmQ was identified as potential copper chaperone due to its similarity with Sco1 and Sco2 from *S. cerevisiae*. Both are copper chaperones of the inner mitochondrial membrane. Sco1 participates in the assembly of copper centers in cytochrome *c* oxidase [179]. Similar to Sco1 and Sco2 YpmQ contains an N-terminal membrane anchor that hinders its efficient heterologous expression in *E. coli* BL21(DE3). After removal of the membrane anchor YpmQ could be expressed, but its coexpression did not increase the copper content or the specific activity of CotA. Equal to Sco1, YpmQ might be involved in the copper center assembly of cytochrome *c* oxidase. In *B. subtilis* the dependence of copper incorporation into cytochrome *c* oxidase on the homologous YpmQ has been shown [180, 181]. Consequently, *B. licheniformis* YpmQ might be specific for copper incorporation into cytochrome *c* oxidase and, due to that, is not able to incorporate copper ions into recombinant enzymes like CotA.

CopZ was identified by similarity to *Enterococcus hirae* CopZ which is a small soluble copper chaperone in the cytosol. *E. hirae* CopZ delivers copper ions to the copper export pump CopB and to the transcription regulator CopY [129]. The sequence of *B. licheniformis* CopZ did not contain any transmembrane or signal sequences and is therefore probably located in the cytosol. The coexpression of CotA and CopZ in *E. coli* BL21(DE3) increased the copper content of CotA by up to 20 % and its specific activity by up to 26 %. This demonstrates that coexpression of copper chaperones is a suitable strategy to increase copper content and specific activity of heterologously expressed laccase. However, coexpression of CopZ did not result in fully copper loaded CotA. CopZ might be involved in copper incorporation to a specific copper center in CotA exclusively. In this case, copper centers that are not a target of CopZ would not be accessible and would remain unoccupied. Or the concentration of CopZ might be too low for full copper incorporation into CotA. With the used expression systems both, CotA and CopZ, are under control of the T7 promoter. As a consequence the expression of CotA and CopZ is induced simultaneously and to the same degree. The use of a second promoter that is under control of an independent repressor would facilitate fine tuning of the coexpression. For instance, a higher CopZ to CotA ratio might be beneficial for copper incorporation. With two independent repressors that could be achieved by addition of different concentrations of inducer. Moreover, the timing of coexpression could be important. Preliminary induction of CopZ might insure an expression

environment with a high copper delivery capacity. Induction of CotA after induction of CopZ would then result in improved copper delivery and in higher copper content of CotA. Nonetheless, the current coexpression system is simple to use, does not require much additional experimental efforts, and can therefore be used in standard expressions of CotA from *B. licheniformis*.

## 5.7 Conclusions

Three recognized limitations of bacterial laccases were addressed within the presented work: the low number of characterized bacterial laccases; their low redox potential; and the difficulty to obtain fully copper loaded bacterial laccases upon heterologous expression. By expression and characterization of Ssl1 from *S. sviceus* a novel bacterial laccase with interesting features for industrial applications has been added to the toolbox of available laccases. Ssl1 demonstrated alkaline activity, thermal robustness, stability in presence of organic solvents, and a low redox potential. At the moment the use of Ssl1 in several processes is under investigation.

Based on the crystal structure of Ssl1 residues that contribute to the redox potential of Ssl1 could be identified. Substitution of these residues allowed tuning of the low redox potential to higher values. Thereby substitutions of amino acids that were located not directly at the T1 center resulted in variants that could degrade industrial dyes more efficiently than wildtype Ssl1. The established crystallization conditions are applicable on Ssl1 variants and will be a useful tool for investigation of structure-function relationships in small laccases. Finally, this could facilitate rational tailoring of Ssl1 for given substrates or process conditions.

The copper content of CotA from *B. licheniformis* heterologously expressed in *E. coli* is low and limits activity yields. Coexpression of the small soluble copper chaperone CopZ from *B. licheniformis* was shown to be a simple and successful approach to increase the copper content and the specific activity of CotA. Whether this approach is universal and is able to increase the copper content of further bacterial laccases in heterologous expression will be tested in further experiments.

In summary, the three addressed limitations could be mitigated and approaches for a further improve of the usability of bacterial laccases were suggested. Finally, this might lead to an extended utilization of bacterial laccases in industrial processes.

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# 7 Abbreviations

| | |
|---|---|
| 2,6-DMP | 2,6-dimethoxyphenol |
| *A. aeolicus* | *Aquifex aeolicus* |
| *A. aegypti* | *Anopheles aegypti* |
| *A. cycloclastes* | *Achromobacter cycloclastes* |
| *A. gambiae* | *Anopheles gambiae* |
| ABTS | 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) |
| Atx1 | anti-oxidant 1 |
| *B. licheniformis* | *Bacillus licheniformis* |
| *B. subtilis* | *Bacillus subtilis* |
| *C. aurantiacus* | *Chloroflexus aurantiacus* |
| *C. bulleri* | *Cyathus bulleri* |
| *C. glutamicum* | *Corynebacterium glutamicum* |
| CCO | cytochrome *c* oxidase |
| Ccs | copper chaperone for superoxide dismutase |
| *cop* | copper resistance operon |
| Ctr1/Ctr3 | copper transporter 1/3 |
| Cox11/Cox17 | cytochrome *c* oxidase subunit 11/17 |
| *cue* | copper efflux system |
| *cus* | copper sensing system |
| *E. coli* | *Escherichia coli* |
| *E. hirae* | *Enterococcus hirae* |
| EC | enzyme class |
| Fet3/Fet4 | iron transporter 3/4 |
| *H. volcanii* | *Haloferax volcanii* |
| HAA | 3-hydroxyanthranilic acid |
| HBT | *N*-hydroxybenzotriazole |
| LccED | Laccase Engineering Database |

| | |
|---|---|
| *M. mediterranea* | *Marinomonas mediterranea* |
| MCO | multicopper oxidase |
| PDB | Protein Data Bank |
| *R. vernicifera* | *Rhus vernicifera* |
| *S. antibioticus* | *Streptomyces antibioticus* |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| *S. coelicolor* | *Streptomyces coelicolor* |
| *S. sviceus* | *Streptomyces sviceus* |
| Sco1 | synthesis of cytochrome *c* oxidase 1 |
| SGZ | syringaldazine |
| SOD | copper,zinc-superoxide dismutase |
| T1Cu | type 1 copper ion |
| T2Cu | type 2 copper ion |
| T3Cu | type 3 copper ion |
| *T. castaneum* | *Tribolium castaneum* |
| *T. hirsuta* | *Trametes hirsuta* |
| *T. versicolor* | *Trametes versicolor* |
| *T. villosa* | *Trametes villosa* |
| *T. zonatus* | *Trametes zonatus* |
| TEMPO | (2,2,6,6-tetramethylpiperidine-1-yl)oxyl |
| TNC | trinuclear copper cluster |

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# 9 Erklärungen zur Dissertation

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf,

Matthias Gunne