

Characterization of novel thermostable bacterial Laccase-like multi-copper oxidases

Brander, Søren; Kepp, Kasper Planeta; Mikkelsen, Jørn Dalgaard

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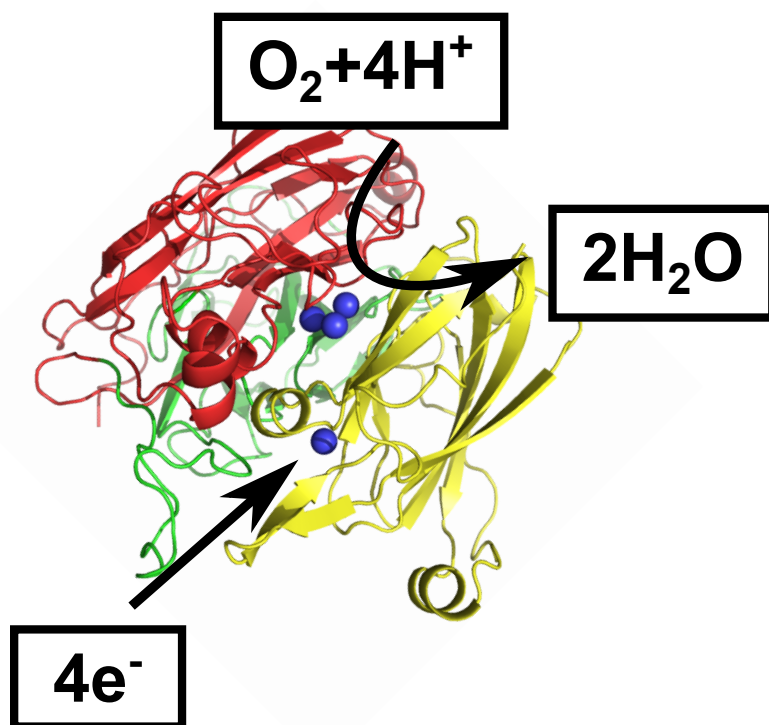
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Søren Brander

Characterization of novel thermostable bacterial Laccase-like multi-copper oxidases

PhD dissertation



Characterization of novel thermostable bacterial Laccase-like multi-copper oxidases

PhD dissertation

Søren Brander
Department of Chemistry
Technical University of Denmark

August 2014

Acknowledgement

This dissertation is submitted to the Department of Chemistry, Technical University of Denmark, in partial fulfillment of the requirement of the PhD degree in the subject of biophysical chemistry. The work was done as collaboration between the Kepp group at the Department of Chemistry and the Center for Bioprocess Engineering at the Department of Chemical and Biochemical Engineering. Time was shared between the two places. A half year research stay with the Solomon group at Stanford University was also part of this work. The project was funded by The Danish Council for Independent Research (FTP).

I would like to thank my supervisors, Prof. Kasper Planeta Kepp and Prof. Jørn Dalgaard Mikkelsen for giving me the opportunity to work on this project. After three years of study, I still find LMCOs to be the most beautiful and inspiring enzymes found in nature.

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Søren Brander, 1st of August, 2014

Summary

Laccase-like Multi-copper Oxidases (LMCOs) catalyze the oxidation of a broad range of substrates in a redox reaction coupled to the complete reduction of molecular oxygen to water. The reaction is catalyzed by four coupled copper-ions that are positioned in the LMCO in such a way, that the substrate bind to one side of the enzyme, while oxygen is recruited and water expelled on the other.

This powerful mechanism makes LMCOs clean enzyme substitutions in all chemical processes that are traditionally driven by the addition of reactive oxygen species such as hydrogen peroxide. E.g. dye decolorization, bleaching of paper pulp, delignification of biomass and remediation of polluted water.

In order to widen the applicability of LMCOs, it is important to establish the properties and substrate specificities of naturally occurring LMCOs. This is especially true for LMCOs from bacteria, whose role in nature is not well-understood. If we want to change a LMCO, to specifically catalyze a man-made reaction, it becomes important to have a diverse and stable starting protein. In this regard bacterial LMCOs are of special interest, because they are intrinsically thermostable and distinct variants can be found in the rapidly increasing number of sequenced bacterial genomes.

This dissertation describes our effort to identify and express novel LMCOs from bacterial origins. Some of these enzymes were also characterized, and special emphasis was put on revealing their substrate specificity and thermostability.

Bacillus clausii KSM-16 is known to produce a potent alkalophilic and thermostable protease that is sometimes used in laundry detergent mixes. We have expressed and characterized the LMCO coded in the genome of the same bacterial strain, and found that it is a thermostable enzyme with substrate specificity similar to that of the well-characterized *Bacillus subtilis* CotA. Stability and catalytic reactivity were both slightly less than *B. subtilis* CotA, while the preferred pHs for both properties were shifted about 1 unit to the more alkaline.

Thermobaculum terrenum is a thermophilic bacterium cultured from a hot dirt patch in Yellowstone National Park. It belongs to the evolutionary interesting phylum Chloroflexi that has been proposed to represent some of the earliest life-forms on Earth. The genome of *T. terrenum* codes for a LMCO, and we have expressed and characterized the enzyme. It is the second most thermostable

characterized LMCO, but was only able to selectively oxidize two out of 57 tested substrates.

Of special interest to the characterization of bacterial LMCOs is the thermostability. Measurement of thermal inactivation of LMCOs is hampered by an often observed heat-induced increase in enzyme activity. We found that heat activation is accompanied by a change in the Electron Paramagnetic Spectroscopy (EPR) spectrum, and used this to characterize the mechanism behind the process. It is a redox transformation, and for the *T. terreum* LMCO it was found to be controlled by temperature and NaCl, while the similar transformation in *B. subtilis* CotA also needed the reducing agent, ascorbate, in order to take place. The discovered mechanism can most likely be expanded to also encompass other LMCOs that have previously been shown to undergo heat-activation.

Dansk resumé

Laccase-lignende Multikobber Oxidaser (LMCOer) katalyserer oxidationen af en bred vifte af substrater i en redox reaktion koblet til den fuldstændige reduktion af molekylært ilt til vand.

Mekanismen katalyseres af fire kobber-ioner, der er placeret i LMCOen på en sådan måde at substrat binder til den ene side af enzymet, mens ilt bliver rekruteret til- og vand udstødt fra den anden side.

Denne effektive mekanisme gør LMCOer til affaldsfrie enzym-substituenten i alle kemiske processer, som man ellers ville fremme ved tilsætning af reaktive oxygenforbindelser såsom brintoverilte. F.eks. affarvning af tekstilfarve, blegning af papirmasse, fjernelse af lignin fra biomasse og rensning af forurenet vand.

For at udvide anvendelses mulighederne af LMCOer, er det vigtigt at fastlægge enzym egenskaber og substratspecificiteter af naturligt forekommende LMCOer. Dette er især tilfældet for LMCOer fra bakterier, hvis rolle i naturen ikke er forstået i detaljer. Hvis vi ønsker at ændre en LMCO til specifikt at katalysere en menneskeskabt reaktion, er det vigtigt at have et diversificeret og stabilt protein at starte fra. I denne forbindelse er bakterielle LMCOer af særlig interesse, fordi de generelt er varmestabile, og diverse varianter kan findes i det hastigt voksende antal af fuldt sekventerede bakterielle genomer.

Dette arbejde beskriver vores indsats for at identificere og udtrykke nye LMCOer fra bakteriel oprindelse. Nogle af disse proteiner er også blevet karakteriseret med særlig vægt på deres substrat specificitet og termostabilitet.

Bacillus clausii KSM-16 er kendt for at producere en potent alkalofil og termostabil protease, der undertiden anvendes i vaskemidler. Vi har udtrykt og karakteriseret en LMCO kodet i den samme bakterie stammes genom, og fandt, at det er et termostabilt enzym med substratspecificitet svarende til den velkarakteriserede *Bacillus subtilis* CotA. Stabiliteten og den katalytiske aktivitet er begge lidt lavere end *B. Subtilis* CotA. I mellemtiden er de foretrukne pH-værdier for begge egenskaber flyttet omkring 1 enhed mod det mere basiske.

Thermobaculum terrenum er en termofil bakterie kultiveret fra en varm jordbunds forsænkning i Yellowstone National Park. Den tilhører det evolutionært interessante phylum Chloroflexi, der er af nogen betragtes som repræsnetanter

for den tidligste livsform på Jorden. Genomet af *T. terrenum* koder for en LMCO, og vi har udtrykt og karakteriseret den. Det er den anden mest varmostabile karakteriserede LMCO, men ud af de 57 testede substrater, var den kun i stand til selektivt at oxidere to. Fra disse substrat tests og en sammenligning mellem relaterede gener og genomer har vi foreslået en specifik rolle for denne LMCO i biosyntesen af antibiotika. Dette er den første karakterisering af et protein fra *T. terrenum*.

Termostabilitet er i særdeleshed interessant for karakterisering af bakterielle LMCOer, men målingen af varme inaktivering hæmmes af en ofte observeret varme-induceret forøgelse i enzymaktivitet. Vi fandt, at varmeaktivering er ledsaget af en ændring i enzymets Elektron Paramagnetiske Spektroskopi (EPR) spektrum og har anvendt dette til at karakterisere mekanismen bag processen. Det er en redox-transformation og vi viser at processen for *T. terrenum* LMCO er kontrolleret af temperatur og NaCl, mens den tilsvarende omdannelse af *B. Subtilis* CotA også skal bruge et reduktionsmiddel for at finde sted. Den beskrevne mekanisme kan sandsynligvis udvides til også at omfatte andre LMCOer, der bliver varme aktiveret.

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1 Introduction

1.1.1 Oxygen chemistry in biology

One of nature's most precious inventions is the utilization of free oxygen in metabolism. In geological time, the emergence of atmospheric oxygen coincides with the formation of the ozone layer, the evolution of photosynthesis and the appearance of most animal groups¹. All are things that are essential for the ecosystem of the Earth in general. Normally we are only concerned about oxygen when there is too little of it. E.g. when we are getting winded from a hard exercise, or getting dizzy in a stuffy office without ventilation. However, Life emerged in an oxygen-free (anaerobic) environment and have since then adapted to increasing concentrations of oxygen¹.

Oxygen chemistry is complicated for cellular organisms, because complete reduction of oxygen into water takes four electrons, and each intermediate step produces a toxic oxygen species as outlined in Figure 1².

Of the intermediate oxygen species, hydrogen peroxide is the most commonly known species. It is widely used as a household chemical in e.g. stain removal and disinfection. It is also the main component in hair lightening mixtures, massively used in paper pulp bleaching and forms the basis of the active oxygen species used in laundry detergents and dishwashing powders. A similar chemical of popular use is bleach and the two are often interchangeable³

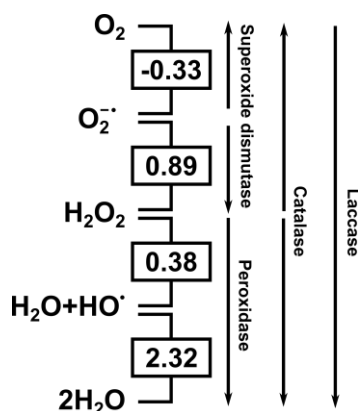


Figure 1 Oxygen species and redox potentials. Stepwise reduction of oxygen to water is outlined and intermediate oxygen species labelled. The redox potential of the non-enzymatic one electron reduction steps are given at pH 7 compared to the standard hydrogen electrode². Enzymatic transformation of the oxygen species is indicated by arrows⁶.

Within living cells, the reactive oxygen species are generated due to e.g. incomplete respiration or photosynthesis, and they cause oxidative stress when not countered. The unspecific reactivity of the oxygen intermediates is mostly unwanted and will damage essential cellular components e.g. lipids, proteins and DNA⁴. As a consequence of the potentially lethal actions of reactive oxygen species, a lot of cellular energy is expended on controlling these species⁵.

Figure 1 shows the relevant oxygen species, that occur as intermediates in reduction of oxygen to water, along with the redox potential of the one electron reduction steps at neutral pH². The redox potential is a relative measure of the ability to absorb an electron from a chemical compound. In chemical terms, such loss of an electron leaves the compound “oxidized”, and conversely the compound receiving the electron is “reduced”. From the redox potentials of the oxygen species, it can be seen that oxygen is relatively unreactive, while superoxide and the hydroxyl radical are very reactive. Figure 1 also outlines the biological mechanisms to remove these toxic species. Superoxide dismutase converts superoxide to oxygen and hydrogen peroxide. Catalase turn hydrogen peroxide into oxygen and water. The hydroxyl radical is so reactive that nature’s best solution is to have a lot of antioxidants, such as Vitamin K and Vitamin C, around to absorb the harmful radical⁶.

1.1.2 Laccases

One class of enzyme that reduces oxygen, but avoids the generation of intermediate oxygen species, is the laccases. They encompass four connected copper atoms that function as a small battery, and only when the enzyme is fully charged (with four electrons) does reaction with oxygen proceed. In popular terms this makes laccases clean machines that can drive a chemical reaction worth of approximately 800mV, powered only by oxygen and with only water as a byproduct (see Figure 2). This is an astonishing feature in itself, but also highly relevant to industrial applications where oxygen is cheap and clean reactions a bonus. The prospect of using laccases as a green and controlled substitute for reactive oxygen species in industry and households has fueled a continued research into laccase and their application.

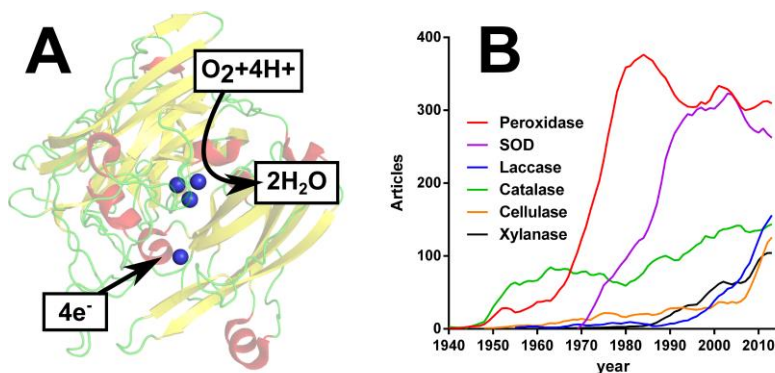


Figure 2 Introduction to laccases. A) The three-dimensional structure of CotA laccase from *Bacillus subtilis* 168 in a cartoon representation with the four copper ions shown as blue spheres (PDB:1GSK). Four substrate molecules react in sequence with the exposed copper in the lower left and donate four electrons. Oxygen will in turn react with the three nuclear copper-cluster and produce water. B) New articles per year in the period 1940-2013 with the words peroxidase, superoxide dismutase or SOD, laccase, catalase, cellulase or xylanase in the title. Data is retrieved from the PubMed database <http://www.ncbi.nlm.nih.gov/pubmed>.

People who have used bleach as cleaning or disinfection agent will most likely be able to tell how even a small spill will discolor their clothes. This goes to show how effective oxidative treatment is in decolorizing dyes, and by a similar mechanism laccases can decolorize textile dyes⁷. When immobilized on a filter membrane⁸ or a bacterial biofilm⁹ laccases are able to persistently decolorize wastewater from e.g. a textile manufactories¹⁰ or olive oil plants¹¹. This is perhaps the best example of an application where the reusable activity of laccases is preferred over the burst activity of adding a chemical oxidant such as hydrogen peroxide or bleach. If the wastewater is to be discharged directly to the sewers or rivers, bleach is even less preferred.

Another interesting application of laccases, is decolorization of lignin in preparation of paper. Most cardboard boxes have a brownish hue due to lignin, and this component has to be removed in the manufactory of e.g. white paper. This process is mostly achieved by a massive addition of hydrogen peroxide or bleach, and these can to some extent be replaced by laccases¹². Lignin constitute about a fourth of the plant cell wall, and a major incentive in laccase research is the possibility of activating this biomaterial for further fermentation into biofuels¹³. In contrast to most enzymes, laccases are not always limited to catalyze a specific reaction. Instead a laccase will catalyze the oxidation of a chemical, if it

comes close enough to the first copper and does not have redox potential much larger than that copper¹⁴.

The beneficial properties of laccases have spurred an amazing amount of scientific effort into characterizing new laccases, both in an effort to extend the applicability, but also to understand the mechanistic details behind the oxygen reduction that is still unmatched in efficiency by synthetic catalysts¹⁵. Laccases has seen an almost exponential rise in publication activity as shown in Figure 2B. while for example the interest in superoxide dismutase has peaked. Xylanases and cellulases have great synergy with laccases in opening biomass^{16,17}, and especially the latter is in a sharp uptrend starting in 2008.

Characterization of novel laccases is interesting both with respect to basic and applied sciences. Bacterial laccases are relative new additions to the laccase family and have attracted growing attention since the first characterization of a potent bacterial laccase-like enzyme from *Bacillus subtilis* in 2001¹⁸. In general the biological substrates of these enzymes are unknown. This can be seen as a potential for new applications, because it implies new and untested substrate possibilities. More important to the applicability is perhaps the inherited stability of bacterial laccases. Especially in continues applications, such as the remediation of dye wastewater, is stability of the laccase preparation paramount. Stability is also advantageous in chemical reactors where high temperatures¹⁹ or an organic co-solvent might be preferable²⁰.

A dream is to enable the advantageous properties of laccase-catalyzed oxygen reduction for a non-biological reaction. In other words, to optimize the protein sequence in such a way, that new functionality arises. However, new enzyme functionality is generally followed by a loss of stability²¹, which necessitates a very stable laccase to start from.

The aim of this project was to identify novel super-stable laccases that can be used for a later laboratory steered optimization. Bacterial laccases was of exclusive focus due the high stability and relatively easy cloning into recombinant protein expression systems. At the same time, bacterial laccases are not well-understood and in the process of identifying new laccase-like proteins, we hoped to extend our general understanding of the functions that govern activity and stability of bacterial laccase.

2 Laccases and Laccase-like multicopper oxidases

2.1.1 Laccase-like Multi-Copper Oxidase

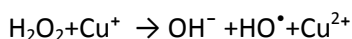
Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are three domain multicopper oxidases (MCOs) that can sequentially abstract one electron from four molecules of substrate and in turn reduce molecular oxygen to water²². The laccase class of enzymes is heterogenous, and the classification as a benzenediol oxidoreductase is only strictly correct for the earliest identified laccases from trees²³. In present time, many enzymes have been identified that have topology and oxygen activation mechanism similar to laccases, but does not show substrate specificity towards benzenediols. Often these enzymes are promiscuous in activity, and it does not make sense to categorize them by one reaction. The term Laccase-like Multi-Copper oxidase (LMCO) has been proposed to encompass these²³, and this term will be used in the rest of this dissertation except when discussing laccases from the *Rhus* family for which the name laccase was originally devised.

This chapter will introduce aspects of LMCO research that are important to the understanding of the scientific results reported here. Focus is on the biological role of LMCOs in general and some mechanistic properties in particular.

2.1.2 Fenton's chemistry and oxidation of transition metals

One LMCO substrate activity is the oxidation of inorganic transition metals such as iron(II) and copper(I). This is fundamentally different from the oxidation of phenolic substrates of laccases and most LMCOs. However, the biological activity is related to the discussion of reactive oxygen species in Chapter 1.

If iron(II) or copper(I) ions are not somehow protected, they will reduce hydrogen peroxide to form hydroxyl radicals in what is known as Fenton's chemistry⁴, e.g.



As discussed in the introduction, hydroxyl radicals are very toxic to cells, and the best biological defense is to mass antioxidants. Selective oxidation of iron(II) and

copper(I) by LMCOs is one way to counter the formation of hydroxyl radicals by removing the precursors.

Ferrous or cuprous oxidase activities of LMCOs are perhaps the most ubiquitously functionality in biological systems. In fact such enzymes have been observed in most forms of Life. Fet3p is a ferroxidase LMCO from the fungus *Saccharomyces cerevisiae* (Baker's yeast) and has been a model enzyme for the characterization of ferrous oxidase activity^{14,24}. Similarly the bacterial CueO from *Escherichia coli* is a model enzyme for cuprous oxidase activity²⁵. The activity is also observed in some trees²⁶, in insects *Anopheles gambiae* (mosquitoes)²⁷ in primitive bacteria²⁸ as well as archaea^{29,30}. The blood of humans and other mammals contain ceruloplasmin, a ferroxidase of similar topology to LMCOs³¹.

2.1.3 Plant laccases

Laccase activity in the sap from Japanese liquor tree *Rhus vernicifera* was first reported in 1883³², making it one of the first known enzymatic processes. The sap contains, among other things, a laccase and the benzenediol uroshiol. If such a tree is damaged and the sap exposed to moist and oxygen, the uroshiol will undergo a laccase catalyzed polymerization that forms a lacquer film and mends the wound³³. Unoxidized uroshiol causes extreme contact dermatitis known from related species that do not produce a laccase in the sap. e.g. *Rhus radicans* (poison ivy)³⁴. The laccase oxidation of liquor tree sap has been exploited for thousands of years in the making of traditional Chinese and Japanese lacquerware³⁴.

Plant LMCOs are rarely reported, but they are expected from gene analysis to be found in the xylem and potentially involved in the formation of lignin³⁵. This functionality is still debated³⁶. A recent study show that deletion of two LMCO genes in *Arabidopsis thaliana* impairs growth of the plant, and significantly decrease the lignin content³⁷.

2.1.4 Fungal LMCOs

Laccase-like activity was first observed in mushrooms in 1896³⁸. LMCO genes are present in all sequenced fungal genomes and show a diverse set of

functionalities³⁹. Some are expressed in plant pathogenic fungi as part of the virulence response. Either as a protectant against chemicals secreted in defense of the plant e.g. tannins, resveratrol and phytoalexin⁴⁰ or as an aggressive virulence factor in the detoxification necrotic plant material⁴¹. Many fungal LMCOs catalyze the formation of melanin pigments that function in protection against UV light and general oxidative stress. This also holds importance as a defense mechanism against oxidative immunoresponses against pathogenic fungi⁴². This is best described for the human pathogen *Cryptococcus neoformans*, that infects the brain and causes fungal meningitis and a visibly melanin colored brain tissue^{43,44}.

LMCOs are often found secreted from fungi in phylum Basidiomycetes, aptly named white-rot fungi for their ability to degrade lignin in decaying wood. This is a highly interesting function for the industrial production of bioethanol, in which lignin and lignin degradation products impair the release of fermentable sugars^{45,46}. Indeed, natural productions of these LMCOs are induced by small molecules that resemble the building blocks in lignin⁴⁷. The possibility of LMCO mediated removal or modification of lignin is perhaps the most important reason for the constant interest in this enzyme class⁴⁸.

The redox potential of the substrate binding copper is approaching 800mV for an isomer of the white-rot fungus *Trametes versicolor* LMCO⁴⁹. This is the highest reported LMCO redox potential and significantly more than the 410mV reported for *R. vernicifera* laccase⁵⁰. Because a high redox potential increases both turnover rates and the range of accessible substrates⁵¹, LMCOs from Basidiomycetes appear to be the most useful class for industrial applications. However, as with most proteins from higher organisms, these laccases needs extensive and specialized post translational modifications, e.g. cysteine bridges and glycosylations, making heterologous over-expression problematic⁵². In addition they are most active and stable at low pH which is not always desirable⁵³.

2.1.5 Bacterial Laccase-like Multi-Copper Oxidases

A LMCO in the plant symbiotic bacterium *Azospirillum lipoferum* was reported in 1993⁵⁴, which makes bacterial LMCOs one of the more recent additions to the LMCO group. They are often found to be involved in formation of pigmentation in sporulating bacteria or have ability to oxidize trace metals, e.g. copper(I), iron(II), manganese(II), manganese(III)⁵⁵.

The biological roles of bacterial LMCOs are generally unknown, however two common laboratory strains, *Escherichia coli* K-12²⁵ and *Bacillus subtilis* 168¹⁸ have been found to naturally produce LMCOs, and these have been characterized in details. Copper efflux Oxidase (CueO) from *E. coli* was characterized in 2001²⁵ and found to be directly involved in oxidation of toxic copper(I) and indirectly in regulation of the storages of copper and iron⁵⁶. The functionality is modulated by the concentration of copper(II) and at high concentrations, the enzyme show laccase-like activity on phenolic substrates²⁵. Spore coat protein A (CotA) from *B. subtilis* was identified in 2001 as a LMCO crucial to the formation of spore pigmentation¹⁸. It is a relative potent LMCO with a redox potential of 525 mV⁵⁷ and broad substrate specificity. Apart from being a pigment synthase, the *B. subtilis* CotA has also been found to efficiently oxidize phenolic compounds²³, bilirubin⁵⁸ and various dyes⁵⁹.

The broad substrate range goes to show the versatility of bacterial LMCOs and makes them a viable alternative to fungal LMCOs in industrial processes⁶⁰. Bacterial LMCOs have the advantage of being non-glycosylated and often possible to produce heterologously, sometimes to a very high yield⁶¹. They are mostly active at neutral or slightly alkaline solutions, which makes them different from fungal laccases, and importantly they are found to be very stable towards heat and organic solvents¹⁹.

2.1.6 Structure of Laccase-like Multi-Copper Oxidases

The first complete structure of a LMCO was reported by Messerschmidt et al. in a series of articles from 1989 to 1993 for the ascorbate oxidase from Zucchini⁶²⁻⁶⁴. As of July 2014 30 crystal structures of sequence-wise different but structural homologue LMCOs have been deposited in the Protein Data Bank, including structures of *B. subtilis* CotA (see Figure 3A). The tertiary structure is defined by three cupredoxin domains that wrap around a central core containing the four coppers. These are arranged in such a way that the tri-nuclear copper center (TNC) is exposed to oxygen on one side of the LMCO, and one copper is open for binding substrate on the other. The two active sites are connected through the backbone of the copper coordinating amino acids as shown in Figure 3B in a manner that allow for electron transfer to take place between the coppers via super-exchange pathways²².

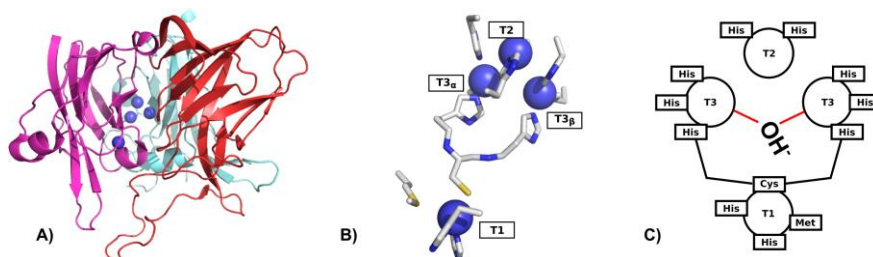


Figure 3: Structure and copper coordination of *Bacillus subtilis* CotA. A) Cartoon structure of *Bacillus subtilis* CotA (3ZDW) with the three cupredoxin domains visualized in cyan, red and purple. The four coppers are represented by blue spheres. B) Close-up on the coordination of amino acids to the coppers in CotA. C) A general diagram of the resting state copper coordination in LMCOs. Black lines between two amino acids indicate that they are connected through the backbone. Red lines show a hydroxide bridge that antiferromagnetically couples the two T3 coppers.

The coppers are named by their spectral features; Type 1 (T1), type 2 (T2) and type 3 (T3) as annotated in Figure 3B and Figure 3C⁶⁴. The T1 copper is positioned in the substrate binding site. It is coordinated by two histidine residues and a cysteine in a slightly distorted trigonal planar geometry which give rise to a characteristic Electron Paramagnetic Resonance (EPR) spectrum²². In addition the cysteine forms a charge transfer complex with the T1 copper and gives rise to an UV-VIS absorption signal around 600nm and the blue color of LMCOs. In LMCOs a fourth T1 ligand is most often methionine, but can be leucine, isoleucine or even phenylalanine in fungal LMCOs^{65,66}. In the oxygen binding site two T3 coppers are bridged by a hydroxide ion which makes them an antiferromagnetic binuclear couple. They are EPR inactive but give rise to an ultra violet absorption peak at 330nm. Adjacent to the T3 binuclear couple is a T2 copper that does not absorb UV or visible light, but has well resolved EPR spectrum²². Together the T2 and T3 coppers form the TNC.

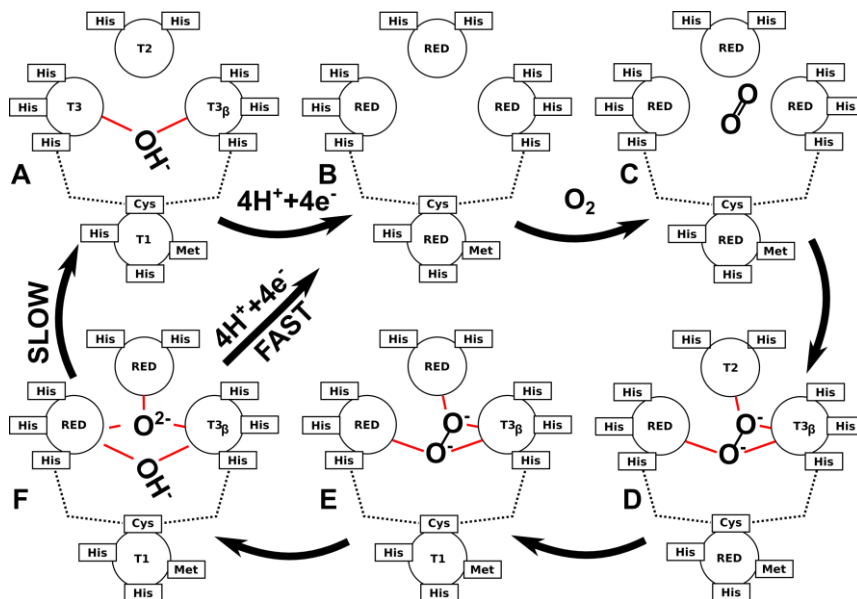


Figure 4: Catalytic cycle of LMCOs. The LMCO copper network is outlined similarly to Figure 3. In addition reduced coppers are labelled with RED and the T3 β is labelled to differentiate between the non-coupled T3 copper sites. The “resting oxidized” state A) can react with reducing agents to form the fully reduced state B) that in turn will bind molecular oxygen C). The di-oxygen is reduced to peroxide in what is known as the Peroxide Intermediate (PI) D) and an electron is transferred from the T1 site to the TNC giving the PI+e state E). The peroxide bond is further reduced to water in the Native Intermediate (F). If more reducing agent is available, the cycle is quickly reentered by going directly to B). Otherwise the F) state will decay into the more stable, but less reactive A). Figure is an adaption from Solomon et al.⁶⁷

2.1.7 Mechanism of Laccase-like Multi-Copper Oxidases

The submolecular mechanism of LMCO catalysis is known in some details from stabilized intermediates that have been characterized by their spectral properties and quantum mechanical calculations⁶⁷. When not in a catalytic cycle, the LMCO is in the “resting oxidized” state that is fully redox active, and will be reduced by four molecules of substrate (See Figure 4). The reduced enzyme recruits an oxygen molecule that binds in the TNC and extracts one electron from the T2 and the T3 β copper and in turn forms what is known as the Peroxy Intermediate. The electron from T1 is then transferred to the TNC to make a very reactive copper(I)-peroxy intermediate that readily cleaves the dioxygen bond. The resulting Native Intermediate has all copper(II) and an unusual μ_3 -oxo ligand bridge between the coppers in the TNC. This complex is speedily reduced if additional substrate is available, otherwise the complex will decay back into the resting state⁶⁸. The reaction rate of the native cycle can reach 650 s^{-1} for the *R. vernicifera* laccase,

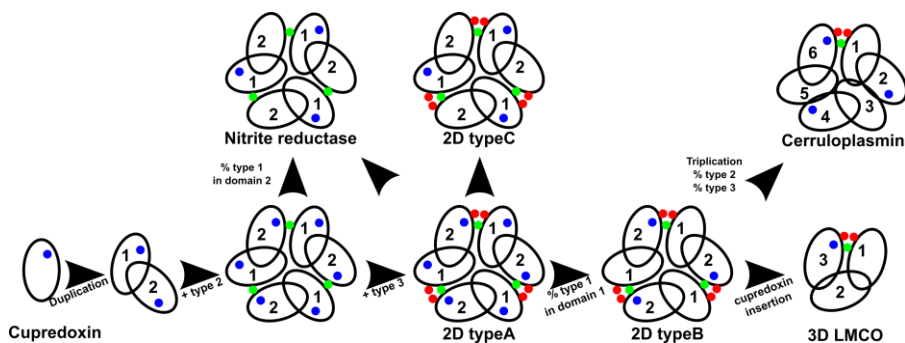


Figure 5: A model of LMCO evolution by domain duplications. An ancient cupredoxin domain is gene duplicated and evolves an inter-molecular T2 copper site. This basic enzyme might have lost one the T1 copper centers similar to the Copper-containing nitrite reductases. It can also acquire a T3 copper pair to form the two domain typeA form which in the trimeric state has all the coppers of known MCOs. This form evolved to typeB and typeC by the loss the T1 copper site in the domain 1 and 2 respectively. TypeB is also known as small-laccases and shares topology and reactivity with LMCOs. Adapted from Nakamura et al.⁷³

while the rate of the decay to the resting state is $0.058s^{-1}$ ⁶⁹. The reduction of the “resting oxidized” state is slow and pH dependent, with a reported lack phase of 25 seconds at pH 7.4 and a delay of 40 seconds for all the enzyme to be fully reduced and entered into the native catalytic cycle⁷⁰.

2.1.8 Evolution of Laccase-like Multi-Copper Oxidases

LMCOs are not the only multi copper oxidases based on cupredoxin domains. Several one domain cupredoxin proteins with one T1 copper has been identified and function as electron shuffles in respiratory pathways, e.g. plastocyanin in plant photosynthesis⁷¹ or pseudoazurin in bacterial denitrification⁷². They use the same set of copper binding ligands as do most LMCOs, i.e. two histidines, one cysteine and one axial methionine. Because the T1 cupredoxin motif is not only found in LMCOs, but also in plastocyanins, nitrite reductases and ceruloplasmin an evolutionary process of cupredoxin domain duplication has been proposed as shown in Figure 5⁷³. This scheme is validated by the later characterization of two domain laccase-like enzymes typeB^{74,75} and typeC⁷⁶. Nitrite reductases and two-domain LMCOs are trimeric in their active form with the T2 or TNC coppers shared between the monomers. This makes the active protein a six cupredoxin domain enzyme similar to ceruloplasmin. In this light, with only three cupredoxin domains, LMCOs are actually the smallest of the multi domain MCOs.

Analysis of general protein evolution suggests that the cupredoxin domain is the earliest evolutionary copper binding protein, emerging about the time when free oxygen became available⁷⁷. Before, bacteria had been living anaerobically with little oxidative stress. The widespread use of the cupredoxin in different enzyme roles suggest an immediate need for the organism to respond to the toxic oxygen, and hence favored diversification, e.g. duplication, of one of the only radical scavenging proteins⁷⁸.

The evolutionary path hypothesized after analysis of the sequential and structural differences of MCOs is outlined in Figure 5. Duplication of a plastocyanin-like protein together with addition of a T2 copper binding site is basically enough to evolve a plastocyanin-like enzyme into a nitrite reductase. Similarly, the other observed enzyme forms can be thought to have evolved in a series of loss or gains of copper binding sites or cupredoxin domains (see Figure 5).

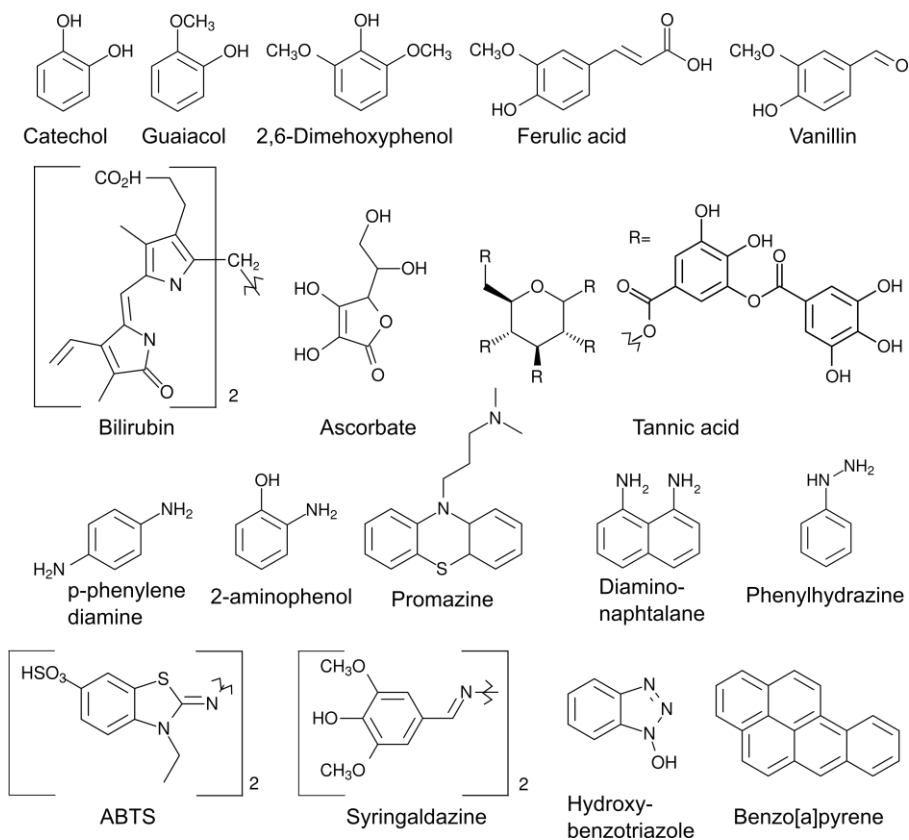


Figure 6: Some organic substrates for LMCOs. All structures were prepared with BKchem 0.13.0

2.1.9 Organic Substrates of Laccase-like Multi-Copper Oxidases

LMCOs are very versatile in their substrate affinity. In principle they will react with anything that fits into the substrate binding site and has lower redox potential than the T1 copper. However, the substrate binding site can make interactions to the substrate such as to change the redox potential¹⁴. LMCOs are known for their ability to oxidize phenolic compounds, with some examples shown in Figure 6 row 1. Catechol, guaiacol and 2,6-Dimethoxyphenol (DMP) are common substrates that resemble lignin monomers and are oxidized by most LMCOs²³. True lignin monomers like ferulic acid are para substituted with an electron-negative group that by mesomerism destabilizes the phenolate anion and increases the redox potential of the molecule⁷⁹ This makes especially vanillin hard to oxidize²³.

Some naturally occurring substrates of possible biological importance are shown in row 2. Bilirubin is a breakdown product of heme complexes and is the culprit of the discolored skin of patients that suffer from jaundice. LMCOs are often capable

of oxidizing bilirubin and it has been suggested to use this activity in a kit to quantify the state of jaundice in patients⁸⁰. Specific ascorbate oxidase activity has been observed for LMCOs in *Cucurbita* plants, e.g. squash⁶⁴. Ascorbate is commonly known as vitamin C, and it is unknown how the ascorbate oxidase activity can be an advantage to the organisms⁸¹. Ascorbate has a low redox potential and reacts with most LMCOs, but does so very slowly. In fact it is has seen use in combination with the favorable substrate benzene-1,4-diol (hydroquinone) in sub second kinetics where the ascorbate readily reduces the colored product of hydroquinone, making it possible to follow the spectrophotometric changes of the copper chromophores⁸². Tannic acid is a condensation of a varying number of gallic acid moieties and glucose. It is a plant produced antibacterial with high chelation strength especially towards iron, which is often the limiting nutrient for microbial growth. Gallic acid is a favorable LMCO substrate, and because degradation of tannic acid is advantageous to soil living microbes, this activity might have a biological role⁸³.

Despite being named for their ability to oxidize phenolic compounds, laccases and LMCOs are quite capable of oxidizing phenylamines as well. Some of these substrates are shown in Figure 6 row 3. P-phenylenediamine is a reactive, low redox substrate. A 2-aminophenol derivate is the natural substrate of a LMCO from *Streptomyces antibioticus* in the formation of a phenoxazinone antibacterial agent⁸⁴. Promazine is a substituted phenothiazine that has been reported to be oxidized by bacterial LMCOs, but not fungal²³. 1,8-diaminonaphthalene is another strong metal chelator that is readily oxidized by LMCOs, and it has been used as a developing agent for LMCO specific zymograms⁸⁵. Phenylhydrazine is a common reagent for chemical synthesis and has recently been reported as a LMCO substrate⁸³.

The last row in Figure 6 show some synthetic substrates of which 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is of immense scientific importance, mainly because it makes a good substrate for activity assays. It is fairly stable, water soluble, has a high LMCO catalyzed turnover and forms a strongly absorbing product. It is symmetrical over a central azine bond, making it rather large and doubly charged. Despite the uncommon geometry of ABTS, some LMCOs are reported to have high affinity for the substrate with K_m values as low as a few μM ⁸⁶. Syringaldazine is another very common substrate with two DMP moieties coupled through an azine linkage⁸⁷.

The oxidation of a LMCO substrate typically involves the loss of a single electron and the formation of a free radical. The radical is in general unstable and may undergo further laccase-catalyzed oxidation or nonenzymatic reactions e.g., hydration, disproportion or polymerization⁸⁸. Alternatively, some substrates like ABTS and 1-hydroxybenzotriazole forms relatively stable radicals, which makes them potent redox mediators in the oxidation of compounds too big to fit into the LMCO substrate binding site⁸⁹. This property is very useful in the oxidation of bulky substrates like benzo[a]pyrene⁹⁰, various azo dyes and even lignin polymers⁸⁹.

2.1.10 Stability of Laccase-like multi-copper oxidases

LMCOs are often thermostable with optimal reaction temperatures above 45°C¹⁹. The most stable characterized fungal LMCO is isolated from *Pycnoporus cinnabarinus* with an estimated 2 hours half time of inactivation at 80°C and some residual activity after 245 days of incubation at 37°C⁹¹. The latter result goes to show that thermostability often translates into general stability and becomes very beneficial for prolonged processes. Prokaryotic LMCOs are often very thermostable. The currently longest lived LMCO is derived from *Thermus thermophilus* with a half time of inactivation of 868 minutes⁹². Thermostability can also be translated into stability against various co-solvents exemplified by the two domain LMCO from *Streptomyces sviveus* that has a half time of inactivation at 80°C of 10 minutes and 60-80% residual activity after overnight incubation in 40% of either DMSO, methanol, ethanol, 2-propanol, acetonitrile⁷⁵.

The activity of a LMCO preparation does not always strictly decrease after incubation at elevated temperatures, but is sometimes first activated. The mechanism behind heat activation is not well understood, but it has been reported for several LMCOs including the fungal *Mycelium thermophila*, the bilirubin oxidase from *Myrothecium verrucaria*⁹³ and CotA from *B. subtilis*⁹⁴. As much as nine times higher activity of the heat activated preparation has been reported⁹⁵.

3 Results and discussion

3.1 Project aims and outlines

This project started out with the intention to rationally or randomly create a LMCO variant of improved stability or activity over the wild-type version. However, we came to realize that this was too much work for a one man project taking place in a laboratory with no previous experience with bacterial LMCOs. A decisive change of strategy was instigated when we realized that heat activation of bacterial LMCOs would make high-throughput screening of stability problematic without a better understanding of the process. Instead we chose to characterize some wild-type LMCOs of potential high thermostability and went on to investigate the mechanism behind heat-activation.

Some of the strategic decisions, made in the planning of this project, were governed by the intention to make LMCOs of unnatural properties. Directed evolution especially holds some promise to achieve this goal. It makes use of error-prone PCR to introduce random mutations into a gene. Subsequently these genes are transformed into an expression host, and a very high number of clones are screened for advantageous changes in enzyme properties. Such experiments have been done excessively for the fungal LMCO from *M. thermophila* by screening for high turnover of ABTS and syringaldazine⁹⁶ in increasing concentration of co-solvents⁹⁷ and at high temperatures⁹⁸.

An attempt to do directed evolution of the *B. subtilis* CotA protein has been reported by Brissos et al. using heterologous expression in *E. coli*⁵⁹. However the experiment is only preliminary. They performed only one round of activity screening without previous diversification of the gene library. A naturally occurring protein has been under much more evolutionary pressure than we can create in the laboratory by simple means such as a single round of random mutagenesis. Any mutant that is found in such a way must necessarily already have been tested in nature⁹⁹. As a consequence, a single mutation must be considered a trade-off between protein properties in which the scientist has a different priority than the wild-type host. An often observed trade-off is between activity and stability¹⁰⁰ and this is one of the main reasons why this project was focused on thermostable LMCOs. Having a very stable starting enzyme, means more possibilities to improve an unnatural activity.

If truly new functions are to be given a protein, it must be relaxed away from the specialized state that is found in wild-type organisms. One way to achieve this it to make a few rounds of random mutagenesis with a selection cut-off that is set to less than the property of the wild type enzyme. After generating a big library of mediocre variants, the selection cut-off can be increased step-wise in a few rounds of directed evolution. One alternative is to use gene recombination after DNase treatment to randomly combine two or more proteins sequences and use the resulting chimeric gene as a starting point for directed evolution¹⁰¹. With the latter process in mind, it is interesting to develop a set of diverse LMCOs for potential recombination.

3.1.1 Outline of dissertation

During the work reported in this dissertation, some novel bacterial LMCOs were identified and characterized. The LMCOs are identified by genome mining in the non-redundant RefSeq database¹⁰² and the work is reported in section 3.2.1. 15 genes were cloned and expressed in *E. coli* and this is reported in section 3.2.2, 3.2.4 and 3.2.5. A few of these have been characterized in detail as discussed in section 3.3 and 3.4 and reported in Paper I and Paper II. Of particular interest was the thermostability and heat activation of these enzymes. Some insight into the associated process is discussed in section 3.5 and 3.6.1 and reported in Paper III

In general this chapter tries to rationalize and explain the work that is not easily reported in journal articles. Two unpublished result of some novelty is the generation of a fluorescent LMCO which is discussed in section 3.2.3 and the preliminary characterization of a unique MCO as discussed in section 3.6.3

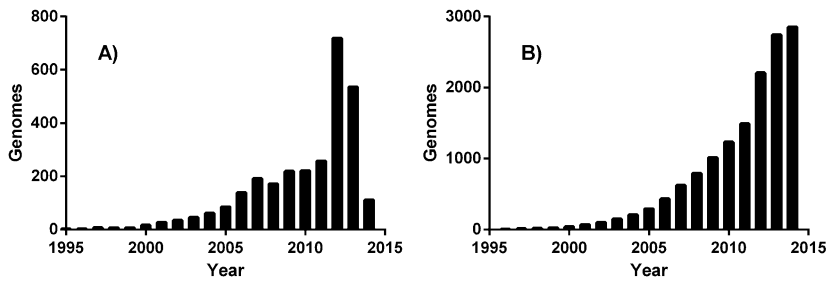


Figure 7: Sequenced prokaryotic genomes 1995-2014. A) Annual additions to the KEGG database. B) Cumulative total of bacterial genomes

3.2 Identification and expression of LMCOs

3.2.1 Identification of LMCO genes

In the beginning of this project in 2011, more than 1400 fully sequenced prokaryotic genomes were available. As of July 2014, this number has increased to more than 2800 as derived from www.genome.jp/kegg/catalog/org_list.html (see Figure 7). In comparison only 259 eukaryotic genomes have been sequenced. The growing number of sequenced genomes is a source of protein diversity and possibly new functions and enzymes. Especially so if protein properties can be estimated from the properties of the organism, e.g. pH profile, stability, salt tolerance¹⁰³

The most reactive bacterial LMCO is of the *Bacillus* CotA type²³. They are remarkably thermostable despite being derived from mesophile bacteria and our first approach to identify new thermostable LMCOs was to search the genome databases¹⁰² for *B. subtilis* CotA homologues from extremophile organisms. Alternatively we also looked for homologues from other niches where laccase-like activity seemed beneficial. We used the CotA protein sequence as a template in a psi-BLAST search for homologues using the on-line tool at blast.ncbi.nlm.nih.gov/Blast.cgi. This is essentially the same thing as building a hidden Markov model (HMM)¹⁰⁴ as was later reported by Ausec et al. for different MCOs, including LMCOs¹⁰⁵. The output of both the psi-BLAST and the HMM method produced a list of more than 1000 homologue sequences and the difference was mostly in the ranking. We went on to manually reduce the list by

handpicking interesting genomes following three selection criteria for the organism: Not anaerobic, not a pathogen or plant symbiotic and must live in an interesting habitat.

Anaerobic bacteria are often found in extreme environments, but they are unlikely to produce oxygen consuming enzymes like the LMCOs and were thus discarded. Pathogen or plant symbiotic bacteria are often sequenced due to their importance in medical or agriculture sciences. However, they are expected to grow in non-extreme environments and thus ruled out.

www.ebi.ac.uk/genomes/bacteria.html hosts a list of the bacterial genomes together with a very short description of the organism and from this information, together with the results from the psi-BLAST, a tentative list of interesting genes were put together. Some highlights with the UniProt accession number in parenthesis is listed here:

- *Acidobacterium* sp. MP5ACTX9 (E8WV80): Acidophilic bacterium
- *Aquifex aeolicus* (O67206): Growth 85-95°C oxygen respiration produces water
- *Bacillus clausii* KSM K16 (Q5WEM6): This particular strain is known for producing extremely alkalophilic proteins, and M-protease which is a common enzyme in washing agents.
- *Halorubrum lacusprofundi* (B9LMQ8): Isolated from hypersaline lake in Antarctica
- *Meiothermus Silvanus* (D7BGT0): Thermophile that grow on stainless steel machinery in a paper mill.
- *Pyrobaculum aerophilum* (Q8ZWA8): Grows optimally at 100°C
- *Stigmatella aurantiaca* (Q08U34): Grows on decaying wood.
- *Thermobaculum terrenum* (D1CEU4): Isolated from soil in Yellowstone National Park. Optimal growth at 68°C
- *Thermus thermophilus* HB27 (Q72HW2): Optimal growth at 65°C

The fact that the LMCOs from *P. aerophilum*, *A. aeolicus* and *T. thermophilus* HB27 showed up on the list, gave us confidence in the method. These three are already characterized and show half-times of inactivation when incubated at 80°C of 330 minutes³⁰, 60 minutes¹⁰⁶ and 868 minutes⁹² respectively. This made them the most thermostable bacterial LMCOs characterized as of 2011.

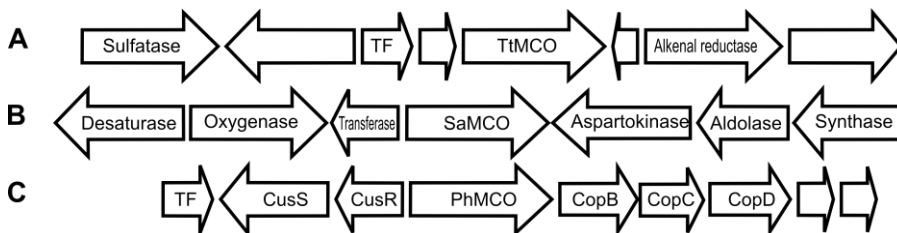


Figure 8 Outline and annotation of genes coded in the vicinity of three selected LMCO coding genes.

A) *Thermobaculum terrenum* is an ancient organism and gene homology is not clear. However, no copper resistance genes were predicted in the vicinity of the LMCO (TtMCO) gene. B) *Stigmatella aurantiaca* LMCO (SaMCO) is coded in putative gene cluster together with genes coding for various synthases and related enzymes. C) *Pseudoalteromonas haloplanktis* LMCO (PhMCO) is coded together with a full set of copper resistance proteins. Transcription factor (TF) binding DNA sequence, Copper sensor kinase (CusS), copper sensing regulator (CusR), Copper resistance protein B (CopB), Copper resistance protein C (CopC), Copper resistance protein D (CopD).

The full list contained 72 interesting organisms with genomes that code for at least one Cota homologue. In choosing genes for further characterization, it was decided to increase the chance of selecting an enzyme with high activity against organic substrates rather than cuprous or ferrous oxidase activity. The cuprous oxidase CueO from *E. coli* contain a methionine-rich loop that binds extraneous copper(II) and modulates the activity of the enzyme¹⁰⁷. Characterized cuprous oxidases from *Rhodococcus erythropolis*¹⁰⁸ and *A. aeolicus*¹⁰⁶ contain a methionine-rich loop of unknown functionality. Methionine-rich loops have exclusively been characterized in LMCOs with cuprous oxidase activity, and even though the position of the loops is not necessarily conserved, we chose to regard such methionine-rich loops as a general indication of cuprous oxidase activity.

Many of the interesting LMCO sequences contain a methionine-rich loop and it was decided to discard these sequences as putative cuprous oxidases. For similar reasons, genes that were encoded in vicinity of other copper resistance, e.g. copper efflux pump proteins were avoided. Some examples of genome regions that encode LMCOs are shown in Figure 8. The gene localization and annotations were retrieved from NCBI, e.g. www.ncbi.nlm.nih.gov/gene/?term=Q08U34

Figure 8 show three LMCO encoding genome regions that are representable for this study. Often the neighboring genes are not annotated or of low homology to characterized proteins as exemplified by the *T. terrenum* gene-region shown in Figure 8A. Sometimes the gene is positioned by itself in a one gene operon where it is unknown if it has any synergy with neighboring genes as shown for *S. aurantiaca* in Figure 8B.

```

B.clausii -----
B.subtilis -----
T.terrenum MSGARLSRRRFMKMGAIGAGLLLACCGTCCGVARLADRF
S.aurantiaca MSLTPRRTERAGIRWLSFSGREPHSREPVLLWSVRA

B.clausii -----MELEK-----FVDPMPIMKTAIPKKTSKDGD
B.subtilis -----MTLEK-----FVDALPIPDILKPVQSKEKT
T.terrenum FDVGLTGTRLQSRIPLREFENPLPIPPVLRVRRDSDTD
S.aurantiaca SVLCVGLLAASG-----AGAASVPNQDVSLELAVKYA

```

Figure 9: Sequence alignment of four LMCOs that were later cloned. The *B. clausii* LMCO is close homologue of *B. subtilis* CotA and the two have similar N-termini. LMCO from *T. terrenum* has a classical Twin Arginine translocation motif and the expected secretion peptide is outlined with a red box. The LMCO from *S. aurantiaca* is predicted to be secreted through the SEC pathway and the secretion peptide is outlined with a red box.

The gene of the characterized LMCO from *P. haloplanktis*¹⁰⁹ takes up the CopA position in a gene-cluster that encodes well-known copper stress proteins as shown in Figure 8C. The gene organization is similar for the LMCO from *A. aeolicus*¹⁰⁶, but neither LMCOs have been explicitly tested for cuprous oxidase activity. This is despite being encoded in the CopA position, having a methionine-rich loop and having better homology to CueO from *E. coli* than CotA from *B. subtilis*.

Looking closer at the protein N-terminal sequences, three groups of CotA homologues were identified by their secretion peptide or lack thereof (see Figure 9). Despite the CotA proteins being localized to the outside of the spore¹¹⁰, the proteins are not preceded by a secretion peptide. This is possible because of the sporulation process where an asymmetric cell-division is followed by the mother-cell engulfing the small daughter-cell which then becomes the spore. The spore has a double membrane, where the outer spore-membrane previously was internal to the mother cell¹¹¹.

Interestingly to the speculations on the biological role of CotA proteins, it has been observed that *B. subtilis* CotA is localized preferable to the distal site of the spore¹¹⁰. It seems reasonable that proteins directly involved in the formation of the spore matrix are evenly distributed and thus a non-specific role of CotA proteins in detoxifying the surroundings is plausible. This would also help to explain the surprising stability of CotA proteins. The spores are dormant until germination starts¹¹², and if the CotA proteins function to detoxify the surroundings until this event sets in, they have to be ideally indefinite.

In contrast most of the predicted LMCOs were preceded by a Twin Arginine Translocation (TAT) peptide as predicted by PRED-TAT¹¹³ www.compgen.org/tools/PRED-TAT/. One such sequence is the LMCO from *T. terrenum* as shown in Figure 9. A few of the sequences were preceded by a SEC secretion peptide as predicted by SignalP¹¹⁴ www.cbs.dtu.dk/services/SignalP/. One such sequence is the LMCO from *S. aurantiaca*. The *S. aurantiaca* LMCO sequence does have the double arginines but lack the rest of the TAT motif [S/T]RRxFLK¹¹³ and scores perfectly for a SEC pathway secreted protein.

3.2.2 Cloning of LMCO expression vectors

It was decided to investigate one LMCO from each group, i.e. CotA from *B. clausii* KSM-16, a TAT pathway LMCO from *T. terrenum* and the SEC pathway LMCO from *S. aurantiaca*.

The protein sequences were submitted to GeneArt who synthesized *E. coli* optimized DNA coding for the LMCOs with restriction sites NcoI and KpnI. Such restriction sites make the genes them compatible with the EMBL pETM series of expression vectors¹¹⁵. www.embl.de/pepcore/pepcore_services/strains_vectors/vectors/bacterial_expression_vectors/index.html

KpnI is part of a multiple cloning site, and other restriction enzymes can be used, but the vector system is explicitly designed to use the NcoI restriction site for insertion of the N-terminal coding part of the gene.

The pETM vectors make it possible to express the protein of interest in a fusion with various expression enhancing peptides. The fusion proteins are removable by the non-commercial TEV protease. The three LMCO genes were cloned in fusions with Maltose Binding Protein (MBP), Glutathione S-transferase (GST), monomeric Green Fluorescent Protein (mGFP) as well as a no-fusion variant. An outline of a MBP-TEV-LMCO vector is shown in Figure 10A. However, expression yields and protein activity was low for all constructs.

It was decided to make a positive control for protein expression and the best characterized LMCO for that purpose was *B. subtilis* CotA. To make a better comparison with other reported expressions of this protein, we chose to use the wild-type gene as found in our laboratory strain *B. subtilis* 168 rather than a synthetic gene. The cloning was complicated by two intrinsic NcoI restriction sites.

Our best solution was to use a type IIS restriction enzyme that cuts outside its recognition site¹¹⁶. The type IIS recognition site can be added by PCR outside the gene of interest, and subsequently cut off by the restriction enzyme. This makes it possible to create all DNA overhangs ready for ligation by varying the PCR primer rather than the restriction enzyme. BsaI is the most common type IIS restriction enzyme, but the recognition sequence was again intrinsic to the CotA gene. Instead the less common alternative BsmBI was used:

```
CotA protein           M T L E K F V . . .
CotA DNA:             ACAGATGACACTTGAAAAATTTGTGG . . .
p#20                  ATCGTCTCTCATGACACTTGAAAAATTTG
```

The above listed PCR primer p#20 was ordered and used to extend the CotA DNA with ATCGTCTCTC as shown above with the BsmBI recognition site written in bold. Digestion with BsmBI leaves a 4 bp overhang 5'CATG identical to a NcoI digestion overhang and thus made it possible to clone the *Bacillus subtilis* CotA gene into the pETM vectors.

3.2.3 Superfolder GFP – CotA fusion protein

At one point we were considering to start a campaign of directed evolution. In making such an experiment, it is important to design the experiment in a way that minimizes handling. LMCOs are easy in this regard, because you can measure activity directly on crude extracts using e.g. ABTS as a substrate. In doing so, the measured activity is actually the Michaelis-Menten parameter V_{max} , while the better measure would be the related k_{cat} . The two are linearly related by the enzyme concentration, $V_{max} = k_{cat} \cdot [E]$ and in this light, the measured activity is hugely dependent on the expression level of the clones. Variability in enzyme concentration can be intrinsic to the expression system, but also rise from variability in the high-throughput cultivation environment, e.g. the 36 wells on the edges of 96-well plate are better aerated than inside wells and will probably grow faster. Gupta et al. solved this issue in their campaign of directed evolution on *B. subtilis* CotA by testing activity for both ABTS and syringaldazine and selecting for clones with a high ratio between the two¹¹⁷. An improvement to the assay would be to normalize enzymatic activity protein concentration. We considered that a fluorescent fusion protein similar to mGFP would ease the problem. E.g. the

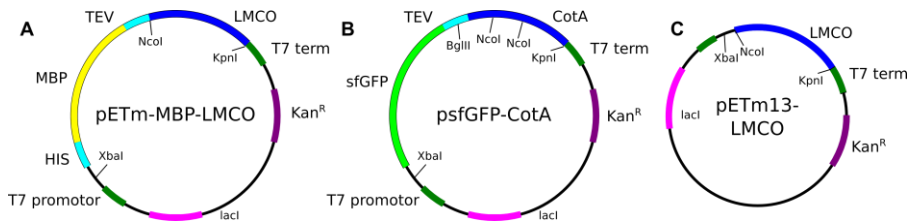


Figure 10: Outline of the cloned expression vectors and important restriction sites. A) An example of a pETM expression vector. Here the fusion protein is annotated as MBP, but it could be any of the fusion proteins mentioned in the main text. B) psfGFP-CotA fusion expression vector as mentioned in the text. C) pETm13 vector without fusion proteins. This is the minimalist pETm expression vector which was chosen for expression of the LMCOs.

activity of the CotA is measured by UV-VIS using ABTS as the substrate, and the relative amount of protein is estimated from the fluorescence of the GFP fusion.

The mGFP vector fusion is not ideal for hard-to-express proteins, but an alternative superfolder-GFP (sfGFP) has been reported to alleviated many of the problems^{118,119}. As the name implies, sfGFP is a variant of GFP that fold reversible, and in this aspect it might have some chaperone effect. Consequently a derivate expression vector was created from pETM using a codon optimized version of superfolder GFP (sfGFP) as the fusion protein.

CotA protein:	M T L E K F
CotA DNA:	ACAGATGACACTTGAAAAATTT
p#1	AA AGATCT GTACTTCCAAATGACACTTG

The above listed PCR primer p#1 was used to extend the CotA DNA to overlap with the sfGFP DNA over a BglIII restriction site. The BglIII restriction site is written in bold.

Complementary oligomers of sfGFP and CotA were generated by PCR, digested with BglIII and subsequently ligated together. After a new round of PCR on the sfGFP-CotA fusion, the oligomer was digested with XbaI and KpnI, inserted into a similar treated pETM vector and named psfGFP-CotA (See Figure 10B). The TEV protease recognition sequence was slightly modified from the pETM amino acid sequence ENLYFQ'GAM into EDLYFQ'M where the apostrophe marks the cleavage site. A canonical TEV protease recognition sequence cannot be coded with a palindromic DNA sequence and thus cannot contain a classical restriction enzyme recognition site. This is probably the reason for the design of the pETM vectors that leaves at least two extra amino acids on the N-terminal of the protein after

removal of the fusion protein. The asparagine to aspartate mutation introduced to the TEV protease recognition site in sfGFP-CotA is in the least specific position in the TEV recognition sequence¹²⁰, and is in fact slightly favored in terms of catalytic activity¹²¹. The modified TEV recognition site can be encoded by DNA with the before mentioned BglIII restriction site. This design makes it possible to do routine cloning into the psfGFP vector without introducing extra amino acids to the produced enzyme.

3.2.4 Heterologous expression of LMCO in *E. coli*

Recombinant expression of LMCOs in *E. coli* was a big concern in this project. It did not seem to make a difference as to which pETM vector was used for heterologous LMCO expression, and all the clones displayed low activity. The exception being the GST fusion vector pETM30 that failed to produce soluble LMCOs. The best results were achieved when using the no-fusion pETM13 vector for LMCO production (See Figure 10C).

Standard *E. coli* heterologous expression conditions produced very little active enzyme even when the expression medium was supplemented with copper, e.g. with vigorous shaking at 30°C, grow culture in LB medium with 250µM CuCl₂ to OD₆₀₀=0.6. Induce with 100µM IPTG and harvest after 4 hours. Much better activities were observed when the same protocol was extended by an overnight incubation without shaking as first proposed by Durao et al.¹²² who used the term microaerobic expression to describe the process.

Microaerobic has since been used in several studies in the expression of LMCOs including *B. clausii*⁸³, *Aeromonas hydrophila*¹²³, *B. pumilus*¹²⁴, *B. amyloliquefaciens*¹²⁵ and *Bacillus* sp. HR03¹²⁶. The microaerobic incubation is rationalized by the higher accumulation of copper inside the *E. coli* cell under these conditions^{127–129}.

However, we found that the LMCO activity after microaerobic expression was not easy to reproduce and would not be viable in a high-throughput screening without normalizing for the expression yield. A standard campaign of high-throughput screening of *B. subtilis* CotA expressed in *E. coli* was attempted by Brissos et al. They could not detect significant LMCO activity after aerobic expression and

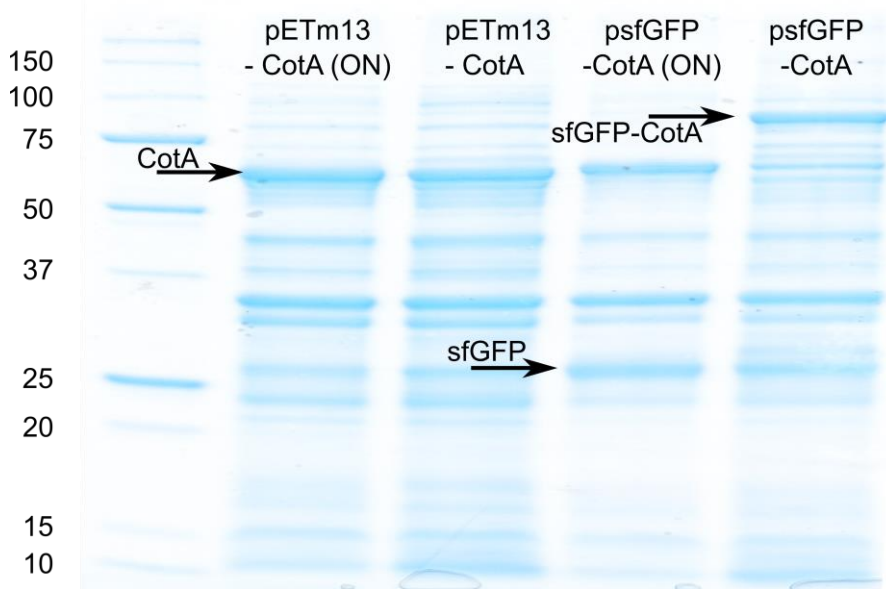


Figure 11: SDS-PAGE gel of a two different *B. subtilis* CotA expressions. Protein expression in autoinduction medium using *E. coli* BL21(DE3) transformed with vectors pETm13-CotA or psfGFP-CotA. Soluble protein was extracted with BPER. Lane 1: Precision Plus Protein™ marker. Lane 3 & 5: Enzyme from aerated expression conditions. Lane 2 & 4: Enzyme from aerated expression conditions followed by additional microaerobic incubation overnight. Bands conferring to *B. subtilis* CotA, superfolder GFP and the fusion sfGFP-CotA are marked with black arrows.

reported 25% uncertainty in their measured activities after microaerobic expression.⁵⁹

Late in this project it was noticed that microaerobic conditions during protein expression was not strictly necessary. Even after prolonged aerated expression, when protein production was minimal and the medium spend, static incubation of the culture overnight activated the protein. This effect might be explained by Duraó et al.¹²² who in conjunction with the characterization of microaerobic expression of *B. subtilis* CotA also reported full recovery of specific activity of the apo-protein after reconstitution with copper(I) in contrast to copper(II). Copper(I) is the form of copper that is expected inside microaerobic *E. coli*¹²⁷ and thus the mechanism behind microaerobic expression and reconstitution with copper(I) might be the same.

Figure 11 show a SDS-PAGE gel of the soluble proteins after expression with the vectors pETm13-CotA and psfGFP-CotA. The two cultures were grown with vigorous shaking at 20°C in ZYM-5052 autoinduction medium¹³⁰ with 250µM CuCl₂

for 3.5 days. At this time the psGFP-CotA culture was bright yellow from the fluorescent fusion protein. The cultures were split in halves, one part being left static overnight, and the other harvested and the cell pellet frozen at -20°C. The next day, the static incubated parts were harvested and similarly frozen. The four pellets were thawed and soluble proteins extracted with BPER according to manufacturer's instruction.

As seen from Figure 11, very little difference in the protein composition was seen by the extra day of static incubation. However, the activity of CotA with ABTS as the substrate was increased five-folds. Interestingly the extra day of static incubation facilitated the proteolytic cleavage of the sfGFP-CotA fusion giving rise to a SDS-PAGE band of slightly higher mass than free CotA and a free sfGFP. The cleaved off CotA and the sfGFP-cotA fusion had similar activities on ABTS only slightly lower than that of the microaerobically incubated pETm13-CotA expression. The latter suggest that the sfGFP fusion might have a beneficial effect on the aerobically expressed CotA.

3.2.5 A small and diverse library of LMCO genes

In our intention to create a LMCO of unique properties, we were looking for LMCOs of high stability that would allow mutational optimization. At the same time we needed diversity and thus it was decided to screen a set of LMCO genes from diverse species. One advantage of synthetic DNA in this regard is that it can be similarly codon optimized and thus increase the DNA redundancy without changing the peptide sequence. E.g. the native LMCO coding genes from *T. terrenum* and *B. clausii* have only 22% similarity, while the synthetic variants used in this project have 42% similarity and large blocks of identical bases can be found in the regions that code the conserved regions of LMCO. Such two genes can be recombined by DNA shuffling^{131,132} and would make a good starting point for further optimizations.

A total of 15 LMCO genes were acquired by different means (see Figure 12). The *B. subtilis* gene was not codon optimized, but acquired from the wild-type *B. subtilis* 168 as discussed previously. *S. aurantiaca*, *T. terrenum* and *B. clausii* LMCO genes were synthesized by GeneArt and naively codon optimized to use only the codons that are otherwise of highest use in wild-type *E. coli* genes. The first attempts to express LMCOs were done with the genes from GeneArt, but the apparent low

UniParc	Origin	sec	homol.	ABTS
P07788	<i>Bacillus subtilis</i>	CotA	100	+
Q08U34	<i>Stigmatella aurantiaca</i>	SEC	20	-
D1CEU4	<i>Thermobaculum terrenum</i>	TAT	39	+
Q5WEM6	<i>Bacillus Clausii</i>	CotA	59	+
D1CHB6	<i>Thermobaculum terrenum</i>	TAT	31	(+)
D2S0F9	<i>Haloterrigena turkmenica</i>	TAT	20	(+)
E8TU66	<i>Alicyclophilus denitrificans</i>	TAT	29	-
E8WV80	<i>Acidobacterium</i> sp.	CotA	44	(+)
B9LMQ8	<i>Halorubrum lacusprofundi</i>	CotA	43	?
A9FLW2	<i>Sorangium cellulosum</i>	SEC	26	(+)
C8XHY8	<i>Nakamurella Multipartita</i>	none	20	NA
E6W7R9	<i>Pantoea</i> sp. At-9b	TAT	19	-
D7BGT0	<i>Meiothermus Silvanus</i>	TAT	31	NA
QOS9P3	<i>Rhodococcus Jostii</i> RHA1	TAT	34	(+)

Figure 12: LMCO library. The LMCOs selected for characterization is listed by their UniProt accession number together with the organism, secretion type and homology to *B. subtilis* CotA. The P07788 coding gene was amplified from wild-type *B. subtilis* 168. Q08U34, D1CEU4 and Q5WEM6 coding genes were synthesized by GeneArt. The rest were synthesized and codon optimized by DNA 2.0.

yields inspired us to try the more convoluted codon optimization offered by DNA2.0. Despite the later optimization of expression protocols and sufficient expression levels and activity of the LMCOs from *B. clausii* and *T. terrenum*, the genes from DNA2.0 never made good protein expressions.

The proteins listed in Figure 12 are three domain LMCOs with the exception of D2S0F9 from *Haloterrigena turkmenica* that belong to the two domain typeB and D1CHB6 from *T. terrenum* that is a unique fusion of a two domains typeB MCO with a cupredoxin domain. The latter protein is discussed in section 3.6.3.

Because of the low expression yields and activities of the DNA2.0 genes, these were not characterized in detail. However, 6 of the LMCOs were found to have some ABTS oxidizing capability while two failed completely (see Figure 12). The rest of the results were inconclusive. The LMCO from *H. lacusprofundi* was expressed in a non-active truncated form and the genes coding LMCOs from *Nakamurella multipartita* and *M. silvanus* were not successfully cloned.

Interestingly, the gene yielding the highest protein expression is the non-codon optimized *B. subtilis* CotA. This hints that there is something more to the codon composition of LMCOs than just the availability tRNA. One theory on *in vivo*

protein folding is that the transcriptional speed is variable, and that the ribosome is purposefully slowed down at positions in the gene to allow for correct folding¹³³. It is possible that in the attempt to increase protein expression by codon optimization, we removed critical pauses in the gene translation and the result is aggregated protein.

Two exceptions to the low expression yield from genes synthesized by DNA2.0 are the unique D1CHB6 from *T. terrenum* and the sfGFP.

3.3 Characterization *Bacillus clausii* CotA

The CotA from *B. clausii* KSM-K16 (Q5WEM6) was characterized as part of this project and is described in details in Paper I. The organism was the most conservative pick in the list of interesting extremophile organisms. It is from the same phylum as *B. subtilis*, it is spore forming and the LMCO have 59% identity and 74% homology to CotA from *B. subtilis*. Everything indicates that this LMCO is another CotA protein in the list of characterized CotAs, e.g. *B. subtilis*⁹⁴, *B. licheniformis*¹³⁴, *B. pumilius*¹²⁴, *B. pumilus* WH4¹³⁵, *B. amyloliquefaciens* 12B¹²⁵, *B. tequilensis* SN4¹³⁶, *B. halodurans*¹³⁷, *B. vallismortis*¹³⁸, *B. subtilis* WPI¹³⁹, *B. thuringiensis* RUN1¹⁴⁰, *B. sphaericus*¹⁴¹, *B. sp.* HR03¹⁴², *B. Brevibacillus sp.* Z1¹⁴³ *B. sp.* ADR¹⁴⁴. Basically, wherever there is dirt or feces, there are *Bacillus* strains with laccase-like activity. In case of *B. sphaericus* you even find it in the stratosphere.

B. clausii has optimal growth at pH 9.0 compared to pH 8.0 for *B. subtilis*. Considering that the relatively higher pH optima for laccase-like activities in bacterial versus fungal LMCOs is one of the main reasons for investigating the CotA type LMCOs⁶⁰, we wanted to investigate if the difference in optimal pH for growth of the native host carries over into enzymatic activities of the CotAs¹⁴⁵.

B. clausii KSM-K16 is known for being the native host of the M-protease, which is one of the original enzymes that saw commercial use as an additive to laundry detergents, because it shows excellent stability towards both heat and high pH¹⁴⁶. It has been proposed that these properties are due to a general increase in arginine over lysine ionic interactions¹⁴⁷ and the *B. clausii* CotA does seem to have substituted many of the charged residues on the surface as compared to structure of *B. subtilis* CotA. These might be involved in a different ionic interaction network and can possibly explain a difference in pH stability. An example of this



Figure 13: Multiple protein sequence alignment of some CotA proteins from four *Bacillus* strains. The first 70 amino acids show some of the highest variability in the protein class. Red lines connects charged amino acids in close vicinity as suggested by the *B. subtilis* CotA structure (3ZDW).

reorganization is shown in the multiple sequence alignment in Figure 13. The T26D and E66K pair of substitutions are in close proximity (3\AA) as determined by homology model of *B. clausii* CotA build on the *B. subtilis* X-ray structure (3ZDW) using Phyre2¹⁴⁸. Together, the sequence and the growth profile of the native host suggested that *B. clausii* CotA was adapted to a higher pH than that of *B. subtilis* CotA.

The *B. clausii* and *B. subtilis* CotAs were characterized in parallel (Paper I) and the inclusion of *B. subtilis* CotA for comparison was chosen for a few reasons. *B. subtilis* CotA is the best characterized bacterial LMCO, and using this as a reference, enables other laboratories to reproduce and validate our work without necessarily establishing the expression system for *B. clausii* CotA. Our group and laboratory, was new to the field of bacterial LMCOs and in our attempt to identify and characterize more extreme LMCOs, it seemed scientifically prudent to develop a well-studied reference system as a positive control. Lastly, it was well-established that the fraction of enzymatically active protein is not necessarily the total amount of protein when characterizing otherwise pure bacterial LMCOs¹²². With this in mind, normalizing to the total concentration of protein, as is done in Michaelis-Menten kinetics¹⁴⁹ seems unsatisfactory. Instead, using a well-known reference like *B. subtilis* CotA allow for at least a relative comparison.

3.3.1 Short summary and discussion

B. clausii CotA was in general slightly less thermostable compared to *B. subtilis* CotA. However, the optimal pH stability was shifted to 9.0 compared to 8.0 for *B. subtilis* and similarly the pH for optimal enzymatic activity was shifted 1-2 pH units towards more alkaline environment for some substrates, i.e. DMP, caffeic acid and bilirubin.

The higher pH of optimal growth for *B. clausii* compared to *B. subtilis* did translate into a CotA protein that is more adapted to alkaline environment when looking at

the enzymatic activity and stability. This confirmed our hypothesis that some LMCO characteristics can be deduced from native biotopes. This ability might be beneficial in screening well-known phyla for the most advantageous homologue of a specific protein. The general lower thermostability of *B. clausii* CotA compared to *B. subtilis* CotA suggest that thermostability of CotA type proteins are either not under the same evolutionary pressure in the two species, or that the adaptation of *B. clausii* CotA to more alkaline environment has happened under the often reported stability-functionality trade-off¹⁵⁰.

3.4 Characterization of *Thermobaculum terrenum* TtMCO

The *T. terrenum* Multi-copper oxidase (TtMCO) with UniProt accession number D1CEU4 was also characterized during this project, and the results are reported in Paper II. TtMCO is a distant homologue to *B. subtilis* CotA and the two share only 50% homology and 39% identity. *T. terrenum* belongs to the Thermomicrobia group within the deeply rooted phylum Chloroflexi¹⁵¹. The organism is not well-understood, exemplified by the current characterization of TtMCO being the first characterized protein from the organism. *T. terrenum* is a gram positive and monoderm bacterium with an unusual thick cell wall¹⁵². In contrast to most members of phylum Chloroflexi, *T. terrenum* and the closest relative *Sphaerobacter thermophilus*¹⁵³ stain gram positive. This discrepancy of gram staining within the phylum goes to show that the cell wall composition is not strictly conserved. LMCOs are often associated with the cell wall. Either adhered to the outside as in the gram positive *B. subtilis*¹¹⁰ or entrapped in the periplasmic space between cell membranes of gram negative cells such as *E. coli*¹²⁷. Because bacteria in Phylum Chloroflexi in general and *T. terrenum* in particular have uncommon cell walls, the genomes of these organisms are good places to look for LMCOs that have properties different from both CotA and CueO.

The varying cell wall composition has also been proposed to indicate that Phylum Chloroflexi evolved before the archetype gram positive (mono-layered) and gram-negative (double-layered) cell compositions evolved. Whether the Chloroflexi represents a very early type of life¹⁵⁴ or species in evolutionary transition from single to double layered cell walls¹⁵⁵ is unknown¹⁵⁶. Phylogeny in these deeply rooted species is very hard to track due to the possibility of multiple instances of horizontal gene transfer¹⁵⁷. However, both phylogenetic positions indicate a very

early evolutionary step. In this aspect, bacteria from phylum Chloroflexi can be considered living fossils that resemble some of the earliest form of Life.

From a basic scientific point of view, LMCOs from *T. terrenum* is very interesting, but since the organism is also thermophile with optimal growth at 67°C, it easily made the list of interesting extremophile bacteria.

3.4.1 Short summary and discussion

TtMCO is the second most thermostable LMCO characterized, showing half-time of inactivation of 350 minutes at 80°C and approximately 2.24 days at 70°C. It is not surprising to see such high stability at 70°C considering the optimal growth of *T. terrenum* at 67°C¹⁵². The LMCO from *P. aerophilum* (McoP)³⁰ was found to have slightly smaller half-time of inactivation of 330 minutes at 80°C, despite the organism having an optimal growth at 100°C¹⁵⁸.

P. aerophilum was a prime candidate on the first list of extremophile organisms with LMCO coding genes discussed in section 3.2.1. TtMCO took the place of McoP when we realized that McoP had already been characterized in details. Fernandes et al. expressed McoP gene in full length³⁰ including the very hydrophobic TAT secretion peptide that is expected to be cleaved off in the mature protein. This might explain why they had to co-express five chaperone proteins along with McoP to express the protein³⁰, and can also explain why the first 39 residues were unresolved in the later reported protein structure (PDBID:3AW5)¹⁵⁹. It is very possible that the extraneous hydrophobic tail has changed the stability of McoP making it more prone to aggregation and thus making the reported 330 minutes half-time of inactivation at 80°C an underestimation of the true value.

57 different substrates of varying properties were tested as substrates for TtMCO, most of which are oxidized by *T. versicolor* LMCO. The only two substrates that was oxidized by TtMCO and behaved according to Michaelis-Menten kinetics were ABTS and 1,8-diaminonaphtalene (DAN). The apparent lack of TtMCO activity on small phenols goes to show that we currently do not understand the biological significance of bacterial LMCOs.

DAN does have an aromatic core with amino substitutions somewhat similar to the common p-phenylenediamine (PPD). Interestingly PPD was not a good

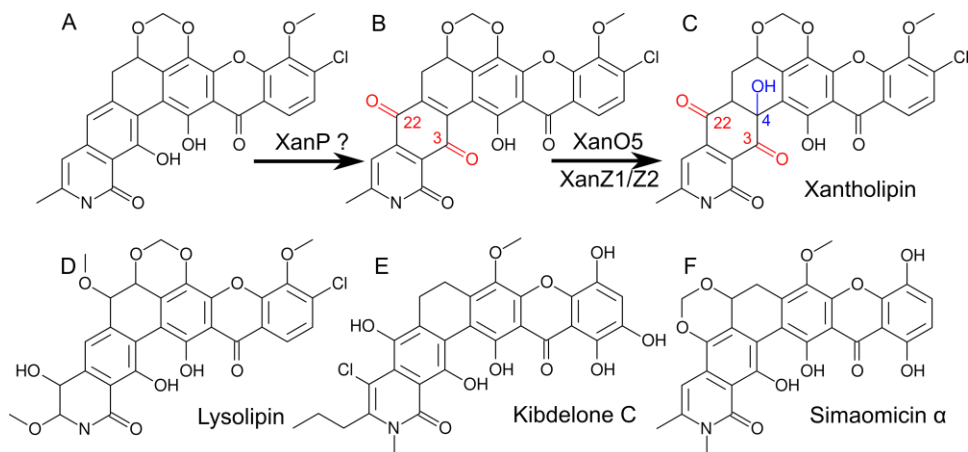


Figure 14: Structures of some xantholipin related species. Zhang et al. reports a conversion of A) to B) in the synthesis of xantholipin C) in *Streptomyces flavogriseus*. The conversion from A to B (highlighted in red) is unassigned while the conversion from B to C (highlighted in blue) is assigned to XanO5 and two dehydrogenases XanZ1 and XanZ2. D) Lysolipin is a related xanthone containing antibiotic for which the biosynthetic pathway has been characterized. Lysolipin has a position 3 hydroxyl substitution and xantholipin has position 3 and 22 oxy substitutions similar to the conversion between A and B. Another difference is the tertiary hydroxyl in position 4 of xantholipin (blue) which is not present in lysolipin. (-)-kibelone C and (-)-simaomicin are two synthetic enantiomeric drugs that can potentially be matured *in situ* by XanP or TtMCO.

substrate for TtMCO and the difference between PPD and DAN suggests that TtMCO prefers bulky substrates.

TtMCO lack close homologues, but one of the best is the XanP LMCO from *Streptomyces flavogriseus*. The XanP protein is uncharacterized, but the gene is positioned in a characterized gene cluster that is otherwise responsible for the biosynthesis of the antibiotic and cancer drug xantholipin. In the characterization of the biosynthetic pathway of xantholipin a step converting a hydroxyl substitution to two oxy substitutions were left unassigned¹⁶⁰ (See Figure 14). The conversion resembles the laccase catalyzed formation of quinones from small molecule phenols, except the active group is nested within a large polyketide. We propose that XanP is involved in this conversion. A similar drug, lysolipin, is synthesized by proteins coded in a gene cluster that does not include a LMCO gene¹⁶¹ and one of the minor differences between xantholipin and lysolipin is indeed the before mentioned oxy substitutions.

Another difference between xantholipin and lysolipin is a tertiary hydroxyl group in position 4 as shown in Figure 14C. This gives additional albeit a bit involved evidence to TtMCO having a role in antibiotic maturation. The insertion of the hydroxyl group in xantholipin is catalyzed by the XanO5 enzyme¹⁶⁰ and the gene of

the TtMCO homologue found in *S. thermophilus* is positioned in a putative gene cluster together with a XanO5 homologue gene.

Due to the distant homology of TtMCO to other LMCOs, we have to use these secondary indications to suggest a possible specific activity. E.g. the preference of bulky substrates, the homology (68%) to a proposed antibiotic maturation protein and the homology (67%) to a LMCO in *S. thermophilus* that is expected to be expressed with a XanO5 homologue. We propose the possibility of TtMCO being involved in maturation of antibiotics.

Gupta has suggested that the uncommon cell walls observed for bacteria in phylum Chloroflexi is an evolutionary response to evolution of the first antibiotics¹⁵⁵. In this light it is possible that *T. terrenum* and *S. thermophilus* also produce an antibiotics and that TtMCO is involved in a function similar to XanP. However, other roles cannot be ruled out.

E. coli periplasmic cell division proteins SufI (P26648), is a well-characterized structural homologue of LMCOs, that has the three cupredoxin domains similar to LMCOs, but does not contain neither coppers nor most of the otherwise conserved copper binding residues¹⁶². The lack of good substrates for TtMCO could also indicate that it is an evolutionary step towards SufI functionality. The homology between the two protein sequences is 45% which is in the range of homology to *B. subtilis* CotA (50%) and *E. coli* CueO (43%). However, we established that TtMCO has the EPR copper signature and thus functionality of a LMCO.

As a curiosity, the full genome of the ancient bacteria *A. aeolicus* V5 was sequenced in 1998 before bacterial LMCOs gained popularity. The LMCO sequence (O67206) is annotated as a SufI protein even though the LMCO has later been characterized to be a copper containing cuprous and ferrous oxidase¹⁰⁶.

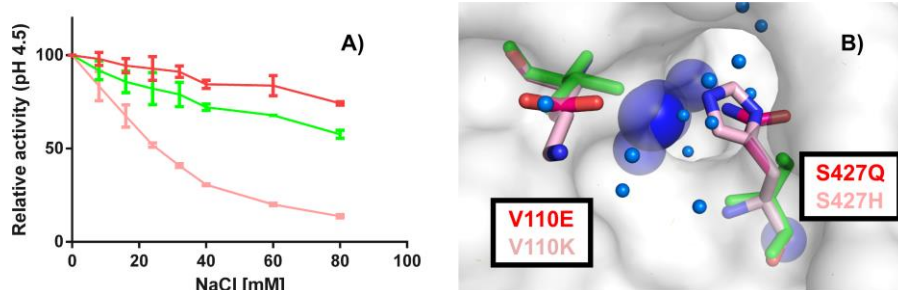


Figure 15: NaCl inhibition and structural model of three bacterial LMCOs. Data in both figures are color-coded to show *B. subtilis* CotA (green), *B. clausii* CotA (red) and *T. terrenum* TtMCO (pink). A) ABTS activity measured at pH 4.5 vs NaCl concentration. Experiments were done in parallel. B) The structure models of TtMCO and *B. clausii* CotA are superimposed on the structure of *B. subtilis* CotA (3ZDW) and two important and variable residues lining the water exit channel are explicitly shown. Amino acid changes are numbered on the *B. subtilis* CotA sequence. The T2 copper is visible through the open channel and resolved water molecules are represented by small blue spheres. This visualization is similar to Brander et al. figure 1C⁸³, but is also showing TtMCO

TtMCO is very intolerant to NaCl, with only 14% activity in 80mM NaCl at pH 4.5 as shown in Figure 15A. In comparison *B. clausii* CotA shows 75% and *B. subtilis* CotA shows 58% activity under similar conditions. Halide inhibition is very pH dependent⁵³ and we have hypothesized that alkaline and halide inhibition of LMCOs is modulated by the amino acids lining the water exit channel⁸³. Residue E107 and Q418 in *B. clausii* were in particular suggested to function as guards against small negatively charged hydroxide and chloride ions by electrostatic repulsion, see Figure 15B. In the case of TtMCO, the two amino acids are substituted with lysine and histidine respectively. Here the opposite argument can be made, that the positively charged water exit channel can attract the negatively charged inhibitors and might explain the strong modulation effect of NaCl on TtMCO activity.

3.5 Heat activation of LMCOs

A preliminary experiment on TtMCO showed that it was indeed a very thermostable protein and because of this we wanted to do bulk purification of the recombinant *E. coli* lysate by precipitation of heat labile proteins at 70°C^{75,134,142}. However, activity decreased rapidly in the process, and we were able to identify NaCl in the lysate buffer as the culprit in the process.

Instead of our standard lysis buffer; 50mM Tris pH 7.6, 200mM NaCl, we turned to use a salt free MOPS buffer. MOPS has three advantages over Tris as a buffer in the TtMCO system. The $d(\text{pKa})/dT$ of MOPS is -0.011 compared to -0.028 of Tris¹⁶³,

making temperature dependent experiments more reliable. MOPS is an acid and is titrated with NaOH to become a buffer. This is in contrast to Tris which is base and often titrated with HCl which will leave surplus chloride anions that can interfere with LMCOs. Lastly, we found that MOPS in contrast to Tris¹⁶⁴ does not form a complex with free copper as measured by EPR and this makes MOPS buffer more useful in spectroscopic characterization of the copper centers. The latter characteristic is shared with the chemically similar MES buffer.

HEPES and PIPES are also common buffers in biological systems, but these are redox active which might be problematic in LMCO studies¹⁶⁵. Phosphate buffers including McIlvaine and Britton-Robinson universal buffers are not recommended for long-term storage of LMCOs as reported by Reiss et al¹²⁴. They observe that the inactivation at pH 7.0 and 4°C, 45°C and 65°C of *B. pumilus* CotA is respectively 144, 25 and 10 times slower in demineralized water compared to phosphate buffered solution. This is most likely explained by the insolubility of $\text{Cu}_3(\text{PO}_4)_2$ in water. Any labile LMCO coppers will precipitate in a phosphate buffer and leave a non-reactive protein.

In the characterization of TtMCO we observed that it was very sensitive to NaCl. In particular a difference in the EPR spectrum was observed when TtMCO was heat incubated either with or without 200mM NaCl. A copper EPR signal characterized by $g_{\perp} = 2.10$, $g_{\parallel} = 2.34$ and $A_{\parallel} = 100 \times 10^{-4} \text{ cm}^{-1}$ was found in the salt treated form, and this changed to $g_{\perp} = 2.5$, $g_{\parallel} = 2.24$ and $A_{\parallel} = 186 \times 10^{-4} \text{ cm}^{-1}$ upon heating without salt. The two spectra resemble those of the “alternative resting” and “resting oxidized” states as characterized for bilirubin oxidases¹⁶⁶. The first is the predominant form of the heterologously expressed bilirubin oxidase, and it has been characterized to be of low catalytic turnover¹⁶⁷.

In connection with the transformation to the “resting oxidized” state, heat incubation without salt increased the activity similar to the often observed heat activation of LMCOs. These observations suggest that the often observed heat activation of LMCOs is a manifestation of the transformation from the “alternative resting” to the “resting oxidized”. We used EPR spectrophotometry as a handle to characterize the transformation as reported in Paper III

3.5.1 Short summary and discussion

The observation that heat-activation of LMCOs is followed by a change of redox state has to our knowledge not been reported before. The result might hold

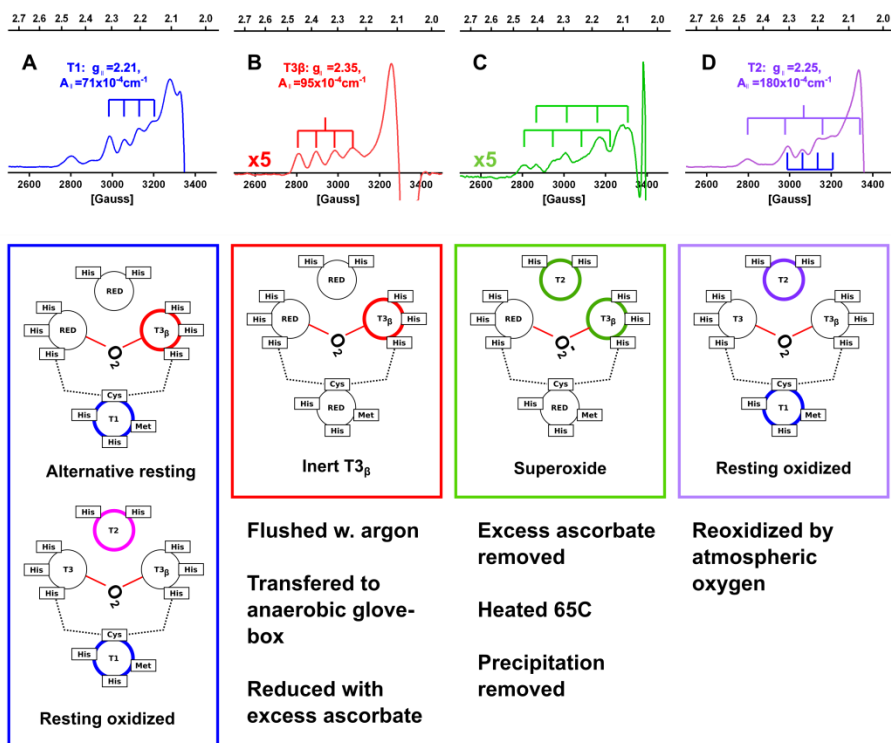


Figure 16: EPR spectra and proposed mechanism for the observed intermediates in heat activation of *B. subtilis* CotA:

EPR spectra of CotA during heat activation with ascorbate shown together with their respective copper conformation. One or two copper hyperfine patterns are color highlighted on each spectrum and the color is repeated in the outline of the copper conformations to show which coppers give rise to the spectrum. A) The starting CotA preparation gives an EPR signal showing features of both the “alternative resting” and the “resting oxidized” states. The T1 copper is common to the two states and can be deconvoluted from the spectrum and is highlighted in blue B) The sample was then flushed with argon and reduced with excess ascorbate in an anaerobic glovebox. An apparently redox inactive copper was still observed by EPR and this signal is highlighted in red. C) The sample was spin-filter dialyzed to remove the excess ascorbate and was then heated to 65°C for 30 minutes. Two new copper hyperfine patterns were observed by EPR and these signals are highlighted in green. D) The sample was opened to the atmosphere and the spectrum was recorded after one hour. The EPR signal now exclusively shows the pattern of the “resting oxidized” state.

implications for other LMCOs that have shown to readily heat-activate, e.g. *M. thermophila*, *Scytalidium thermophilum*, *M. verrucaria*⁹³, *Bacillus* sp. HR03¹⁶⁸, *A. lipoferum*¹⁶⁹, *Melanocarpus albomyces*¹⁷⁰.

When Martins et al. first characterized *B. subtilis* CotA they reported an EPR spectrum showing the profile of a LMCO in the “alternative resting” state⁹⁴. Six years later the same group introduced microaerobic expression and reported an EPR spectrum showing the profile of a LMCO in the “resting oxidized” state¹²². This

suggests that CotA exists in the two forms. However, when Martins et al. tested thermostability, the purified CotA protein was not heat activated. This seemed to disconfirm the above mentioned mechanism of heat activation.

However, the same study did show that the crude spore extract from *B. subtilis* was indeed heat-activated⁹⁴. This was a determining clue to our understanding of the conversion to the “resting oxidized” state of *B. subtilis* CotA. It turned out that the transformation was accelerated in reducing environment as simulated by addition of ascorbate, and it made it possible to completely transform *B. subtilis* CotA from the low activity “alternative resting” state to normal “resting oxidized” state. See Figure 16.

We have captured the intermediate state of an “alternative resting” *B. subtilis* CotA after reduction of the T1 copper and thermal incubation, but before reoxidization. The EPR spectrum showed two different EPR active copper centers together with a sharp radical signal as shown in Figure 16C. There are no tyrosine residues in the vicinity of the copper sites, and because the sample was thoroughly dialyzed before heating, we propose that the sharp signal is due to a small molecule that binds strongly to the tri-nuclear copper center and is subsequently reduced after heating.

A likely candidate for this role is molecular oxygen and the sharp signal would then be due to superoxide. The “resting oxidized” state is normally bridged by a hydroxide, but this is not clear for the “alternative resting” state. X-ray crystallography is not ideal for characterizing the active copper sites in MCOs, because the coppers will reduce under irradiation of the synchrotron beam¹⁷¹. However, while most structures are reported to have hydroxide as the bridging ligand between T3 coppers, *B. subtilis* CotA¹⁷² and the LMCO from *M. albomyces*¹⁷³ clearly show the coordination of molecular oxygen.

In this light we propose that heating a LMCO in the “alternative resting” state will oxidize one of the redox inactive coppers coupled to the reduction of molecular oxygen to superoxide anion. This three electron reduced state is able to enter the catalytic turnover described in section 2.1.7.

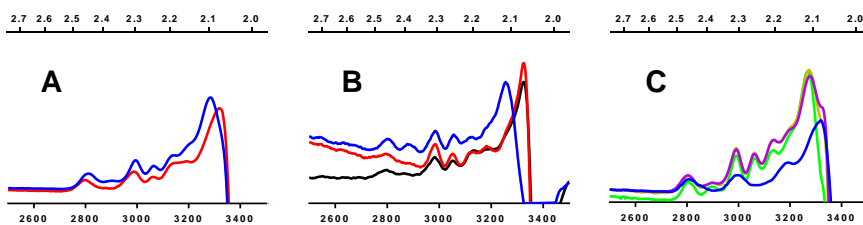


Figure 17: Conversion between “resting oxidized” and “alternative resting” states of *Bacillus subtilis* CotA as probed by EPR. A) Two preparations of *B. subtilis* CotA purified to homogeneity on a HiTrap-SP column with (blue) and without (red) preceding heat purification in 200mM NaCl containing lysis buffer. B) EPR spectra of *B. subtilis* CotA before (black) and after overnight incubation in 1mM ascorbate with (blue) or without (red) 200 mM NaCl. C) EPR spectra of *B. subtilis* CotA after overnight incubation with 25 mM (blue), 50 mM (purple), 250 mM (yellow) and 500mM (green) NaCl.

3.6 Preliminary results

3.6.1 Inactivation of LMCOs

Another interesting aspect of the transformation between “resting oxidized” to “alternative resting”, that is only briefly touch upon in Paper III, is the opposite transformation from “resting oxidized” to “alternative resting” and specifically, how not to trigger the transformation during expression and purification. Some preliminary progress towards understanding this process will be presented and discussed here.

As reported in Paper III, TtMCO transforms to the “alternative resting” state when heat incubated with 200mM NaCl. Figure 17A shows a similar experiment on *B. subtilis* CotA that is purified to homogeneity either with or without heat precipitation of heat labile proteins in a buffer containing 200mM NaCl. It is clear that the heat purification in NaCl containing buffer transforms the CotA to the “alternative resting” state similarly to what has been described for TtMCO.

The bilirubin oxidase from *Magnaporthe oryzae* has been reported to be completely transformed to the “alternative resting” state by incubation in 100 mM NaCl for 48 hours¹⁷⁴ which suggests that high temperature is not always necessary to drive the transformation. Seeing how ascorbate was important in heat activation of *B. subtilis* CotA, it was tested whether ascorbate was similarly involved in the opposite transformation to the “alternative resting” state. Figure 17B shows the EPR spectra of two *B. subtilis* CotA samples that have been

incubated overnight at 4°C in 1mM ascorbate with or without 200mM NaCl. The latter sample is clearly transformed to the “alternative resting” state.

In another experiment, CotA was incubated overnight with varying concentrations of NaCl and the EPR spectra are shown in Figure 17C and it is indicated that the transformation to the “alternative resting” state is also happening without ascorbate but at lower rate. The EPR spectrum after incubation with only 25mM NaCl is different and will be discussed shortly.

As briefly discussed in Paper III, transformation to the “alternative resting” state is accelerated by oxygen turnover of the CotA. Together these preliminary data suggest that in order to avoid inactivation of *B. subtilis* CotA and probably other LMCOs, storage in a solution with a reducing agent or chloride anions should be avoided.

Besides chloride anions, reducing agents and temperature, a parameter that is likely to influence the transformation between the “alternative resting” and “resting oxidized” states is the pH. These parameters and their combinations have to be tested in further experiments before any decisive conclusions can be made on what controls the transformation process.

3.6.2 Purification of some LMCOs

B. subtilis CotA easily precipitate during desalting and this was taken to imply that NaCl has a solubilizing effect on the protein. Because of this perhaps false understanding, heat purification of CotA was not attempted in salt free lysis buffer. *B. subtilis* CotA can be purified easily because it has a rare ability to binds to both anion and cation exchange columns in an apparently non-specific manner e.g. HiTrap-Q and HiTrap-SP at pH 8.

More specifically, in preparation of the protein used to collect the EPR spectra shown in Figure 17A, unpurified *B. subtilis* CotA in lysis buffer containing 200mM NaCl was diluted four times with 50mM MOPS pH 8.0 and applied to a HiTrap-SP column using an ÄKTA system. The protein formed a sharp and deep blue band on the column that slowly leaked out when buffer containing 0 or 50mM NaCl was applied. The blue CotA was eluted close to homogeneity in a broad peak containing fractions of 100-200mM NaCl. This protocol is very similar to the one

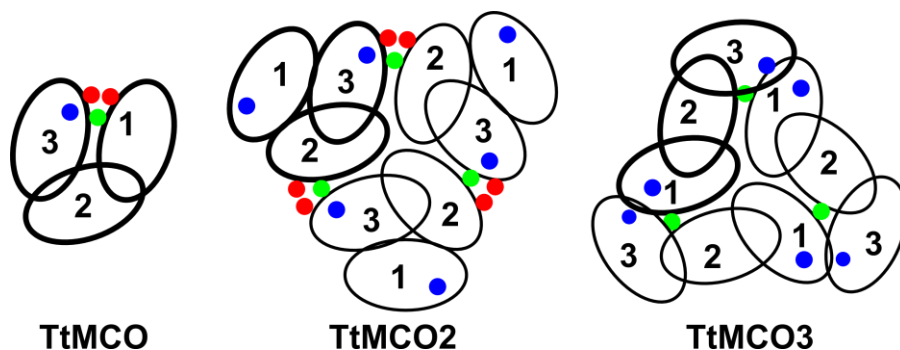


Figure 18: Outline of the 3 domain MCOs from *T. terrenum*. Uniparc codes for the protein sequences are written in paranthesis. TtMCO (D1CEU4) is a three domain LMCO. TtMCO2 (D1CHB6) is a two domain typeB MCO with an extraneous cupredoxin domain fused to the n-terminal. C) TtMCO3 (D1CH29) is a nitrite reductase with an extraneous cupredoxin domain fused to the c-terminal.

used by Bento et al.¹⁷² It was often observed that *B. subtilis* CotA had a tendency to aggregate during buffer exchange or desalting. The odd EPR spectrum in Figure 17C for the sample incubated with 25mM NaCl is the attempted resuspension of aggregated CotA protein. The apparent non-specific binding to sepharose columns and elution with 100-200mM NaCl, combined with the observation that CotA protein easily precipitates during desalting, suggests that CotA is actually precipitated on the column and solubilized by NaCl during elution.

3.6.3 Characterization of *Thermobaculum terrenum* TtMCO2

Interestingly, *T. terrenum* has three types of three domain MCOs. Following the nomenclature used in the section 2.1.8⁷³ TtMCO is a 3D LMCO, TtMCO2 is a two domain typeB with an N-terminally fused cupredoxin domain, TtMCO3 is a copper-containing nitrite reductase with a C-terminally fused cupredoxin domain. See Figure 18. This unique diversity of MCOs in an ancient species suggests that some major steps of MCO evolution happened in a bacterium similar to *T. terrenum*.

TtMCO2 was also expressed and subjected to characterization in this project. However, the most interesting observations are perhaps on the in vivo protein as expressed in *E. coli*. Some pictures from the expression steps are shown in Figure 19. The protein is readily expressed in such quantities that the *E. coli* culture turns visibly blue. The picture of the aerated culture in Figure 19B does not give the blue color full credit. Interestingly, the color slowly fades away in approximately 10

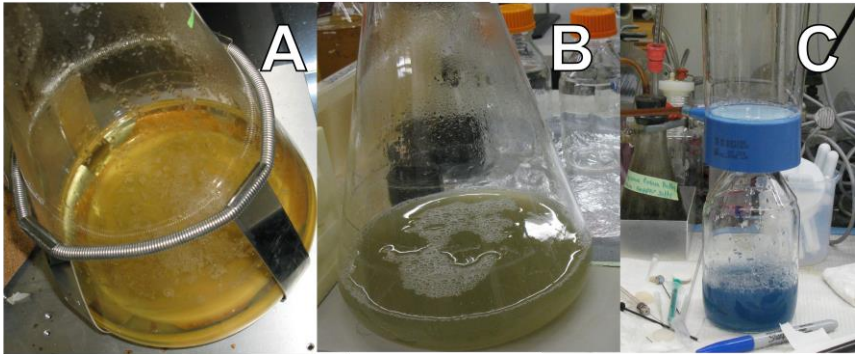


Figure 19: Expression of TtMCO2. A) TtMCO2 expressed in *E. coli* BL21(DE3) under microaerobic conditions. The cells are pale yellow. B) Same flask after swirling. Cells are slightly blue. C) Sterile filtration of TtMCO2 containing lysate. The crude protein extract is intensively blue.

minutes if the culture is left under microaerobic conditions, but it readily returns after swirling the flask. This change in color can be explained by a redox change of a T1 copper and goes to show that oxygen is quickly depleted in the microaerobic *E. coli* culture and that LMCOs in such environment can be expected to be predominantly in the reduced form.

Similarly to the observed color change in suspended medium, harvested TtMCO2 expressing *E. coli* cells form a thin but deep blue layer where they are exposed to air. If this layer is broken with a spatula, the newly exposed cells will turn blue in a matter of seconds.

TtMCO2 can easily be extracted after sonication of *E. coli* in lysis buffer containing 200mM NaCl. However, such a preparation shows no activity on ABTS. If sonication is performed in a lysis buffer without NaCl, the TtMCO2 is insoluble, but the blue cell debris can oxidize ABTS, albeit slowly. The soluble preparation of TtMCO2 precipitates upon addition of extra copper (50 μ M) or desalting.

Of the characterized cupredoxin proteins, the N-terminally fused cupredoxin in TtMCO2 had highest sequential homology to auracyanin B from *Chloroflexus aurantiacus*. Auracyanin B function as an electron shuttle in the anaerobic photosynthesis¹⁷⁵.

The soluble preparation of TtMCO2 has been characterized by UV-VIS and EPR as shown in Figure 20. The UV-VIS shows features similar to auracyanin B, including the absorption peak around 450 nm¹⁷⁶, but has in addition an weak absorption

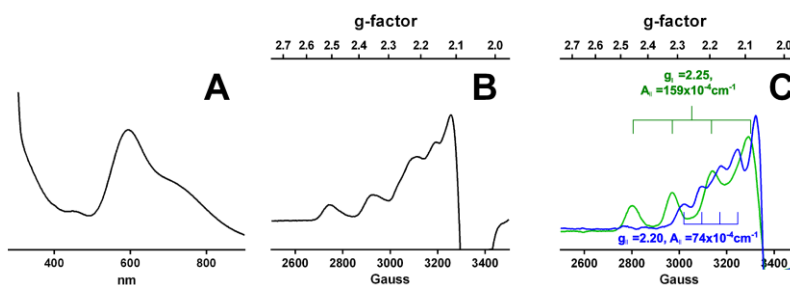


Figure 20: UV-VIS and EPR spectra of soluble TtMCO2 as discussed in the text. A) UV-VIS spectrum. B) & C) EPR spectra. TtMCO2 was anaerobically reduced with ascorbate and an EPR spectrum was measured just when the blue color disappeared (green). Subtracting this spectrum from the spectrum of the fully oxidized sample B) gives an EPR spectrum of the blue copper(s) (blue).

signal around 300 nm. The EPR is more peculiar and does not easily deconvolute into normal copper spectra with four hyperfine bands. Titration of TtMCO2 with ascorbate until the blue color disappeared was performed in an anaerobic glovebox. The EPR spectrum of this sample is a clean copper signal characterized by $g_{||}=2.25$ and $A_{||}=159 \times 10^{-4} \text{ cm}^{-1}$ as shown in Figure 20C. These values indicate that the remaining oxidized copper(s) is of the normal Type 2. The T2 copper signal subtracted from the oxidized spectrum is also shown in Figure 20C. This spectrum shows hyperfine splitting $g_{||}=2.20$ and $A_{||}=74 \times 10^{-4} \text{ cm}^{-1}$ which is characteristic of a type 1 copper²². The two spectral features are of roughly the same intensity as judged by the double integrated EPR spectra, however, neither of the spectra are easily simulated and it is possible that more than one copper contributes to the signal.

T. terrenum does not grow anaerobically and neither does it utilize photosynthesis. So the function of TtMCO2 must be unrelated to that of auracyanin B. The closest homologue sequence to the N-terminal cupredoxin fusion is the before mentioned c-terminally fused cupredoxin domain in TtMCO3. This MCO has strong nitrite reductase homology and the fused cupredoxin domain makes sense in this case, because cupredoxin mediated electron transport to nitrite reductase is well-described for e.g. pseudoazurin in *Achromobacter cycloclastes*⁷². It is possible that the unusual cell membrane of *T. terrenum* makes it necessary for the nitrite reductase and its electron transporter to be fused. Recently the structure of a copper-containing nitrite reductase from *Ralstonia picketti*¹⁷⁷ has been solved and reveals a c-terminally fused cytochrome c domain

– an iron based electron transporter. The extraneous domain is aligned with the trimeric nitrite reductase complex in such a way that the cytochrome c makes inter-domain electron donations to the T1 copper of the nitrite reductase. This structure and functionality has inspired the domain outline of TtMCO3 in Figure 18.

One electron acceptor in the periplasmic space that sometime share electron donor with nitrite reductase is the Nitrous Oxide Reductase¹⁷⁸ (NOR). These enzymes catalyze the reaction $N_2O + 2 e^- + 2H^+ \rightarrow N_2 + H_2O$. Because of the shared electron donor to TtMCO2 and TtMCO3, reduction of nitrous oxide is a likely functionality for TtMCO2. In support of this hypothesis, the archaeal LMCO from *P. aerophilum* was reported to have NOR activity³⁰.

The loss of enzyme activity of TtMCO2 after purification halted the characterization of this novel and very interesting enzyme. It is a protein without close homologues, derived from the genome sequence of an obscure organism and the catalytic activity is not obvious. For all we know, the gene might be silent and the protein sequence void.

Some preliminary and negative experiments were made in an attempt to establish the function. Characterizing TtMCO2 in a heterologous expression system and then deducing the function would have been a fantastic achievement, but such a campaign could easily turn out to be a waste of time. A more rational approach would have been to grow the actual bacterium, *T. terrenum*, under different conditions and then measure the levels of TtMCO2 encoding mRNA by real-time PCR. Only if a pattern emerges does characterization of the protein make sense.

4 Concluding remarks and future perspectives

The main objective of this project was the identification and characterization of thermostable LMCOs. The first approach was to investigate a close homologue of *B. subtilis* CotA from the more alkaline adapted *B. clausii* KSM-16. The characterization of this LMCO revealed an enzyme where the preferred pH for stability and catalytic activity was shifted about 1 unit to the more alkaline compared to *B. subtilis* CotA (Paper I). This result suggests that pH adaptation of LMCOs can be deduced from the habitat of the natural host and it directly

validates our approach to search for extreme LMCOs in bacteria adapted to extreme environments.

One such extreme environment is a hot depression in the ground in Yellowstone National Park, Wyoming, USA, where *T. terrenum* was cultivated. The genome of this organism codes for a LMCO, TtMCO, which we have expressed and characterized. It is the second most thermostable characterized LMCO, and was only able to selectively oxidize two out of 57 tested substrates. Based on the substrate screen, and a comparison of related genes and genomes, we have proposed a specific role for this LMCO in maturation of antibiotics. This is the first characterization of a protein from *T. terrenum* (Paper II).

The specificity of TtMCO activity is in contrast to the broad substrate spectrum of e.g. *B. clausii* CotA and goes to show that the advantageous mechanism of LMCOs can be coupled to a specific chemical reaction. It would be interesting to investigate the activity of TtMCO on the two synthetic drugs (–)-kibdelone C and (–)-simaomicin α that have the properties of the proposed substrate. It is possible that TtMCO can selectively mature these drugs for enhanced activity. Even if TtMCO only show low activity on these drugs, it might be possible to create a specific enzyme by directed evolution.

In the effort to identify and characterize thermostable LMCOs, we also made some headway into understanding the often reported heat-activation of LMCOs. We observed that the process is followed by a change of the EPR signal, and we used this handle to characterize the heat-activation of *B. subtilis* CotA and TtMCO. Specifically we observed that heat activation – or inactivation - is controlled by temperature, salt, reducing agent and oxygen (Paper III). This is an important observation that might have implications for many more LMCOs, especially those of bacterial origin. Still, the characterization is not complete and more effort should be put into determining the interplay of these properties, especially in regards to LMCO inactivation. One parameter that was not investigated, but is likely to have an effect on inactivation is the pH.

Another interesting result is that CotA expressed as a fusion with sfGFP was more active than the fusion-free version when expressed without microaerobic incubation. In this project the derived vector psfGFP-CotA and the expression clone was mostly used as a positive control for LMCO expression, but preliminary result suggests that the sfGFP fusion is very desirable in e.g. high-throughput

screening of LMCO activity (section 3.2.3). This could be confirmed in a relatively quick study by investigating the EPR signal of CotA and sfGFP-CotA after aerobic expression and calculating the z-factor for both.

The last of the results presented in this dissertation is the preliminary characterization of TtMCO2, a unique MCO from the obscure organism *T. terrenum*. When this protein was expressed heterologously in *E. coli* the color of the cells visibly changed between blue and white according to the availability of oxygen. Despite this visual indication of an active enzyme, we did not identify a substrate (section 3.6.3). The protein is most likely coupled to the metabolism, and a future measurement of the redox potential for the blue color-change might give a clue to where it fits in. In addition it might be interesting to express the cupredoxin and the type2B MCOs on their own.

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Paper I

Characterization of an Alkali- and Halide-Resistant Laccase Expressed in *E. coli*: CotA from *Bacillus clausii*.

Søren Brander, Jørn D. Mikkelsen, and Kasper P. Kepp*

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Characterization of an Alkali- and Halide-Resistant Laccase Expressed in *E. coli*: CotA from *Bacillus clausii*

Søren Brander^{1,2}, Jørn D. Mikkelsen¹, Kasper P. Kepp^{2*}

1 Technical University of Denmark, DTU Chemical Engineering, Kongens Lyngby, Denmark, **2** Technical University of Denmark, DTU Chemistry, Kongens Lyngby, Denmark



Abstract

The limitations of fungal laccases at higher pH and salt concentrations have intensified the search for new extremophilic bacterial laccases. We report the cloning, expression, and characterization of the bacterial cotA from *Bacillus clausii*, a supposed alkalophilic ortholog of cotA from *B. subtilis*. Both laccases were expressed in *E. coli* strain BL21(DE3) and characterized fully in parallel for strict benchmarking. We report activity on ABTS, SGZ, DMP, caffeic acid, promazine, phenylhydrazine, tannic acid, and bilirubin at variable pH. Whereas ABTS, promazine, and phenylhydrazine activities vs. pH were similar, the activity of *B. clausii* cotA was shifted upwards by ~0.5–2 pH units for the simple phenolic substrates DMP, SGZ, and caffeic acid. This shift is not due to substrate affinity (K_M) but to pH dependence of catalytic turnover: The k_{cat} of *B. clausii* cotA was 1 s^{-1} at pH 6 and 5 s^{-1} at pH 8 in contrast to 6 s^{-1} at pH 6 and 2 s^{-1} at pH 8 for of *B. subtilis* cotA. Overall, k_{cat}/K_M was 10-fold higher for *B. subtilis* cotA at pH_{opt} . While both proteins were heat activated, activation increased with pH and was larger in cotA from *B. clausii*. NaCl inhibited activity at acidic pH, but not up to 500–700 mM NaCl in alkaline pH, a further advantage of the alkali regime in laccase applications. The *B. clausii* cotA had ~20 minutes half-life at 80°C, less than the ~50 minutes at 80°C for cotA from *B. subtilis*. While cotA from *B. subtilis* had optimal stability at pH~8, the cotA from *B. clausii* displayed higher combined salt- and alkali-resistance. This resistance is possibly caused by two substitutions (S427Q and V110E) that could repel anions to reduce anion-copper interactions at the expense of catalytic proficiency, a trade-off of potential relevance to laccase optimization.

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* E-mail: kpj@kemi.dtu.dk

Introduction

Laccases (EC 1.10.3.2, *p*-diphenol dioxygen oxidoreductases) belong to the class of multi-copper oxidases, characterized by four redox-active copper ions organized into three spectrally distinct sites (T1, T2, and T3). Laccases catalyze the one-electron oxidation of four equivalents of a reducing substrate, and in turn reduce one dioxygen molecule completely to water [1,2]. They oxidize a wide range of substrates, and this power can be further enhanced by the use of mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). These properties, along with their substantial stability, have made laccases widely explored as biocatalysts in diverse industrial applications such as delignification, stabilization of beverages, degradation of chlorophenols and dyes, in fine biochemicals and pharmaceutical industries (green organic chemistry), biosensors, and fuel cells [3–6].

A major challenge of laccase engineering is that the catalytic proficiency and stability of enzymes is dramatically dependent on environmental variables such as pH, temperature, ionic strength, and co-solvents. Industrial processes are often best employed at non-natural conditions, e.g. stirring conditions or extreme pH, temperature, or ionic strength. Fungal laccases are often active at acidic pH and low ionic strengths, where the proteins are much less stable [7]. Thus, recent efforts have been directed both towards identifying new alkalophilic and halophilic laccases [8–

12], and towards improving the alkalophilicity of fungal laccases by e.g. directed evolution [7].

While most investigated laccases are of fungal origin, the last decade has seen a range of bacterial laccases being reported [13,14]. The first characterized bacterial laccase, the spore coat protein A (CotA) from *Bacillus subtilis* [15] displayed high thermostability and a middle-range redox potential. The laccase has later been shown to oxidize a large variety of substrates, such as substituted aromatic compounds [16] and a number of important dyes [17]. In addition, it can function as a bilirubin oxidase [18], a low-affinity nitrous oxide reductase [19], and a manganese oxidase [20].

These capabilities of *Bacillus subtilis* cotA, combined with its impressive stability and the well-established bacterial expression protocols, have initiated a wealth of studies on cotA proteins. The orthologs from *Bacillus pumilus* [21], *Bacillus halodurans* [22], *Bacillus sp. HR03* [23], and *Bacillus licheniformis* [24] have been expressed heterologously in *E. coli*, and those of *Bacillus sp. ADR* [25], *Bacillus amyloliquefaciens LC02* [26], *Bacillus SF* [27,28], *Bacillus sp. VUS* [29], *Bacillus sp. XJT-7* [30], *Bacillus vallismortis* [31], and *Bacillus thuringiensis* [32] have been expressed from the native host.

A general tendency of the bacterial laccases in comparison to fungal laccases is higher stability at variable pH and temperature, however usually at the cost of having reduced redox potentials than their fungal orthologs [13], an interesting example of

functional trade-offs that are often a challenge to protein engineering. Whereas fungal laccases are strongly inhibited by hydroxide ions [33], bacterial laccases are known to often have higher optimal pH-values (pH_{opt}) of catalysis. For example, pH_{opt} for *Trametes versicolor* laccase oxidation of 2,6-dimethoxy phenol (DMP) is 3.4 [34] while it is 7.0 for *Bacillus subtilis* cotA [35].

Laccases could be used in a number of future applications where neutral or alkaline pH is desirable, including e.g. hair dyeing, in alkaline organic synthesis reactions, in laundry detergents, for oxidation of natural and synthetic dyes, bioremediation at neutral pH, and for direct oxidation of phenol-containing biopolymers, with phenolic substrates typically having pK_a values near 9–10 [36,37].

This paper reports the cloning, expression in an *E. coli* strain BL21(DE3), and characterization of a previously uncharacterized cotA from the gram-positive *Bacillus clausii* KSM-K16. Showing optimal growth near pH 9 [38], this species is known to produce alkali-, heat-, and detergent-resistant proteases such as the laundry M-protease with optimal enzymatic activity at pH 12.3 [39]. Since expression and handling protocols affect protein activation and consequently observed activities, as shown in this paper, to enable a strict benchmark for characterization, we simultaneously characterized the cotA from *Bacillus subtilis* 168 after expressing it in the same *E. coli* strain.

We found that the cotA from *B. clausii* has activity profiles shifted towards higher pH compared to *B. subtilis* cotA, notably for DMP, SGZ, and caffeic acid. This finding is in accordance with the optimal growth conditions of *B. clausii*. Both enzymes displayed substantial heat activation, as previously reported for *B. subtilis* spores [15], but for *B. clausii* cotA, the activation was much larger at higher pH. At pH 8, both enzymes displayed substantial activation in NaCl before inhibition became dominant. However, at lower pH, strict inhibition was observed as seen previously for fungal laccases usually assayed at low pH. The new cotA is more resistant to alkaline and high salt conditions, but at the expense of a ~10 fold loss of catalytic proficiency, as estimated from k_{cat}/K_M , providing a new example of trade-off in laccases.

Materials and Methods

Cloning protocol

The gene of *B. clausii* cotA (see sequence alignment in Figure S1 in File S1) was identified using protein BLAST with *B. subtilis* cotA as template against the genomic sequence of *B. clausii* KSM-K16. The codon-optimized gene was synthesized with restriction sites NcoI and KpnI by GeneArt Invitrogen in a pMK vector, and amplified by PCR with primers pMK-f 5'-GTG CTG CAA GGC GAT TAA GT-3' and pMK-r 5'-GAG TCA GTG AGC GAG GAA GC-3'. The 1806 bp PCR product was digested with NcoI HF and KpnI HF (Fermentas). The vector pETM13 (kind gift of Dr. Günther Stier, Heidelberg University) was digested with NcoI and KpnI (Fermentas), treated with shrimp alkaline phosphatase (Fermentas) and resolved in a 1% agarose gel with 0.1% crystal violet (Sigma-Aldrich). A band from the linearized vector was identified on a white light table and subsequently cut out.

The gene and linearized vector were purified with Illustra GFX PCR DNA and Gel Band Purification Kit (Illustra) and ligated with a T4 ligase (Fermentas) to yield the *Bacillus clausii* cotA expression vector p133. The cotA gene from *Bacillus subtilis* 168 was prepared by PCR amplification from genomic DNA using the primers 5'-ATC GTC TCT CAT GAC ACT TGA AAA ATT TG-3' and 5'-GTC GGT ACC TTA TTT ATG GGG ATC AG-5' to yield a 1542-bp PCR product. The gene was digested in two steps with BsmBI (New England Biolabs) and KpnI HF

(Fermentas) which allowed for cloning into the previously prepared pETM13 to produce the *Bacillus subtilis* expression vector p130. P130 and P133 were propagated in DH5 α , confirmed by DNA sequencing, and sub-cloned into BL21(DE3), allowing IPTG-induced expression of cotA proteins with no fusions or tailing amino acids.

Expression protocol

Protein expression was carried out in semi-anaerobic conditions [40], which have been shown to enhance copper loading into cotA type laccases. The expression strains were grown in LB medium to OD 0.6 at 180 rpm and 30°C followed by induction to 100 μM IPTG and 250 μM CuCl_2 . Incubation was extended at 120 rpm and temperature 25°C for four hours followed by 16 hours of static incubation at 25°C.

After the static incubation cells were harvested by centrifugation for 20 minutes at 12000 g and re-suspended in 20 mL cold lysis buffer 50 mM MOPS pH 7.8, 200 mM NaCl, 5% glycerol, 0.5 mM Pefabloc SC (Fluka), 0.01% Lysozyme. Cells were sonicated for five minutes on ice (UP400S with microtip H7, Hielscher) in pulses of 30 seconds at maximum power.

The cell lysate was incubated in a water bath for 30 minutes at 70°C, which improved cell lysis and precipitated thermo sensitive proteins. The protein solution was clarified by twice decanting the supernatant after centrifugation 30 minutes at 12000 g and finally filtered through a 0.45 μm cellulose filter. The protein was concentrated on a Vivaspin20 with 30 kDa cutoff (Sartorius), and the buffer was changed to 20 mM MOPS pH 7.6, 50 mM NaCl, and purified on a source Q15 anion exchange column with a linear gradient between 50 mM and 1 M NaCl in MOPS pH 7.6 on an ÄKTA purifier. Fractions were collected based on the absorption at 280 and 600 nm and buffer was changed into 20 mM MOPS pH 7.8 during the final concentration of the samples, using the Vivaspin20 system (for an SDS page gel, see Figure S2 in File S1).

Activity assays

Optimal pH for catalytic activity was determined for eight diverse substrates by measurement of product formation rates in 50 mM McIlvaine buffer with pH range 2.6–8.0, MOPS with pH-range 7.25–8.6, and CHES with range 8.6–10. All measurements were performed on a Tecan Pro Infinite 200 multiplate reader at 27°C by monitoring the kinetic trace for five minutes in order to calculate the initial rate by linear regression. The eight substrates and absorption wavelengths were 200 μM ABTS, 420 nm; 1 mM 2,6-dimethoxyphenol (DMP), 468 nm; 50 μM syringaldazine, 530 nm; 1 mM caffeic acid, 485 nm; 1 mM promazine, 520 nm; 200 μM tannic acid, 355 nm, 1 mM phenylhydrazine, 400 nm, and 0.0025% bilirubin mixed isomers, 440 nm.

All measurements were done in triplicates along with duplicate measurements of auto-oxidation with milliQ-water added instead of enzyme. ABTS and SGZ exhibited no auto-oxidation in the investigated pH range, whereas DMP, caffeic acid, and tannic acid showed steeply increasing auto-oxidation above pH ~8.5, and phenylhydrazine turned yellow below pH 4 which complicated the readings. Promazine displayed substantial auto-oxidation across the entire pH range. Bilirubin oxidase activity was measured as the depletion of substrate rather than the formation of product. Despite using substrate concentrations relevant to the full range of the spectrometer, the reaction was not linear for all pH. The reported pH activity profiles are shown after subtraction of the auto-oxidation rates (full data in Figure S3 in File S1). Furthermore, activity also depended to some extent on the buffer, affecting the activity curves across pH ranges where several buffers

were used. In order to fully understand this effect, activities were measured at overlapping pH values using both relevant buffers, enabling us to quantify the buffer effects and correct for these. The raw data are shown in Figure S3 in File S1, whereas the activities reported in the main paper were corrected for the buffer effect.

Stability estimation

The protein stability was investigated at 50°C and 70°C for the *B. clausii* and *B. subtilis* laccases in parallel, by incubating both enzymes at these temperatures and at pH 8, 9, or 10 for variable amounts of time. 200 μ L laccase solutions in 20 mM buffer was incubated in a 1.5 mL centrifuge tube on a temperature calibrated Eppendorf thermomixer, and at different time points, 10 μ L was retracted and immediately placed in a chilled 96-plate well with 90 μ L milliQ water. The activity was measured in a new 96-well plate by adding 180 μ L 500 μ M ABTS in 200 mM McIlvaine buffer pH 5 to 20 μ L chilled protein sample and measuring the initial rate at 420 nm. A secondary thermal stability experiment was carried out at temperatures 50°C, 70°C, and 80°C with samples being retracted during a 25-hour session and immediately assayed on a Perkin Elmer Lambda 20 spectrophotometer fitted with a temperature controlled cuvette changer. Additional residual activity curves for estimating optimal pH were produced at 5 hours incubation at 50°C are shown in Figure S4 in File S1, and after 20 hour incubation at 30°C in Figure S5 in File S1.

Michaelis-Menten analysis

Since Michaelis-Menten parameters will depend on pH, ten individual Michaelis-Menten analyses were performed based on DMP oxidation, i.e. for both enzymes at pH 5, 6, 7, 8, and 8.5. The concentration range used for DMP was 0–12.5 mM. Data were analyzed in GraphPad Prism 6 using a non-competitive substrate inhibition model for the full substrate range or a regular Michaelis-Menten model for a truncated data set, giving overall similar results (see Figures S6 and S7 in File S1). Protein concentrations were estimated from the UV/VIS absorption maximum at 600 nm (specific to the T1 site). We used the extinction coefficient as previously reported for *B. subtilis* cotA of 4000 $M^{-1}cm^{-1}$ [40], which is similar to those of other bacterial laccases (e.g. 3900 $M^{-1}cm^{-1}$ [19]), making this assignment robust. Total protein concentration from the UV absorption at 280 nm (extinction coefficient 79300 $M^{-1}cm^{-1}$) suggested a purity of approximately 70%, consistent with minor impurities observed on the SDS-PAGE gel (Figure S2 in File S1).

A Michaelis-Menten experiment was performed at pH 8.5 (\sim pH_{opt} for *B. clausii* cotA) using an apparent enzyme concentration of 1 nM. The resulting data were used to derive the kinetic data, based on an estimated protein concentration in the main kinetic analysis of 10 nM. When calculating this number, we accounted for the heat activation described later in this paper using a factor of 3.

Investigation of salt effects

The effect of NaCl concentration on enzymatic catalysis was investigated in a series of measurements using variable salt concentrations and a pH range of 6–9. Final concentration of buffer was 50 mM, whereas NaCl was varied between 0–1 M. The enzyme was applied to a 96-well microtiter plate, and the enzyme assay was initiated by addition of SGZ to a concentration of 50 μ M. SGZ was dissolved in methanol, and to reduce evaporation, fresh SGZ was used as retracted from a sealed volumetric flask for each assay. Activities were monitored as the initial rate of product formation, quantified from the absorption change at 530 nm.

Results and Discussion

The cotA gene from *B. clausii*

This study aimed to identify a protein that extends the positive characteristics of the *Bacillus subtilis* cotA in comparison to fungal laccases, with particular focus on activity and stability at alkaline pH. The impressive characteristics of *B. subtilis* cotA are surprising in the context of the native host being a mesophile, and we thus aimed at identifying orthologs from more extremophilic hosts. The cotA production is a late process of the *Bacillus* sporulation response, which is induced during stress conditions, but since sporulation is limited by the same factors as growth [41], the optimal growth conditions were used to identify high pH stability of related orthologs.

B. clausii KSM-16 encodes a cotA gene, has optimal growth at pH 9 at 40°C [38], compared to optimal growth at pH 8 for *B. subtilis*, suggesting a slightly higher alkaline tolerance. Another well studied sporulation enzyme from *Bacillus* is the subtilisin type protease with pH optima ranging from 8–13 [42], which is used in several industrial applications. The M protease from *B. clausii* belongs to the high-alkaline subtilisin group with maximum activity at pH 12.3. In contrast, the subtilisin proteases from e.g. *B. subtilis*, *B. licheniformis*, etc. have pH optima at pH 9–10. These two biological observations suggest higher spore stability at higher pH values and prompted our cloning, expression, and characterization of *B. clausii* cotA.

Figure 1A shows a sequence-derived nearest neighbor tree of homologous cotA enzymes created using MEGA 5.2 [43] started from the first 100 BLAST hits of *B. clausii* cotA against the Uniref90 database, and then reduced to only the high-scoring branches after a 1000-step bootstrap validation. It can be seen that the *B. clausii* cotA clusters together with a *B. alcalophilus* cotA in a different clade from that of *B. subtilis* cotA, showing a closer homology to this protein from an extremophilic bacteria. The *B. clausii* KSM-K16 cotA protein sequence was found to have 59% identity and 74% homology (BLOSUM62) with *B. subtilis* cotA, with most differences positioned within surface loops. Also, the network of ion pairs are not generally conserved between the two sequences (see Figure S1 in File S1). The most notably difference between the two primary sequences is that *B. clausii* cotA lacks the cystine forming loop present in *B. subtilis* cotA. Previously characterized cotA orthologs have all contained this cysteine bridge of unknown function [44].

Figures 1B and 1C show the superpositions of a structure of *B. clausii* from homology modeling (purple) on the *B. subtilis* cotA structure 3ZDW [45], (green). The tertiary structure of *B. clausii* cotA was constructed with Phyre2 [46], giving a homology model that was overall similar in tertiary and secondary structure to structure 3ZDW of *B. subtilis* cotA. Figure 1B shows the substrate binding pocket of cotA in complex with ABTS (3ZDW). The two loops that are connected by the cystine bridge in *B. subtilis* cotA are generally similar for the two proteins, apart from the tight GCGGD turn that allows the cysteines to bind. Two residues in contact with the substrate are substituted in *B. clausii* cotA; G417V and A227F, using the residue numbering from *B. subtilis* cotA. Both of these changes reflect substitutions from small aliphatic to bulky hydrophobic side chains, and they are positioned on opposing sides of the substrate binding pocket and close to the catalytic site.

Figure 1C shows the superposed water exit channels. Laccases use oxygen as co-substrate and produce water, both of which need to be transported via channels to the interior T2/or T3 copper sites, and thus variations in these part of the proteins could affect turnover. Two variations occur in sites that flank this channel, i.e.

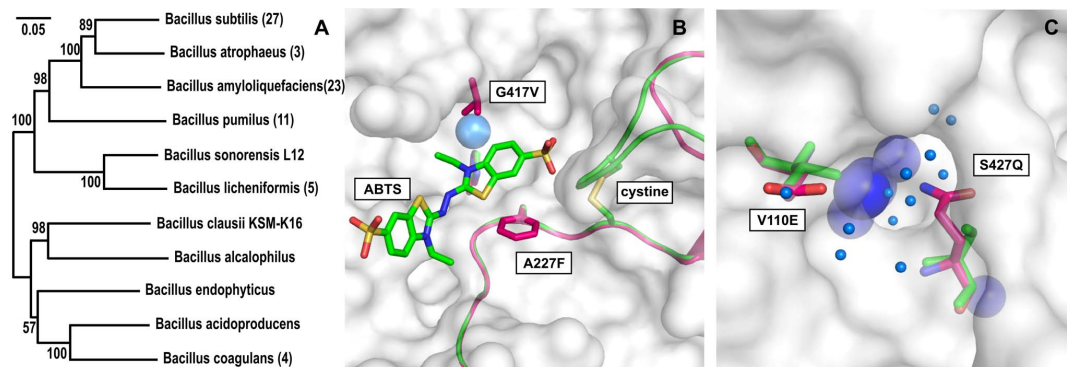


Figure 1. The cotA from *B. clausii*, compared to its ortholog from *B. subtilis*. (A) Nearest-neighbor tree of *B. clausii* cotA as described in the text. The bracketed number is the number of sequences represented by each node. Bootstrap values are given at the base of each branch. (B) The substrate binding pocket of cotA highlighting the differences between *B. subtilis* (green) and *B. clausii* (purple) cotA as described in the text. Amino acid changes are numbered on the *B. subtilis* cotA sequence and annotated with the *B. clausii* cotA amino acid last. (C) The water exit channel of cotA with similar highlighting. The T2 copper site is visible through the open channel. Small blue spheres represent water from pdb structure 3ZDW. Structures were visualized using PyMol 1.5 and the tree was generated using MEGA 5. doi:10.1371/journal.pone.0099402.g001

S427Q and V110E. There are no significant differences within the oxygen entrance channels of the two protein structures.

Activity studies and pH optima

The pH-dependent activities were measured as initial linear rates, averaged over triplicate measurements for both laccases, using eight different substrates in the pH-range of 3–10: ABTS, SGZ, DMP, caffeic acid, promazine, phenyl hydrazine, and the more bulky tannic acid and bilirubin: Apart from hydrazine, all these substrates have previously been shown to be oxidized by some cotAs [16,18]. The data are shown with standard deviations in Figure 2. Since we used three standard buffers to cover the studied pH intervals, we could identify a direct effect of buffer on the activity that has generally been ignored in previous work: Proteins prepared in the high-pH buffer CHES displayed higher activity for the same pH (8.6) than the medium-range buffer MOPS, and MOPS gave a higher activity than the low-pH range McIlvaine buffer at pH 8; the buffer systems affected rates up to ca. 40% of total activity. We thus performed multiple measurements at the limiting, common pH values of the buffers (full data in Figure S3 in File S1); the data presented in Figure 2 are corrected for the buffer effect by scaling the data points of the high-activity buffers to align with data points from the McIlvaine buffer at their common pH values.

We first compare our obtained activity of the cotA from *B. subtilis* with previous work, in particular the pH optima with ABTS, SGZ, and DMP [15,35]. As seen in Figure 2A, the ABTS oxidation rate of cotA from *B. subtilis* has maximum near pH~4. This value is consistent with the $\text{pH}_{\text{opt}}(\text{ABTS})$ of ~4 obtained previously for cotA from *B. subtilis* [35]. Furthermore, the activity behavior vs. pH is quite similar for the new cotA across the pH range, given similar pH_{opt} for this substrate.

The relative activity on SGZ is shown in Figure 2B. While other phenolic substrates exhibited substantial auto-oxidation, to be discussed below, we found no measurable auto-oxidation of SGZ in the pH-range 3–10 despite its phenolic ring system. The difference could indicate that the abstracted SGZ electron is not localized on the hydroxy group but more likely the azo group. The $\text{pH}_{\text{opt}} \sim 7$ is also consistent with that reported previously for *B.*

subtilis cotA [35]. The notable difference between the two enzymes is that the laccase from *B. clausii* remains active at a higher pH than its ortholog from *B. subtilis* and keeps 50% of the activity at pH 9 at which the *B. subtilis* cotA displays almost no activity.

In terms of the phenolic substrate DMP (Figure 2C), we found an optimal turnover for *B. subtilis* cotA at pH~7, which is in agreement with previously reported pH_{opt} for this protein and substrate [35]. In contrast, the cotA from *B. clausii* was most effective above pH 8, showing a significant shift in pH_{opt} (~1.3) compared to the cotA from *B. subtilis*, further indicating enhanced alkalophilicity of the cotA from *B. clausii*.

Caffeic acid is a dihydroxy cinammic acid and is one of the major natural oxidants mainly found in leaves. It is a general phenolic laccase substrate [16], but without the substituted methoxy groups of SGZ and DMP. The first phenolic proton has a pK_a 8.6, but the compound is already charged through the full pH range from deprotonization of a carboxylic acid. The pH activity profile (Figure 2D) shows an optimal pH shifting from 6 for *B. subtilis* cotA to 7 for *B. clausii* cotA. Similar to SGZ, the activity of *B. clausii* cotA broadens into the alkaline range with 40% activity at pH 8 compared to none for *B. subtilis* cotA.

We also investigated the activity of the two enzymes on promazine, a synthetic non-phenolic phenothiazine drug. The observed oxidation was not catalyzed by 1 mM CuCl_2 . In a large screening of laccase substrates, promazine was previously found to be oxidized by laccases of bacterial but not fungal origin [16], making it an interesting substrate to probe potential new oxidation capabilities of bacterial laccases. As seen in Figure 2E, the two enzymes displayed very similar activities across pH for this substrate against a high background of auto-oxidation (full data in Figure S3 in File S1). The pH_{opt} was found to be in the range 4–5 for both enzymes on this substrate, i.e. slightly higher than for ABTS.

We further tested activity of the enzymes on phenylhydrazine, a potent reducing agent with $E^\circ = 410$ mV [47] that readily reacts with Cu(II) (Figure 2F). It has previously been described as both an inhibitor and substrate for a tyrosinase [48], ultimately resulting in an *o*-quinone. Our reaction with laccases produced a similar UV-VIS spectrum, indicating that the same reaction occurs, and both

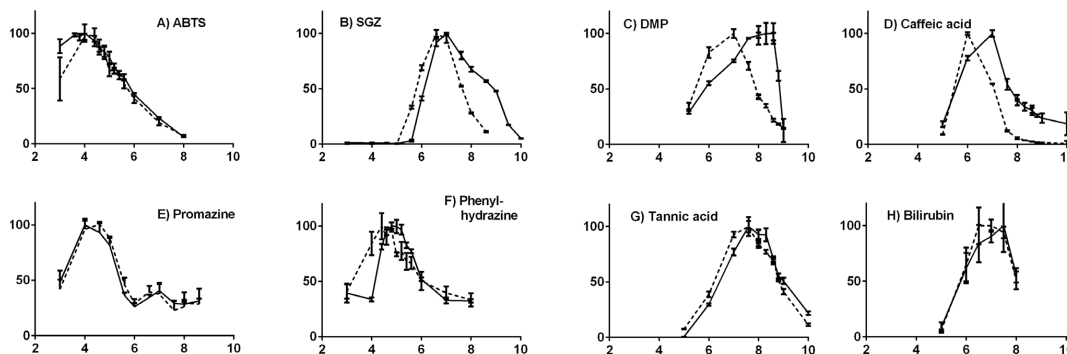


Figure 2. Activity vs. pH of the *cotA* from *B. clausii* (solid lines) on eight substrates, compared with that of *cotA* from *B. subtilis* (broken lines). Activities are averages with standard deviations from triplicate measurements of initial rates of product formation, corrected for buffer effects (see text) and normalized to 100. (A) ABTS; (B) SGZ; (C) DMP; (D) Caffeic acid; (E) Promazine; (F) Phenylhydrazine; (G) Tannic acid; (H) Bilirubin.

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laccases were found to oxidize this compound. The laccase from *B. clausii* and *B. subtilis* were found to have slightly different pH_{opt} shifting from ~ 4.5 in *B. subtilis* *cotA* to ~ 5 with *B. clausii* *cotA*.

We also investigated the activity on an unusual phenolic substrate, tannic acid (Figure 2G). Tannic acid is a plant tannin consisting of branching polygalloyl sugar esters. The tri-hydroxy phenyl moieties make the compound acidic with a first pK_a of ~ 4 [49], and it is a potent metal chelator. In addition, tannic acids precipitate proteins and are considered a general plant defense against herbivores and bacteria. Tannic acids have been reported to inhibit tyrosinases [50]. The optimal pH activity profiles for oxidation of tannic acid (Figure 2G) are similar for both enzymes, with pH optima of $\sim 7.5 \pm 0.5$, although the curve of *B. clausii* *cotA* shifted ~ 0.5 pH units towards more alkaline conditions.

Finally, to further expand the chemical diversity, we also investigated the activity on bilirubin, which has been shown to be a substrate for several laccase-like enzymes, and also for *cotA* [18]. The results (Figure 2H) indicated a similar upper and lower pH for activity for the two enzymes, and the exact pH_{opt} are similar within the uncertainties of the assay and comparable to that previously reported [18]. Thus, the bilirubin assay confirms that the alkaline advantage of the new *cotA* mainly manifests in the phenolic substrates.

An important factor affecting activity across pH is the changing difference in redox potential between the substrate and enzyme [33]. For each decreased pH unit, the difference in redox potential will increase by 60 mV viz. the Nernst equation, driving oxidation up [33]. Another effect comes from the pK_a value of phenolic substrates whose deprotonized forms have much lower redox potentials, also accelerating oxidation when pH increases. These two activity-increasing effects at low and high pH are countered at extreme pH by protein stability and at high pH, the direct OH^- inhibition, most likely due to OH^- binding to the T2/T3 site. However, these effects, general to all laccases, cannot explain the protein-specific differences that we observe. The decreasing redox potential of proton-labile substrates with increasing pH also gives rise to a substantial auto-oxidation at higher pH values (see Figure S3 in File S1).

There are three important trends in the pH profiles. First, promazine, ABTS, bilirubin, and to some extent phenylhydrazine and tannic acid show similar activity profiles vs. pH for the enzymes, with similar pH_{opt} , despite some enhanced loss of *B.*

clausii *cotA* activity at low pH for ABTS and phenylhydrazine. These substrates are not simple phenols and perhaps except phenylhydrazine, they do not change charge in the measured pH range.

Second and in contrast, caffeic acid, SGZ, and DMP (and to some extent the polyphenol tannic acid) all have their active pH range increased for *B. clausii* *cotA*, compared to *B. subtilis* *cotA*. The pK_a values of these compounds are 8.6, 8.8, and 10.0 [51,52], i.e. they change charge in the monitored pH range. Interestingly, the pH optima follow the pK_a values with 7, 7, and 8 for *B. clausii* *cotA* and 6, 6.6, and 7 for *B. subtilis* *cotA*, with a general trend of the *B. clausii* laccase being more active at higher pH.

A third trend, potentially related to the second one, is a shoulder in the activity profile in the pH range 8–9 for *B. clausii* *cotA*, which makes the activity profiles skewed towards higher pH. This is seen for SGZ, caffeic acid, DMP and to some extent for tannic acid, showing that this is a protein-specific effect. As this effect was seen for all the measured substrates that have activities in this pH range and since the effect did not follow the pK_a of substrates, it suggests that *B. clausii* *cotA* has adapted to a generally more alkaline environment, consistent with an optimal growth at pH 9 of its host organism [38]. This non-substrate-specific activity effect coincides with the emerging OH^- inhibition at above-neutral pH. Figure 1C shows the structural changes around the water exit channel of the two *cotAs*. *B. clausii* *cotA* has a glutamic acid (V110E) on the brim of the exit channel, which in itself, via electrostatic repulsion, might reduce OH^- affinity for the channel, or it can possibly make an interaction with the other variable amino acid 427Q to reduce OH^- entry during catalytic turnover. This mechanism would be similar to that suggested for an alkali-tolerant mutant of *T. versicolor* laccase derived from directed evolution, which had a N109S mutation suggested to affect OH^- entry [7].

In conclusion, the activity of the newly characterized *cotA* from *B. clausii* is in some ways similar to that of the *cotA* from *B. subtilis*, but its increased pH_{opt} and the broadened activity profiles up to 1–2 units higher pH for the phenolic substrates DMP, caffeic acid, and SGZ suggest a mechanism of alkali-tolerance that is explored further below.

Incubation at variable pH and T: Activation processes and stability

The stability of bacterial laccases is one of the key properties rendering them attractive in diverse applications where protein life time is of major importance. To investigate the stability of the newly characterized cotA from *B. clausii* we first measured the activity of protein samples that had been stored in buffers at pH 3–10 and incubated at 30°C and 50°C for five hours (See Figure S4 and Figure S5 in File S1). From these experiments, the stability of *B. subtilis* cotA appears to be maximal in the range 7.6–8.6, while the *B. clausii* cotA shows a monotonic increase in activity as the pH becomes more alkaline. Data suggests that there is a significant difference in the interplay between pH and thermostability between the two enzymes. A similar profile of residual activity after incubation has also recently been seen for *Streptomyces viveicus* laccase [10].

To investigate the temperature behavior of the newly characterized cotA from *B. clausii* in more detail, we studied its residual activity after incubation at various times at 50°C, 70°C, and 80°C, as described in the Methods section. Again, we performed all measurements completely in parallel also for the cotA from *B. subtilis*, to enable a strict benchmark for characterization. Furthermore, we did all the incubations and the subsequent activity measurements at three pH values, 8, 9, and 10, because pH variations, although rarely reported, markedly influence residual activity or apparent thermostability upon incubation, and we had observed that pH significantly affects the relative apparent stability of the two enzymes. The combined data for these measurements are shown in Figure 3 and Figure 4. We found thermal activation at short incubation times (shorter for higher temperature) before thermal protein degradation dominated the subsequently measured initial rates. This was especially prominent for *B. clausii* laccase and complicates the analysis of the thermostability.

Figure 3 shows the longer-time-frame data of thermal incubation at variable pH. At 50°C (Figure 3A, 3D), none of the proteins displayed any significant degradation within the first 1500 minutes of incubation. At 70°C (Figure 3B, 3E), the degradation of protein was clearly evident, but so was heat activation and the fate of the proteins is a non-trivial mixture of protein degradation and heat activation. An attempt to fit an exponential decay to the *B. subtilis* cotA data is given in Figure 3B, where only the data after 120 minutes have been used in the fitting procedure. *B. subtilis* cotA show best stability at pH 8 with an approximate $t_{1/2}(70^\circ\text{C})$ of 250 minutes compared to 110 minutes at pH 9 and pH 10. A similar fit was not possible for *B. clausii* due to its substantial heat activation but it is clear that its stability at pH 8 and 9 is slightly higher than at pH 10. At 80°C (Figure 3C, 3F), there is no visible thermal activation, because the degradation is faster than the heat activation. Still, a plateau resulting from combined activation and degradation is visible within the first 20 minutes for both laccases except at pH 10 for cotA from *B. clausii*, which is degraded quickly at this high temperature. The exponential decay function for *B. subtilis* cotA where the first 20 minutes have been disregarded in the fitting procedure is given in Figure 3C. The $t_{1/2}(80^\circ\text{C})$ was measured to be 50 minutes at pH 8 and 22 minutes at pH 9 and pH 10. This compares well with the 56 minutes that can be read from the data in the original *B. subtilis* cotA characterization from 80°C incubation at pH 7.6 [15]. We estimated the corresponding $t_{1/2}(80^\circ\text{C})$ of *B. clausii* cotA to be ~20 minutes at pH 8 and substantially less pH-dependent than cotA from *B. subtilis*.

From all measurements, *B. clausii* cotA had similar apparent stabilities in pH 8 and pH 9 and slightly lower stability in pH 10. In contrast, *B. subtilis* cotA exhibited highest apparent stabilities at

pH 8. The stabilities are best compared at 80°C where heat activation does not substantially affect the short-time data. At pH 8 *B. subtilis* cotA has 30% activity after 100 minutes of incubation at 80°C. A similar reading of *B. clausii* cotA at pH 9 gives 18%. These data thus show that the new cotA from *B. clausii* is slightly less thermostable than the cotA from *B. subtilis*, but has traded some of this thermostability for a higher stability at pH 9–10, whereas cotA from *B. subtilis* is found to be clearly most stable at pH 8.

From the short-time interval measurements shown in Figure 4, we can see that at 50°C, cotA from *B. subtilis* (Figure 4A) displayed no thermal activation at any pH whereas the laccase from *B. clausii* (Figure 4C) was activated three-fold within the first 300 minutes at pH 10, and two-fold at pH 8. The unusual, higher activation at higher pH for *B. clausii* cotA was consistent across the measured rates at pH 8, 9, and 10, and thus significant. At an incubation temperature of 70°C, the cotA from *B. subtilis* began to display some thermal activation up to 140% at pH 10, but no activation at pH 8 (Figure 4B). At 70°C, the cotA from *B. clausii* (Figure 4D) displayed activation up to 220% within 20–40 minutes and then started to degrade. These data show the importance of pH for reporting thermal activation and thermostability.

We found a general trend for both cotAs of more heat activation at higher pH. This is not a result of higher stability which levels off below pH 10 as discussed previously, and must thus be a true property of the intrinsic cotA activation. Thermal activation is commonly observed for laccases and is not generally understood [53]. However, it is likely to either imply a removal of partly inhibiting molecules from substrate binding sites or channels prior to the first catalytic cycles, or alternatively, involve a protein conformational change, where the active form of the protein is (entropically) favored at higher temperature. Alternatively, we suggest that heat favors release of small inhibitors or water in the channels of the enzymes, as their release would be entropically favored and thus more pronounced at high temperature. This heat-favored release may further be more pronounced in proteins with specific residues in the channels that favor anion release, such as the negative charge of *B. clausii* cotA.

Activation and inhibition of cotA vs. pH and NaCl concentration

Laccases are well-known to be inhibited by small anions, as observed for fungal laccases at low pH [2,3]. However, there are also previous reports that NaCl can activate proteins, as observed in another alkalophilic bacterial laccase [22], which displayed pH_{opt} values of 7.9 for SGZ and 7.5 for DMP [54]. This effect seems to be complex and pH-dependent, as discussed previously: OH^- and Cl^- are likely to compete as inhibitors at the T2/T3 site of a laccase, as has been observed for fluoride [33,55]. Also, both the Na^+ and Cl^- ions may affect the stability and turnover in some pH ranges due to non-specific electrostatic interactions with the proteins, e.g. via Hofmeister effects. Thus, to optimize catalytic performance, it is of importance to understand how NaCl and pH play together to activate or inhibit laccase activity.

Figure 5 shows the activity (initial rates of SGZ product formation) of cotA from *B. subtilis* and *B. clausii* as a function of both NaCl concentration and pH. It can be seen from Figures 5A and 5B that both proteins are inhibited at acidic pH, as observed before for F^- [33,56] and typically seen in fungal laccase assays where activity is highest at low pH [53]. However, we also observe a substantial activation of both proteins at higher pH, even though these measurements were done at room temperature.

To further confirm the estimates of the alkali-tolerance of the proteins, the intersections at 400 mM NaCl and 1000 mM NaCl

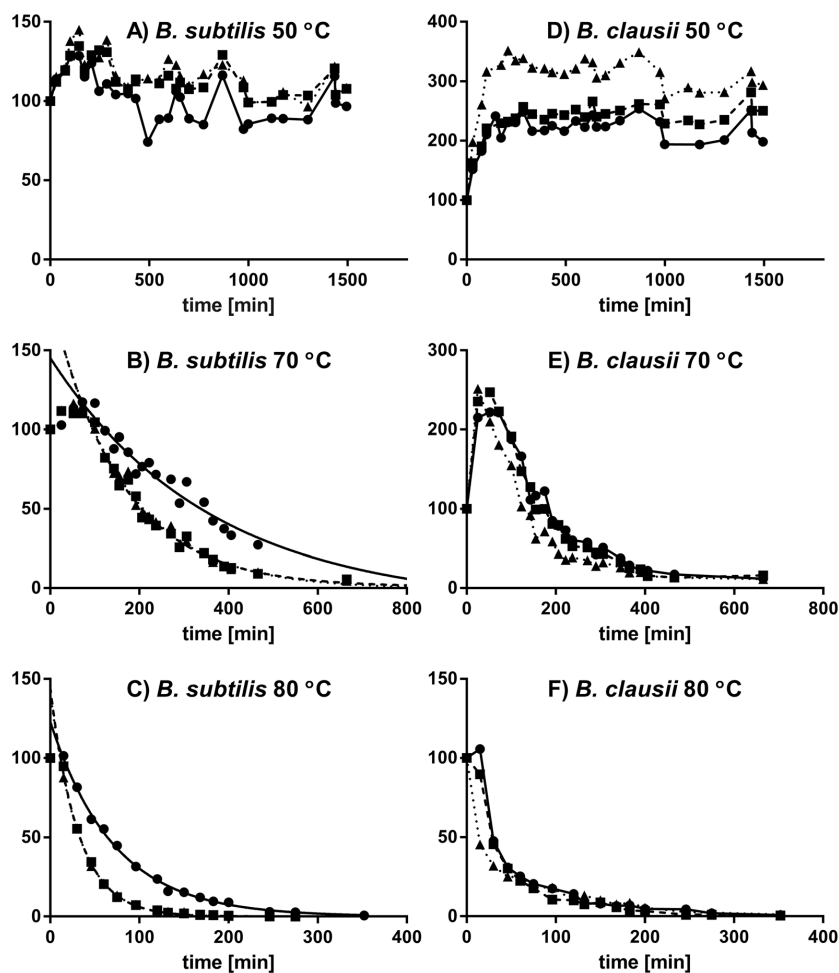


Figure 3. Stabilities of the cotAs from *B. clausii* and *B. subtilis* vs. temperature and pH 8 (circles, solid line), 9 (squares, broken line) and 10 (triangles, dotted line). (A) *B. subtilis* cotA at 50°C; (B) *B. subtilis* cotA at 70°C; (C) *B. subtilis* cotA at 80°C; (D) *B. clausii* cotA at 50°C; (E) *B. clausii* cotA at 70°C; (F) *B. clausii* cotA at 80°C. Activities were normalized to the starting activity before heat incubation. doi:10.1371/journal.pone.0099402.g003

are shown in Figures 5C and 5D, respectively. From these figures, it is more clear that the cotA from *B. subtilis* loses activity quickly above pH 8, whereas the cotA from *B. clausii* preserves substantial activity at higher pH, consistent with the results from the activity profiles vs. pH, Figure 2.

The most important conclusion from these experiments seems to be that chloride inhibition of laccases at low pH is countered at high pH by activation, meaning that higher pH is very desirable also for this reason in laccase applications. It is reasonable that chloride inhibition would be electrostatically favored at low pH where the protein channels are likely less negatively charged, if electrostatics affects the OH⁻ and Cl⁻ affinity. The strong dependence of halide inhibition on pH means that the halide tolerances of different laccases, which are commonly described at only one pH and compared to each other at variable pH [57], should be quantified over a range of pH to be commensurable.

Heat activation is common for laccases, but for *B. clausii* we also observed similar effect from high pH incubation and NaCl. These three observations can potentially be derived from the same mechanism. A simple explanation is that the laccases are produced in a non-functional form in the heterologous host. However, activation has been observed up to 150% for natively expressed cotA in *B. subtilis* spores at 80°C [15], and activation up to 130% has been observed at 37°C for *B. pumilus* laccase [58], which is within the optimal temperature range for growth of the native host. Thus, it seems likely that cotA activation occurs *in vivo* as part of the function of this enzyme in the sporulation process.

Introduction of a lysine into *B. subtilis* HR03 CotA [59] has been shown to significantly increase the potential heat activation to an extreme 900%. This was explained by the introduction of a new ionic interaction between two domains. It was hypothesized that this interaction is broken at high temperature, creating a more

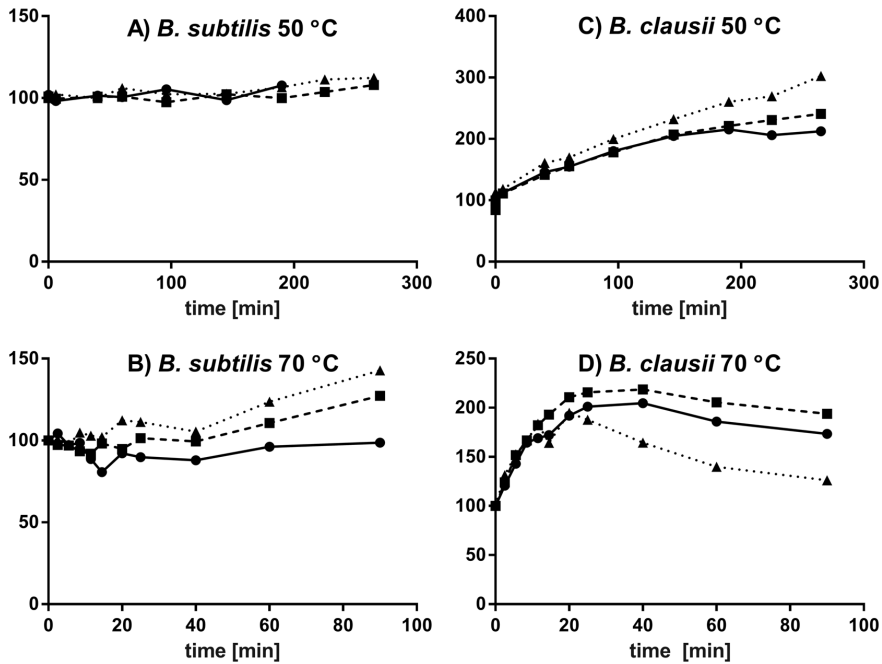


Figure 4. Short-term residual activity of the proteins after incubation. Heat activation is visible at pH 8 (circles, solid line), 9 (squares, broken line) and 10 (triangles, dotted line). (A) *B. subtilis* cotA at 50°C; (B) *B. subtilis* cotA at 70°C; (C) *B. clausii* cotA at 50°C; (D) *B. clausii* cotA at 70°C.
doi:10.1371/journal.pone.0099402.g004

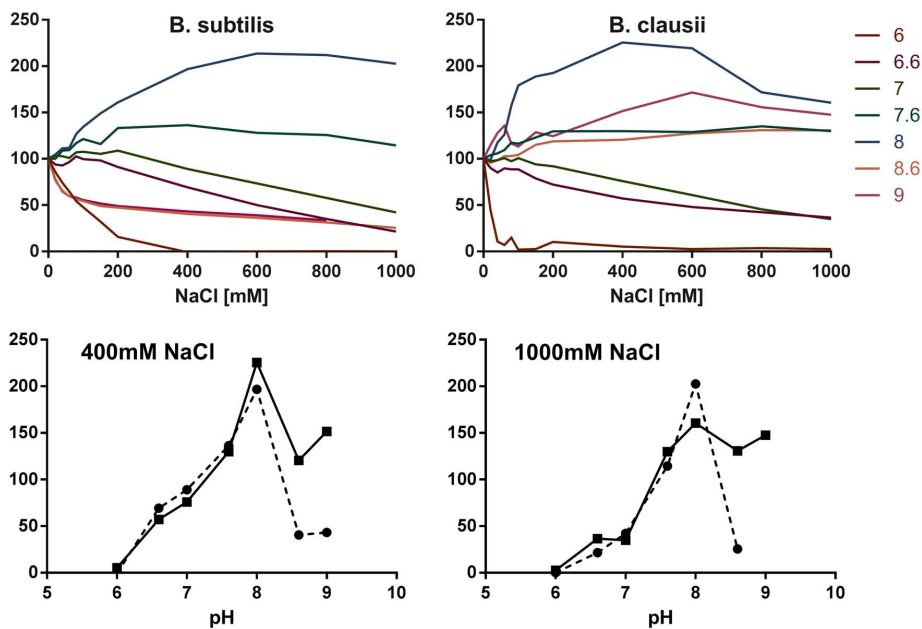


Figure 5. Activity of cotA from *B. subtilis* and *B. clausii* as a function of NaCl concentration and pH. (A) *B. subtilis* cotA; (B) *B. clausii* cotA; (C) Intersection at 400 mM NaCl and (D) intersection at 1000 mM NaCl, with *B. clausii* cotA in full line and *B. subtilis* cotA in broken lines.
doi:10.1371/journal.pone.0099402.g005

active enzyme. A similar change in ionic interactions as pH approaches the pK_a of an important salt bridge might explain our observed enzyme activation at higher pH. Since NaCl activation occurred in combination with pH activation, it is possible that high ionic strength may destabilize labile salt bridges to give a similar response, but understanding the complex interplay of temperature, pH and salt on activation-inhibition of laccases clearly requires more mechanistic investigations.

Variation in K_M and k_{cat} as a function of pH

To understand the causes of the pH-dependent activity differences between the two enzymes, which is most pronounced in DMP (Figure 2), we additionally performed a Michaelis-Menten analysis for both enzymes at pH values of 5, 6, 7, 8, and 8.5 (full data in Figures S6, S7, and S8 in File S1). Figure 6 shows the behavior of k_{cat} , K_M , and k_{cat}/K_M as a function of pH resulting from this analysis.

All parameters vary substantially with pH, suggesting that the use of one pH for Michaelis-Menten analysis is insufficient. While the K_M s behave largely similar vs. pH (Figure 6B), there are substantial differences in the pH-dependence of k_{cat} for the two enzymes (Figure 6A). The ratios of the two parameters depend similarly on pH (Figure 6C), although the optimum for cotA from *B. clausii* is at ~ 0.4 higher pH, as obtained from a Gaussian fitting. From this, we conclude that the pH-dependent differences measured from the assays of phenolic substrates, including DMP, are largely due to differences in k_{cat} , i.e. the electron transfer catalytic step, and not substrate binding (K_M). This finding confirms that the performance of cotA from *B. clausii* at higher pH is enhanced by activity associated with the copper sites, not the substrate binding step, and is consistent with an enhanced ability to prevent anion inhibition. Our K_M derived from non-linear fitting at pH 7 was 0.34 mM for cotA from *B. subtilis*, somewhat larger than the 0.23 mM determined by Duraõ et al. [40]. As outlined in the supporting information (Figures S6 and S7 in File S1), DMP kinetics give rise to substrate inhibition. Thus, the difference could result from substrate concentration effects or different amounts of active enzyme, which is a major challenge to laccase benchmarking. The measured K_M for *B. clausii* cotA was 1.02 mM, similar to cotA from *B. pumilus* with reported K_M 0.822 mM towards DMP at pH 7 and 37°C [58].

We observed substantial conversion between active and inactive forms of the enzymes, as evident also partly from the heat activation in Figure 3, rendering a definite assignment of active enzyme amount error-prone. We accounted for this activation in the calculation of protein concentration and k_{cat} for *B. clausii* using a factor of 3 as indicated by Figure 3 and used absorption at

600 nm for concentration estimation (File S1, Figure S8). The values of k_{cat} deduced from the analysis (Figure 6) are for *B. subtilis* cotA $\sim 6 \text{ s}^{-1}$ at pH 6 and $\sim 2 \text{ s}^{-1}$ at pH 8 and for *B. clausii* cotA 1 s^{-1} at pH 6 and 5 s^{-1} at pH 8. The reported k_{cat} of *B. subtilis* cotA is in the lower end of the previously reported range 7–30 s^{-1} at pH 7 [40]. The k_{cat}/K_M curves generally follow the pH-dependent activities of Figure 2. The k_{cat}/K_M values resulting from Gaussian fitting (Figure 6) are 10-fold higher for *B. subtilis* cotA, suggesting that the alkali-salt tolerance discussed above has been achieved at the expense of catalytic proficiency. A more detailed kinetic analysis requires a protocol to fully convert between inactive and active protein forms – We are currently exploring work in this direction.

In addition to conversions between active and inactive forms, auto-oxidation, and buffer effects, which should be carefully accounted for during laccase characterization, we also observed a substantial non-competitive inhibition of both enzymes starting at DMP concentrations of $\sim 2.5 \text{ mM}$. The Michaelis-Menten data were thus derived also from non-linear fitting taking inhibition into account, given similar results (See Figure S6 and Figure S7 in File S1, last panels). Moreover, since we performed the Michaelis-Menten analysis at various pH-values, we also observed that this inhibition is absent at acidic pH but initiates at pH ~ 6 for *B. subtilis* cotA and at pH ~ 7 for *B. clausii* cotA. This inhibition further increases with increasing pH. Thus, the pH-dependence of the non-competitive inhibition is also consistent with the alkaline advantage of cotA from *B. clausii*.

Conclusions

We have reported the cloning, expression and characterization of a cotA from *B. clausii*, strictly benchmarked against its well-known ortholog from *B. subtilis*, expressed heterologously and characterized fully in parallel. We find that cotA from *B. clausii* has a shift in optimal pH for oxidation of phenolic substrates and a high salt resistance at high pH, compared to its ortholog from *B. subtilis*. We also identify important, pH-dependent effects of buffers, auto-oxidation, thermal activation, and non-competitive substrate inhibition that, together with the general changes in relative redox potentials of substrates and laccases, confirm and expand the previously noted [60] complex behavior of these enzymes under variable pH and NaCl concentration.

The stabilities of the two enzymes were substantial, with half-times of inactivation of 50 and 20 minutes for cotAs from *B. subtilis* and *B. clausii*, respectively, at pH 8 and 80°C. In comparison, the newly reported alkali-stable laccases from *actinomyces* [12] and *Trametes sp.* [11] had similar half-lives (60 minutes) at only 60°C

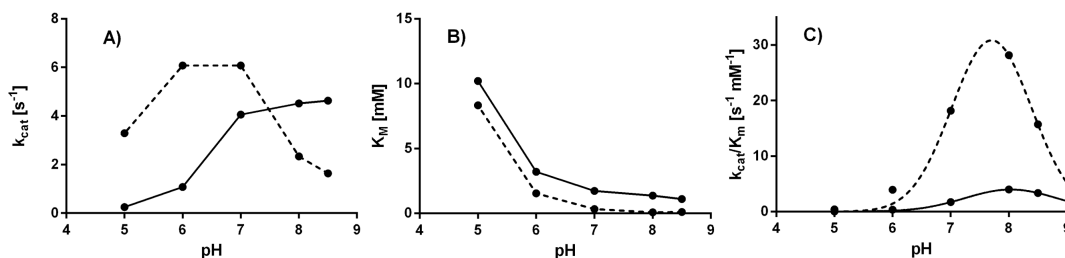


Figure 6. k_{cat} and K_M for DMP oxidation as a function of pH for *B. subtilis* cotA (broken lines) and *B. clausii* cotA (full lines). (A) k_{cat} in s^{-1} . (B) K_M in mM (C) k_{cat}/K_M showing the effect on total pH-dependent activity. The profiles were fitted with a Gaussian using a mean pH of 7.7 and an amplitude of 30.8 for *B. subtilis* cotA and 8.1 and 4.0 for *B. clausii* cotA, respectively. Please see File S1 for full data. doi:10.1371/journal.pone.0099402.g006

and pH 8, showing the substantial advantage of the characterized bacterial laccases over fungal orthologs if stability is preferred over reactor potential. This advantage is, as shown in this work, further enhanced by the interplay of salt and pH at alkaline conditions where the cotA enzymes, in particular the cotA from *B. clausii*, work well.

The additional proficiency of cotA from *B. clausii* could be due to substitutions relative to its *B. subtilis* ortholog that may repel anion inhibitors in the water exit channel (notably V110E, Figure 1), although this requires further investigation, as does the complex interplay between pH and salt. From Michaelis-Menten analysis, we found that the alkaline activity shift of the new cotA is not due to K_M but mainly to k_{cat} , suggesting changes at the copper sites rather than the T1/substrate binding sites. While this shift is consistent with higher alkali-salt tolerance and potentially explained by increased anion repulsion in the water exit channel, this tolerance has been at the expense of 10-fold lower k_{cat}/K_M (DMP) compared to cotA from *B. subtilis*. The observed trade-off in the new cotA between alkaline stability and catalytic proficiency

should be of interest when optimizing bacterial laccases for applications under neutral or alkaline conditions.

Supporting Information

File S1 File S1 contains supporting data on sequence alignment of cotA orthologs (Figure S1), SDS page (Figure S2), activity profiles vs. pH without correcting for buffer differences and without subtraction of auto oxidation (Figure S3), data for pH-stability from activity measurements after incubation (Figure S4 and Figure S5), and data for the Michaelis-Menten kinetics of cotAs from *B. subtilis* (Figure S6) and *B. clausii* (Figure S7). **UV-VIS absorption profile of *B. clausii* cotA.** (Figure S8). (PDF)

Author Contributions

Conceived and designed the experiments: SB KPK. Performed the experiments: SB. Analyzed the data: SB KPK. Contributed reagents/materials/analysis tools: SB JDM. Wrote the paper: SB KPK JDM.

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Paper II

TtMCO: A highly thermostable laccase-like multicopper oxidase from the thermophilic and ancient *Thermobaculum terrenum*

Søren Brander, Jørn D. Mikkelsen, and Kasper P. Kepp*

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TtMCO: A highly thermostable laccase-like multicopper oxidase from the thermophilic and ancient *Thermobaculum terrenum*

Søren Brander, Jørn D. Mikkelsen, and Kasper P. Kepp*

Technical University of Denmark, DTU Chemistry, DK-2800 Kongens Lyngby, Denmark

*Correspondence: Email: kpj@kemi.dtu.dk

Abstract

This paper reports the identification, characterization and heterologous expression of TtMCO, the first protein from the thermophilic bacterium *Thermobaculum terrenum* and the first Laccase-like Multi-Copper Oxidase (LMCO) from Phylum Chloroflexi, which is notorious for its diverse morphologies and distinct bacterial phylogeny. TtMCO has only 39% identity to its closest characterized homolog, CotA from *Bacillus subtilis*, but sequence and spectrophotometry confirmed copper coordination similar to that of LMCOs. TtMCO is extremely thermophilic with a half-time of inactivation of 2.24 days at 70°C and 350 minutes at 80°C and pH 7, consistent with an ancient and hyperthermal habitat. TtMCO was screened for activity against 56 chemically diverse substrates: It displayed limited activity on classical LMCO substrates, such as e.g. phenolics, transition metals, or bilirubin. Highest activities were observed for nitrogen-containing aromatic compounds, i.e. 1,8-diaminonaphthalene ($K_m = 0.159 \text{ mM}$, $k_{cat} = 0.295 \text{ s}^{-1}$) and ABTS ($K_m = 0.844 \text{ mM}$, $k_{cat} = 2.13 \text{ s}^{-1}$). The combined data suggest a central role of TtMCO in MCO evolution and a substantial trade-off between activity and stability, compared to other characterized bacterial LMCOs. Based on activity, gene cluster analysis, and homology to LMCOs of close relatives, a possible role in synthesis of antibiotics is suggested.

Keywords: bacterial evolution, multi-copper oxidase, laccase, cotA, hyperthermophile, stability-activity trade-off, LMCO, *Thermobaculum terrenum*, Chloroflexi

Introduction:

Multi-copper oxidases (MCOs) is a versatile class of enzymes that use several redox-active copper sites in cupredoxin-like domains to oxidize substrates by one-electron transfer while reducing electron acceptors such as dioxygen. The cupredoxin domain is the most basic copper-containing domain, which evolved approximately with the emergence of trace dioxygen in the atmosphere[1]. The cupredoxin domain can appear alone as in electron transfer proteins such as plastocyanin or auracyanins, as dimers in nitrate reductase, or as trimers or hexamers in various oxidoreductases[2].

The 3-domain MCOs contain four copper ions: One T1-type copper in the substrate-binding site and a tri-nuclear cluster of two T3 coppers and one T2 copper, which bind to oxygen[3]. Several of these 3-domain MCOs are involved in the protection against oxygen stress by oxidizing cuprous or ferrous ions that could otherwise produce toxic hydroxyl radicals by Fenton-like chemistry[4]. The metal oxidase activity of MCOs is found in all domains of life, exemplified by CueO from *Escherichia coli*[5], Fet3P from *Saccharomyces cerevisiae*[6] and more recently, McoP from the archaea *Pyrobaculum aerophilum*[7].

Another important class of 3-domain MCOs is the laccases (EC 1.10.3.2, *p*-diphenol dioxygen oxidoreductases) that contain a substrate binding site evolved specifically to oxidize aromatic compounds rather than inorganic ions. White-rot fungi produce laccases of substantial industrial interest due to their high stability and ability to perform controlled oxidations using only dioxygen as a co-substrate while producing water as the only side product. They exhibit very high redox potentials (up to ~0.8 V), conferring exquisite oxidating power towards a wide range of substrates[8]. Potential and established applications include decolorization, oxidation in organic synthesis, and lignin degradation.[9]

A major obstacle to using laccases for industrial purposes is the cost of enzyme production. A solution is identification of cheap biological waste materials to support the growth of the laccase producing fungi[10,11]. An alternative is to improve the durability of the laccase and a quest towards identifying and characterizing stable laccases has been initiated[12]. Notably, bacterial 3-domain MCOs with high sequence similarity to fungal laccases are now increasingly being characterized, revealing important roles of such enzymes across bacterial phyla. These generally show intrinsically high stability and wide activity vs. pH profile compared to fungal laccases, which can be beneficial in many applications[13]. In

addition, bacterial laccases can be produced overnight in *Escherichia coli* compared to the often reported two weeks for fungal fermentations⁹. As an example, the spore coat protein A (CotA) from *Bacillus subtilis* displays high thermostability and activity vs. phenolic, non-phenolic and inorganic substrates[14–16]. The success of CotA as an alternative to fungal laccases has inspired a wealth of studies on homologs[15–19]. CotA is expressed as part of the *Bacillus* sporulation response[20] and is critical for the development of spore pigmentation[21]. Another well-described bacterial 3-domain MCO is the *Escherichia coli* CueO, which is a dedicated cuprous oxidase[5] that can function as a laccase at high Cu(II) concentrations[22]. Apart from free transition metals, e.g. Cu(I), Fe(II) and Mn(II,III)[23], natural substrates for specific bacterial MCOs are generally unknown. However, a laccase from *Streptomyces antibioticus* has been reported to catalyze a step in the synthesis of actinomycin D[24]. Also, several bacterial 3-domain MCOs have been characterized as bilirubin oxidases. These widely different activities demonstrate substantial versatility and suggest that modern bacterial MCOs have undergone major evolutionary adaptations.

The term “laccase” was originally used for 3-domain MCOs from plants with activities resembling the lacquer synthase in *Rhus vernicifera*[25]. With the rapid discovery of new “laccases” with diverse activities from all kingdoms of life, the definition has become blurred. Thus, the name Laccase-like Multi Copper Oxidase has been used to describe the group of non-plant 3-domain MCOs, based on the conserved sequential features rather than substrate specificities[26].

This paper reports the cloning, expression, and characterization of a new LMCO, hereafter named TtMCO, identified from the genome sequence of *Thermobaculum terrenum*[27], a bacterium cultivated from extreme thermal soil in Yellowstone Park[28]. *Thermobaculum terrenum* belongs to the class Thermomicrobia within the Chloroflexi phylum[29]. Phylum Chloroflexi is one of the most diverse bacterial phyla mostly known for hosting *Chloroflexus aurantiacus*, a remarkable bacterium that utilizes anoxygenic photosynthesis and respiration proteins otherwise unique to either purple bacteria or green sulphur bacteria.[30] This duality suggests an ancestral heritage to the cyanobacteria photosynthesis system[31,32] and a possible evolutionary link between oxygenic and anoxygenic life. Chloroflexi also presents rare phenotypes relating to e.g. its cell membrane structure, composition, and pigmentation, and the phylum has been suggested to be a branching point between gram-negative and gram-positive bacteria[33,34]. Morphology suggests that evolution of Chloroflexi bacteria is

related to the Last Universal Common Ancestor of all kingdoms of life[35], but this is not supported by phylogenetic analysis of genomic sequences[34,36]. It is very complicated to establish deep bacterial evolution due to a lack of genomic sequences and the frequency of horizontal gene transfer, and the root, if it can be determined at all, remains debated as reviewed elsewhere[37], but everything implies that Chloroflexi represents highly distinct life forms and a critical stage in bacterial evolution. We identified TtMCO as the earliest instance of a Chloroflexi protein with a significant homology (~50%) to CotA from *Bacillus subtilis* and thus decided to investigate it as a model for LMCO evolution.

CotA from *B. subtilis* is already very thermostable, with a half-time of inactivation ($t_{1/2}$) of about an hour at 80°C[14] despite the mesophilic origin. In contrast, *Thermobaculum terrenum* is a hyperthermophile, and TtMCO would thus be anticipated to be particularly stable[38]: As shown in this work, it turns out to be one of the most stable MCOs ever characterized. Also, TtMCO is the first characterized protein from *Thermobaculum terrenum* and the first characterized LMCO from Phylum Chloroflexi, and its activity patterns suggest substantial promiscuity and stability-activity trade-offs consistent with an ancient role in MCO evolution.

Materials and methods:

Gene identification and analysis

The online BLAST database (<http://www.ncbi.nlm.nih.gov>) does not include *Thermobaculum terrenum* in the Chloroflexi phylum, but has it listed as an unclassified bacterium. The TtMCO gene, Tter_0532, was identified by running pBLAST specifically against the *Thermobaculum terrenum* YNP1 genome using the *Bacillus subtilis* 168 CotA protein sequence as template. The TtMCO protein had 39% identity, 50% homology, and 16% gaps compared to *Bacillus subtilis* 168 CotA. It contained all the conserved residues of a bacterial laccase and no methionine-rich stretch (*vide infra*). The sequence begins with a TAT secretion motif, and the cleavage site was predicted using PRED-TAT[39] to cut between residues 37 and 38.

Evolutionary inheritance of the TtMCO protein was investigated by blasting the sequence against all major branches in the 16S rRNA derived “The All-Species Living Three”[40] as well as the most recent phylogenetic tree of *Thermobaculum terrenum*[29,41]. The trees are tied together at the Chloroflexi

node and visualized in Figure 1A. In addition, the sequence of TtMCO was blasted against the full non-redundant protein sequence database to identify alternative evolutionary pathways. Multiple alignments were constructed using *T-coffee*[42] and visualized with *ClustalX*[43]. The homology model was prepared using Phyre2[44] on the basis of the truncated sequence of TtMCO.

Cloning and expression

The DNA sequence for TtMCO without the secretion peptide was codon-optimized for expression in *Escherichia coli* and the DNA was synthesized by GeneArt® Invitrogen, and then cloned into the pETM13 vector as previously described [17]. The cloned vector was transformed into *Escherichia coli* BL21(DE3) to allow for IPTG-inducible expression of TtMCO starting with the peptide sequence MDRFFD. Protein expression was carried out under semi-anaerobic conditions as described by Durao et al.[45]

Cells were harvested by centrifugation for one hour at 5000 g and the cell paste was re-suspended in 20 mL cold lysis buffer 50 mM MOPS buffer (pH 7.0) and frozen at -20°C. The cell suspension was thawed and adjusted to 0.5 mM Pefabloc® SC (Fluka) and 0.01% Lysozyme. Cells were sonicated for five minutes on ice (UP400S with a microtip H7, Hielscher) using pulses of 30 seconds at maximum power. In order to further improve cell lysis and precipitate thermo-sensitive proteins, the cell lysate was incubated in a water bath for 45 minutes at 90°C. The pink cell debris was removed by two rounds of centrifugation for 30 minutes at 12000 g followed by decantation and finally pushed through a 0.45-µm cellulose syringe filter. The extract, still in the lysis buffer, was loaded onto a 5mL HiTrap Q FF column using an ÄKTA purifier and the flow-through was collected. The protein solution was then diluted into 50 mM MOPS buffer at pH 7.8 and reloaded on a clean HiTrap Q FF column, from which TtMCO was eluted in homogeneity with a gradient of 0–0.5 M NaCl. Fractions were collected based on the absorption at 280 nm and 600 nm, pooled and buffer exchanged into 50 mM MOPS, pH 7.0 using a spin-filter with 30kDa cut-off. (See supporting information Figure S1 for a SDS PAGE gel).

Spectroscopic characterization

The purified TtMCO was heat activated by incubation at 70°C for one hour in 50mM MOPS at pH 7.0. The purpose of this procedure is to convert the as-isolated enzyme into fully active protein, since heat activation is crucial for obtaining reliable spectroscopic and kinetic data for laccases[17]. UV/VIS spectra were recorded on a Shimadzu UV-1800 using a 0.5-mL quartz cuvette. X-band EPR was measured on a Bruker EMX at with the sample submerged in liquid nitrogen. EPR spectra were analyzed in EasySpin. All figures were finalized in GraphPad Prism 6.0 and Inkscape 0.48.4.

Enzymatic activity

Potential substrates listed in **Table 1** were tested similar to Reiss et al.[26] Stock solutions of 10 mM were prepared and diluted to 1 mM at pH 5 and 8 in 96-multiwell plates. The full spectra were recorded on a Biotek Synergy microplate reader at 0, 1, and 20 hours after addition of 0.25 μg TtMCO. To evaluate non-enzymatic auto-oxidation, all reactions were also measured by substitution of enzyme with water. In addition to the spectral test, manganese oxidase activity was tested by addition of leucoberberlin blue[46]. Similarly, ferrous and cuprous oxidase activities were tested by addition of ferrozine[47][48]. Substrates were classified as “good” if the catalyzed reaction was faster than auto-oxidation within the first hour and “poor” if they only displayed activity above auto-oxidation levels after 24 hours.

Optimum pH for reactivity of heat activated TtMCO towards 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), syringaldazine (SGZ), bilirubin, 1,8-diaminonaphthalene, and 2,6-dimethoxyphenol (DMP) was measured in Britton-Robinson buffer pH 3–10. Michaelis-Menten kinetics experiments were carried out with ABTS 0–8 mM at pH 4.5 ($\epsilon_{420} = 36 \text{ mM}^{-1}\text{cm}^{-1}$), SGZ 0–40 μM ($\epsilon_{525} = 65 \text{ mM}^{-1}\text{cm}^{-1}$) at pH 8.5 and 10% methanol, bilirubin 0–5 mM at pH 7.5 ($\epsilon_{440} = 60 \text{ mM}^{-1}\text{cm}^{-1}$), 1,8 diaminonaphthalene pH 4.5 ($\epsilon_{530} = 8.3 \text{ mM}^{-1}\text{cm}^{-1}$, see Supporting Information Figure S2) and DMP 0–25mM at pH 8.5 ($\epsilon_{486} = 49.6 \text{ mM}^{-1}\text{cm}^{-1}$) using the extinction coefficient in brackets. Initial rates of reaction were determined by a linear fit to the time-dependent absorption change at the mentioned wavelengths, except bilirubin activity, which was determined by the decrease of substrate signal at 440 nm and fitted with a logarithmic function[49].

Stability measurements

The apparent stability of TtMCO was measured after incubation under various conditions as the residual activity in 100mM acetate buffer at pH 4.5 using 200 μ M ABTS as substrate: First, pH stability was estimated by monitoring residual activity after 60 minutes of incubation at 90°C and at variable pH. Thin-walled PCR microtubes were prepared with 0.4 μ g TtMCO in 30 μ L 10 mM Britton-Robinson buffer at pH 5–10 and incubated in a TC-3000X thermal cycler. A 10-fold dilution of the samples was used to test residual activity.

The time dependency of thermal activation and inactivation was then tested at 70°C, 80°C and 90°, using 48 thin walled PCR microtubes prepared with 0.4 μ g TtMCO in 30 μ L 10 mM Britton-Robinson buffer at pH 7.0 and incubated in a BioRAD C1000 or TC-3000X thermal cycler. At specific time points, a microtube was removed from heat incubation and placed on ice. Residual activity was measured in the same manner as above.

Results and discussion

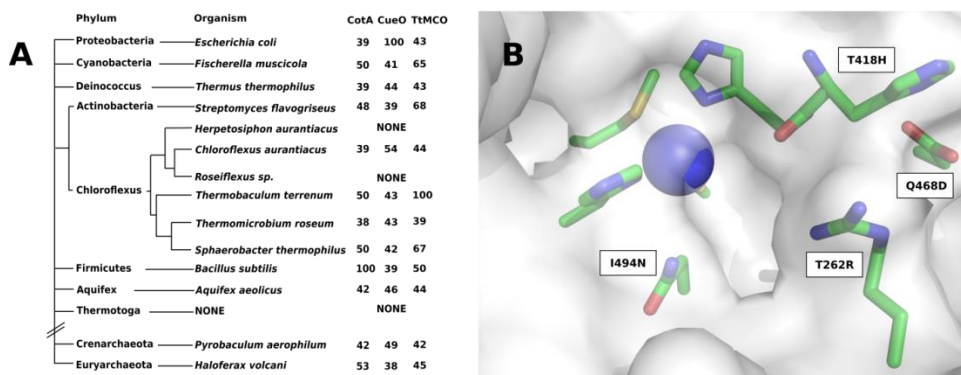


Figure 1. Comparison of the TtMCO sequence to phylogenetically important LMCOs. (A) A general phylogenetic tree with the major prokaryotic phyla plotted on a straight line. The double dash between Thermotoga and Crenarchaeota marks the transition between Bacteria and Archaea. When applicable, each phylum is represented by an organism that produces a LMCO and the sequence homology to *Bacillus subtilis* CotA, *Escherichia coli* CueO and *Thermobaculum terrenum* TtMCO is listed. The Chloroflexi phylum is expanded according to Gupta et al.[41] to show the heterogeneity

of the LMCOs as discussed in the text. Phylum Thermotoga and some Chloroflexi organisms are labeled NONE to show that the genomes do not encode a LMCO. **(B)** Structure homology model of the TtMCO active site. The T1 copper is visible as a blue sphere surrounded by canonical T1-type amino acid ligands. Four amino acids in the vicinity of T1 are substituted between TtMCO and *Bacillus subtilis* CotA and are visualized and numbered according to the CotA sequence. (See Supporting Information, figure S3 for sequence alignment)

TtMCO: Genetic and structural homology

Thermobaculum terrenum is a deeply rooted Chloroflexus bacterium with optimal growth at 67°C and neutral pH[27]. Figure 1A shows a general prokaryotic tree[40] joined with the most recently suggested phylogeny of the Chloroflexi phylum[29,41]. *Thermobaculum terrenum* clusters together with *Sphaerobacter thermophilus* and *Thermomicrobia roseum* in the Thermomicrobia group. Each phylum in Figure 1A is represented by a single organism with a characterized LMCO, except Cyanobacteria from which no LMCO has been characterized. *Fischerella muscicola* LMCO represents the cyanobacteria in Figure 1A because it has the highest homology to TtMCO. The fully genome-sequenced bacteria in the Thermotoga phylum do not code for a LMCO and neither do most Chloroflexi strains such as *Herpetosiphon aurantiacus*[50] that only make two-domain MCOs and *Roseiflexus* sp that only make single-domain cupredoxin proteins. This led us to look for the rare LMCOs in Chloroflexi as the earliest instances of LMCO evolution. For each of the identified LMCOs, the sequence homology to CotA, CueO and TtMCO is listed (see Supporting Information, Figure S3 for sequence alignment and accession codes).

It can be seen that TtMCO has 50% homology to CotA from *Bacillus subtilis* and 43% to CueO from *Escherichia coli*. The LMCO from *Chloroflexus aurantiacus* has only 39% homology to CotA, but 54% homology to CueO and has a methionine-rich stretch suggesting cuprous oxidase activity[22]. Similarly, other thermostable LMCOs from deeply rooted *Thermus thermophilus*, *Aquifex aeolicus* and *Pyrobaculum aerophilum* showed higher homology towards CueO than CotA. Interestingly, the LMCO from the archaea *Haloferax volcanii* shows the highest inter-phylum homology towards CotA, suggesting a possible horizontal gene transfer event at an early stage of LMCO evolution.

The TtMCO protein itself is peculiar in its lack of closely homologous sequences even among related species. The highest scoring homologs have at

most 70% homology (BLOSUM45) and 58% identity. We interpret this as due to the heterogeneity of the Chloroflexi phylum. In fact, the only close intra-phylum homolog is the LMCO from *Sphaerobacter thermophilus* (67%). The other high-scoring homologs belong to Phylum Actinobacteria. In Figure 1A, these are represented by a MCO from *Streptomyces flavogriseus* with 68% homology to the peptide sequence of TtMCO, *vide infra*.

It is possible that TtMCO is an evolutionary ancestor of the Actinobacteria LMCOs. *Sphaerobacter thermophilus* is the phylogenetically closest related organism to *Thermobaculum terrenum* and was originally suggested as one of the deepest nodes in the Actinobacteria phylum[51]. Another indication of an evolutionary heritage of TtMCO is found in the genome of *Thermobaculum terrenum*[27]. Two additional 3-domain MCOs are found, one being a nitrite reductase with a C-terminally fused cupredoxin domain encoded by tter_2149. This is a rare MCO combination also found in some proteobacteria. The other is a novel 2-domain laccase-like protein with a N-terminally fused cupredoxin encoded by tter_2238. Characterization of the latter protein will be reported elsewhere, but preliminary data show that it has only minimal laccase activity. The unique MCOs in *Thermobaculum terrenum* show that this bacterium can utilize unusual combinations of cupredoxins. This makes *Thermobaculum terrenum* a candidate for the critical evolutionary step of fusing a cupredoxin domain with a two-domain MCO, a general mechanism of MCO evolution suggested by Nakamura et al.[2] Thus, multiple indications support TtMCO as an early LMCO that gave rise to the modern Actinobacteria LMCOs. Our characterization described below, in particular the extreme thermostability and the modest classical laccase activity, is in line with this hypothesis.

Figure 1B shows the active site of a structure homology model of TtMCO built using the CotA structure as template. CotA has a disulphide bridge adjacent to the T1 site, but no such close cysteines are present in the TtMCO model. A triad of charged amino acids substitutions within 10 Å of the T1 site (T418H, Q468D, and T262R, using CotA numbering) is conserved for the highest scoring Actinobacteria homologs of TtMCO. These substitutions suggest a different substrate affinity compared to *Bacillus* CotAs. In addition, TtMCO contains the often reported second-sphere substitution I494N, which has been attributed to evolution of bilirubin oxidase activity due to its presence in *Myrothecium verrucaria* bilirubin oxidase[52].

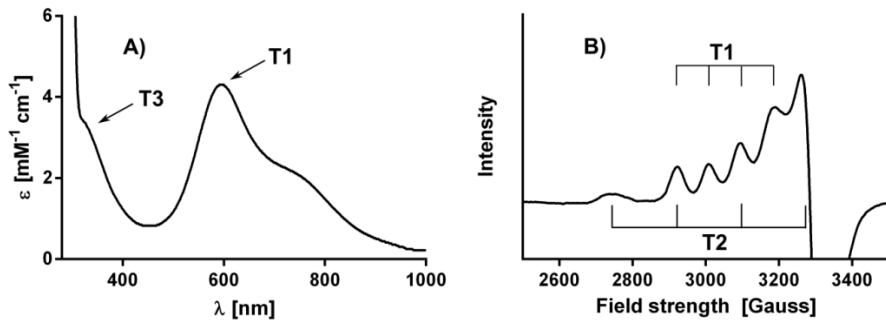


Figure 2 Spectroscopic characterization of TtMCO. (A) UV/VIS spectrum with the two highlighted features assigned to T3- and T1-sites, confirming a copper-loaded 3-domain MCO structure. **(B)** EPR spectrum with highlighted hyperfine splittings assigned to T1- and T2-sites, as discussed in the text.

Spectroscopic characterization of TtMCO

The absorption spectrum of heat activated TtMCO is reported in Figure 2A. The shoulder at 330 nm is normally assigned to the T3 site and is relatively well resolved for TtMCO. A Gaussian fit suggests that the absorption band is slightly shifted upwards to 338 nm. The band at 600 nm and the broader band at 717 nm are due to the T1 copper site[8]. Figure 2B shows the corresponding EPR spectrum, with indications of T1- and T2-specific hyperfine splittings. From the EPR spectrum, we obtain $g_{||} = 2.21$, $A_{||} = 88 \cdot 10^{-4} \text{cm}^{-1}$ for T1 and $g_{||} = 2.24$, $A_{||} = 186 \cdot 10^{-4} \text{cm}^{-1}$ for T2. These values are fairly similar to those reported for *Bacillus subtilis* CotA[45] and other LMCOs[8], suggesting a similar chemical structure around the copper sites. This is supported by sequence alignment where all copper chelating amino acids are conserved (See Supporting Information S3). Thus, spectrophotometric characterization and sequence alignment of TtMCO show that it has all the fingerprint spectral features of a LMCO, with all copper sites present in the heat-activated, characterized protein[8].

Enzymatic activity of TtMCO

Table 1. TtMCO activity towards tested substrates. Substrates that were oxidized within one hour are categorized as “good” substrates. Substrates oxidized within 24 hours are categorized as “poor”. Potential substrates with no TtMCO specific reaction are categorized as “inactive”.

Good	ABTS, Bilirubin, 1,8 diaminonaphthalene, N-(1-naphthyl)ethylendiamine, phenylhydrazine, SGZ, quinine
Poor	p-aminobenzoic acid, aniline, caffeic acid, DMP, dopamine, gallic acid, 5-hydroxy isoquinoline, mandelonitrile, metol, 1-naphtol, p-phenylenediamine
Inactive	acetovanilone, acriflavine, 2-aminophenol, 2-amino-4-methylphenol, arbutin, ascorbate, catechol, 2,5-dimethoxybenzyl alcohol, diphenylamine, ferulic acid, guaiacol, hydroquinone, indole, methyl catechol, 4-methylumbelliferone, nicotine, p-nitrophenol, pyridine, sinapinic acid, syringaldehyde, syringic acid, sulfanilamide, tannic acid, thiamine, o-toluidine, tryptophane, tyrosine, vanilic acid, 2,5-xylidine, crystal violet, indigo carmine, phenolphthalein, reactive black 5, safranin O Cu(I), Fe(II), Mn(II)

To investigate the enzymatic activity of the new enzyme, we subjected it to a range of assays. Substrate selectivity was tested using a protocol similar to that of Reiss et al.[26], and the corresponding results are listed in Table 1. We observed enzymatic oxidation of ABTS and SGZ within minutes. In contrast, activity was not observed with the standard fungal laccase substrate guaiacol, which is not an uncommon observation for bacterial LMCOs[26]. Furthermore, only slow or insignificant catalysis was observed for classical laccase substrates such as DMP, caffeic acid and p-phenylenediamine. The three atypical substrates N-(1-naphthyl)ethylendiamine, phenylhydrazine, and quinine were categorized as good substrates, but oxidation of these compounds still took days before completion.

Together, these observations show that TtMCO is not a laccase and only fits in the wider category of LMCOs[26].

The transition metals Mn(II), Fe(II), Cu(I) were also tested as substrates, but they did not show higher oxidation rates than the control experiments, confirming that TtMCO is not a dedicated metal oxidase. Furthermore, TtMCO activity was not enhanced by supplementing with copper (See Supporting Information, Figure S4). This observation is consistent with the absence of a methionine-rich stretch, a structural motif for copper modulation of CueO activity[53]. The methionine-rich stretch is often found in bacterial cuprous oxidases, including the characterized LMCOs from the thermophilic bacteria *Thermus thermophilus*[54] and *Aquifex aeolicus* [55] that display high metal oxidase activity.

Table 2 TtMCO Michaelis-Menten kinetic data. Bilirubin and SGZ did not show strict Michaelis-Menten kinetics in the measured concentration range. DMP was measured at pH 8.5 because of significant auto-oxidation at higher pH. ABTS and 1,8-diaminonaphthalene are proper TtMCO substrates (See supporting Information, Figure S2, S5 and S6 for further details).

	pH _{opt}	K _m [mM]	k _{cat} [s ⁻¹]	k _{cat} /K _m [s ⁻¹ mM ⁻¹]
ABTS	4.5	0.844 ±0.084	2.13 ±0.091	2.5 ±0.36
Bilirubin	7.5	9.5 ±2.6	0.57 ±0.12	0.06 ±0.03
1,8-diaminonaphthalene	4.5	0.159 ±0.011	0.295 ±0.008	1.9 ±0.18
DMP	>8.5	14 ±0.6	0.175 ±0.004	0.013 ±0.001
SGZ	7	>0.04	NA	0.11

Michaelis-Menten kinetic analysis was subsequently performed on classical laccase substrates as well as some substrates with high apparent turnover (Table 2). Parameters measured for standard laccase substrates confirm that TtMCO is not a classical laccase (See Table 2 and Supporting Information, Figure

S6). TtMCO had only weak affinity towards DMP and SGZ, exemplified by the high $K_m = 14$ mM for DMP oxidation. Analysis of the SGZ oxidation was hampered by precipitation of substrate at concentrations higher than 40 μ M, and no sign of substrate saturation was observed in the used range. The largest k_{cat}/K_m was achieved with ABTS and 1,8-diaminonaphthalene. These two compounds are both bulky aromatic compounds with no hydroxyl groups. $K_m = 159$ μ M for 1,8-diaminonaphthalene indicates tight substrate binding, in particular considering that the maximal activity is at pH 4–4.5 where 1,8-diaminonaphthalene is positively charged ($pK_a \sim 4.6$). ABTS has been shown to bind CotA as the radical[56]. Thus data suggest a preference for substrates with charged aromatic compounds having non-phenolic functional groups such as amines or azo groups. One biological substrate that may be oxidized by LMCOs is bilirubin, a heme derivative containing four pyrroles: Several LMCOs have been classified as putative bilirubin oxidases[57]. We thus performed Michaelis-Menten analysis of the activity of TtMCO towards this substrate. Although the enzyme does oxidize bilirubin, it does so with an approximate K_m of 10 mM, suggesting poor substrate-protein interaction and indicating that TtMCO is not a bilirubin oxidase.

In conclusion, the lack of strong phenol-oxidation suggests that TtMCO is even further from classical fungal laccases than the CotAs, and the absence of strong oxidation of metal ions and bilirubin suggests a biological role different from that of CueO and bilirubin oxidase, with its activity mainly targeting larger, aromatic, charged non-phenolic substrates. Recently, the characterized LMCOs from Phylum Actinobacteria have been reviewed[58]: While neither of these are close homologs of TtMCO, they share a high $K_m \sim 0.4$ mM towards ABTS, and the 3-domain LMCOs have only limited oxidation of phenolic substrates.

Potential role of TtMCO in antibiotic synthesis

TtMCO did not show any significant activity towards 2-aminophenol, which is used as a model substrate for actinomycin synthase activity of a LMCO from *Streptomyces antibioticus*[24]. When screening for homologous proteins with putatively assigned functions, we identified XanP from *Streptomyces flavogriseus* as having 69% homology and 60% identity to TtMCO when disregarding the secretion peptide. XanP is encoded by the gene cluster that codes for enzymes involved in synthesis of xantholin, a polyketide antibiotic[59]. Similarly, the *pks7* gene in *Streptomyces ansochromogene* codes for a LMCO with 65% homology to

TtMCO and is located in the gene cluster that codes for oviedomycin synthesizing enzymes[60]. Thus, while our Michaelis-Menten analysis suggests a function distinct from classical laccases, CotAs, and bilirubin oxidases, close homologs of TtMCO suggest a potential role in antibiotic synthesis.

Among the 16 genes in the putative TtMCO gene cluster, only three corresponding proteins have more than 60% identity to any other protein sequence as reported by BLAST. Because of this general lack of close homologs to *Thermobaculum terrenum* proteins, it is not possible to accurately estimate the functions of proteins translated from genes in the vicinity of the TtMCO gene. The LMCO from *Sphaerobacter thermophilus* is the only TtMCO homolog of more than 45% homology from a closely related species (See Figure 1A). This gene is encoded within an operon that also includes a homolog of the characterized monooxygenase XanO5 from the xantholipin gene cluster and an ABC transporter generally found in antibiotics producing gene clusters[61]. Assigning TtMCO to antibiotics synthesis based on sequence homology to proteins that are only characterized by gene position is hypothetical. However, the proposed synthetic pathway of xantholipin in *Streptomyces flavogriseus* has an unassigned conversion of a “hydroxy-isoquinolinone” moiety to “dioxy-isoquinolinone”[59]. Thus, XanP LMCO seems an ideal candidate for catalyzing this conversion, especially since the closely related antibiotic, lysolipin I does not have the di-oxo modifications and the corresponding characterized gene cluster does not include a LMCO[62].

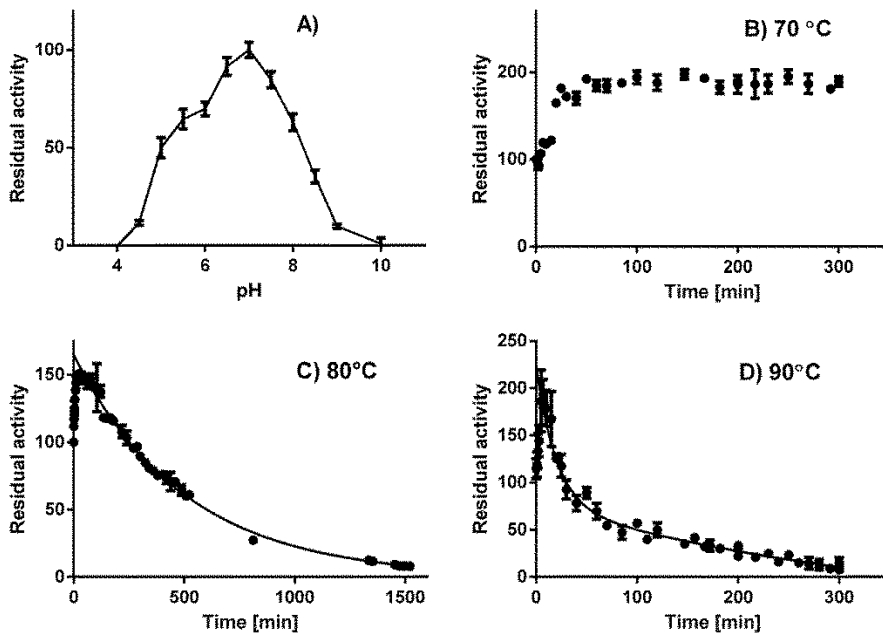


Figure 3 Stability of TtMCO vs. temperature and pH. (A) pH dependency of TtMCO stability when incubated at 90°C. (B) TtMCO incubated at 70°C showing heat activation to 200% activity within the first hour. (C) TtMCO incubated at 80°C showing a single exponential inactivation with $t_{1/2}$ =350 minutes. (D) TtMCO incubated at 90°C showing a double exponential inactivation, with the fastest process having $t_{1/2}$ = 14 minutes.

TtMCO is among the most stable LMCOs ever characterized

Due to its phylogenetic indications and the habitat of the host organism, characterizing the thermostability of TtMCO was of particular interest. We studied the pH dependency of TtMCO heat inactivation by measuring the residual activity of TtMCO after one hour of incubation at 90°C in different pH buffers (see Figure 3A). The optimal pH for stability was pH 7.0, which is identical to the pH of optimal growth condition for *Thermobaculum terrenum*[28]. In Figure 1A, the most thermostable prokaryotic LMCOs have been mapped on the phylogenetic tree, i.e. *Thermus thermophilus*[54], *Pyrobaculum aerophilum*[7], *Aquifex aeolicus*[55] and *Bacillus subtilis*[14]. These have all been characterized by their half-time of

inactivation at close to neutral pH which allows us to directly compare the reported half-time of inactivation for these proteins with those of TtMCO measured at pH 7.0.

First, the activity of TtMCO after incubation at 70°C was investigated. Interestingly, under these conditions, the protein was activated up to 200% within the first hour of incubation, and the protein remained stable for hours at this elevated temperature (See Figure 3B). After 24 hours of incubation, it still had 150% of the initial activity, suggesting a half-time of inactivation $t_{1/2}$ of 2.24 ± 0.12 days at 70°C. Activity was still measurable after two weeks of incubation, consistent with the optimal growth conditions of *Thermobaculum terrenum*. Heat activation is commonly reported for bacterial LMCOs and some bilirubin oxidases[63] and activations up to 900% has been reported[64], rendering inactive as-isolated protein conversion or proper benchmarking accounting for this dual-state situation critical to LMCO characterization[17].

We then subjected the protein to incubation at 80°C and performed similar tests of activity after variable incubation times. At 80°C, the protein became inactivated in an apparent one-phase exponential decay with $t_{1/2} = 350$ minutes, as shown in Figure 3C.

The most thermostable prokaryotic LMCOs have all been characterized by their $t_{1/2}$ at 80°C, rendering this parameter central to the benchmarking of LMCO stability. The highly stable *Bacillus subtilis* CotA was originally reported to have $t_{1/2} \sim 120$ min at 80°C [14] but this number was later updated to 80–170 min depending on copper load.[45] Critical assessment of the published heat inactivation graphs suggest $t_{1/2}$ about 56min in all cases, which is comparable to the 50 min recently reported by independent benchmarking[17]. The two most hyperthermophilic LMCOs characterized to date are those of *Thermus thermophilus* ($t_{1/2} = 868$ min[54]) and *Pyrobaculum aerophilum* ($t_{1/2} = 330$ min[7]), with a third contender being a LMCO from *Aquifex aeolicus* ($t_{1/2} = 60$ min[55]). This ranks TtMCO second only to *Thermus thermophilus* LMCO and comparable to *Pyrobaculum aerophilum* LMCO. The remaining MCOs mapped in Figure 1A also show significant thermostability, but these were only reported at 70°C. The archaea laccase from *Haloferax volcanii* has $t_{1/2} = 10$ min[65] and CueO from *Escherichia coli* has $t_{1/2} = 15$ min[66]. *Streptomyces flavogriseus* XanP is still uncharacterized, but a LMCO from *Streptomyces lavendulae* was found to have a $t_{1/2}$ at 70°C of 100 min[67].

Figure 3D shows the thermal inactivation of TtMCO at 90°C. The decrease in activity was fitted with a two-phase exponential decay with the fastest process having $t_{1/2} = 14$ min. The two-phase decay suggests that a secondary and fast inactivation process is active at this elevated temperature. Similarly, a secondary inactivation process was indicated for the LMCOs of *Pyrobaculum aerophilum*[7] and *Aquifex aeolicus*[55][68] at elevated temperatures. The fast inactivation process at 90°C of *Aquifex aeolicus* LMCO was determined to have $t_{1/2} = 25$ min[55].

Concluding remarks

In this paper, we have reported the cloning, expression, and characterization of the only LMCO from the thermophilic bacterium *Thermobaculum terrenum* belonging to the evolution- and morphology-wise notorious Chloroflexi phylum: It is the first characterized LMCO from Phylum Chloroflexi and the first protein characterized from *Thermobaculum terrenum*. Sequence homology and species phylogeny suggest that it is an evolutionary ancient LMCO more similar to *Bacillus subtilis* CotA than *Escherichia coli* CueO. The highest sequence homology was found to some putative LMCOs of actinobacteria whose genes belong to gene clusters involved in antibiotic synthesis.

TtMCO contains all the conserved amino acids specific to copper binding of LMCO proteins and the spectrophotometry of the purified protein clearly supports that TtMCO is a LMCO with four coppers in laccase-like coordination. However, TtMCO displays only modest activity on a range of standard laccase substrates, including transition metals, phenolic compounds, and bilirubin. The highest apparent substrate affinity (lowest K_m) was observed for 1,8-diamino naphthalene. Together with the ability to oxidize aniline, these observations support a distinct function of TtMCO, probably in maturation of secondary metabolites containing aniline-like moieties, and potentially in the synthesis of antibiotics.

Consistent with phylogenetic indications and host organism lifestyle, the protein was found to be extremely thermostable: It had a half-time of inactivation at pH 7 of 2.24 days at 70°C and 350 min at 80°C, substantially more than the ~1 hour at 80°C estimated for *Bacillus subtilis* CotA and second only to the extreme

hyperthermophilic *Thermus thermophilus* LMCO. Both the extreme thermostability and the low substrate activity are consistent with properties of an ancient protein, and these observations support a special position of TtMCO in MCO evolution.

The preference of TtMCO for charged, bulky N-containing aromatic compounds suggests a distinct, so far unidentified function, or possibly, a very promiscuous protein for which the most selected property is stability. The substantial trade-off in TtMCO between stability and turnover proficiency indicates that evolutionary later, more specialized LMCOs and laccases have been positively selected for specific functions at the expense of thermostability, as their host organisms adapted to less extreme environments.

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Supporting Information. The Supporting Information file contains the following information: Figure S1: SDS PAGE gel of TtMCO purification and activation. Figure S2: 1,8-diaminonaphthalene kinetic traces and standard curve. Figure S3: Alignment of LMCO sequences mentioned in the main text. Figure S4: Influence of inorganic salts CuCl_2 and NaCl on the TtMCO oxidation of ABTS at pH 4.5. Figure S5: pH optimum for the four substrates showing highest turnover. Figure S6: Michaelis Menten kinetics of TtMCO.

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Supporting Information

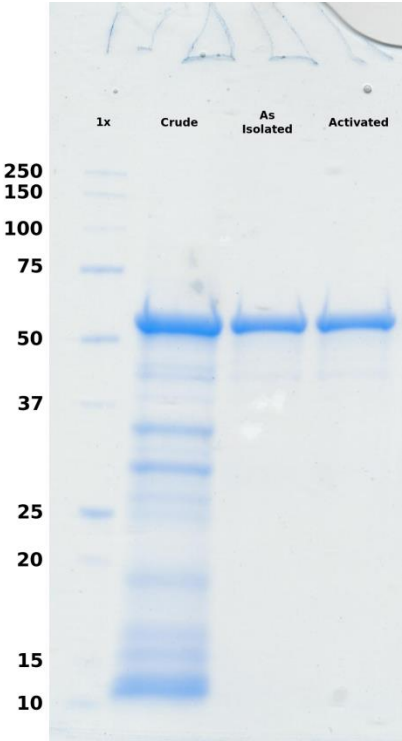


Figure S1. SDS PAGE gel of TtMCO: purification and activation. Lane 1: Molecular marker. Lane 2: Extract after heat purification and removal of cell debris. Lane 3: TtMCO after two rounds of HiTrap Q purification. Lane 4: TtMCO after heat activation for 1 hour at 70°C.

T262R

Table of amino acid sequences for T262R across various species including T. terrenum, S. flavogriseus, and others. The sequences are aligned and color-coded by amino acid type.

Table of amino acid sequences for T262R across various species including T. terrenum, S. flavogriseus, and others. The sequences are aligned and color-coded by amino acid type.

Table of amino acid sequences for T262R across various species including T. terrenum, S. flavogriseus, and others. The sequences are aligned and color-coded by amino acid type.

T418H

Table of amino acid sequences for T418H across various species including T. terrenum, S. flavogriseus, and others. The sequences are aligned and color-coded by amino acid type.

Q468D

I494N

Table of amino acid sequences for Q468D and I494N across various species including T. terrenum, S. flavogriseus, and others. The sequences are aligned and color-coded by amino acid type.

Figure S3. Alignment of LMCO sequences mentioned in the main text. These sequences are diverse as would be expected for protein sequences found in organisms spread over all kingdoms of Life. The strictly conserved histidine and cysteine residues correspond to the copper binding ligands. The four substitutions in the vicinity of the T1 copper site as discussed in the main text are marked with a blue box and numbered according to the *B. subtilis* CotA sequence. Uniprot accession numbers for the protein sequences are: *Thermobaculum terrenum* D1CEU4, *Streptomyces flavogriseus* I1SKV1, *Sphaerobacter thermophilus* D1C7N4, *Bacillus subtilis* P07788, *Haloferax volcanii* D4GPK6, *Myrothecium verrucaria* Q12737, *Aquifex aeolicus* O67206, *Chloroflexus aurantiacus* A9WHU3, *Pyrobaculum aerophilum* Q8ZWA8, *Escherichia coli* P36649, *Thermus thermophilus* Q72HW2, *Thermomicrobium roseum* B9L364. REFSEQ for *Fischerella muscicola* is WP016867746 and GenBank accession number for the *Streptomyces ansochromogene* pks7 cluster is KF170327

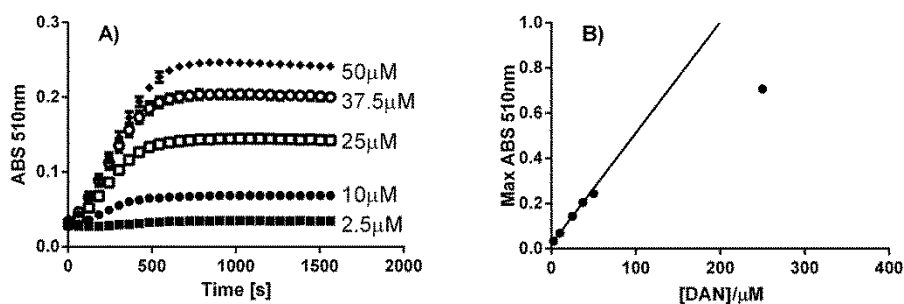


Figure S2. 1,8-diaminonaphthalene kinetic traces and standard curve. A) The kinetic traces of low concentration 1,8-diaminonaphthalene oxidation stabilized after complete product formation. At higher concentration, a secondary product is formed after some time

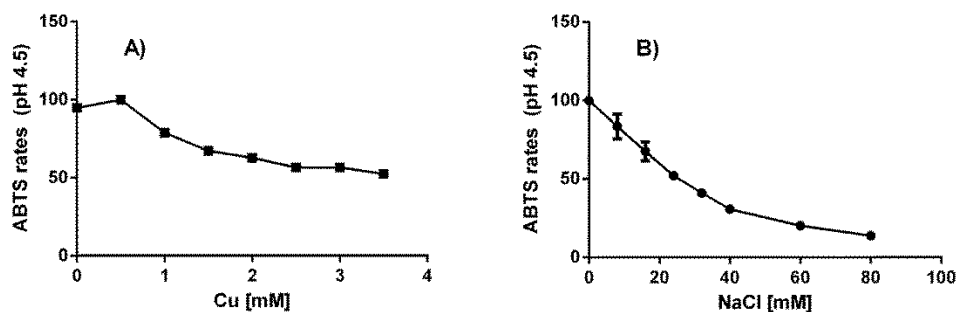


Figure S4. Influence of inorganic salts CuCl₂ and NaCl on the TtMCO oxidation of ABTS at pH 4.5. A) TtMCO was slightly inactivated by the addition of extra CuCl₂ to the reaction mixture. B) TtMCO was easily inhibited by NaCl at pH 4.5.

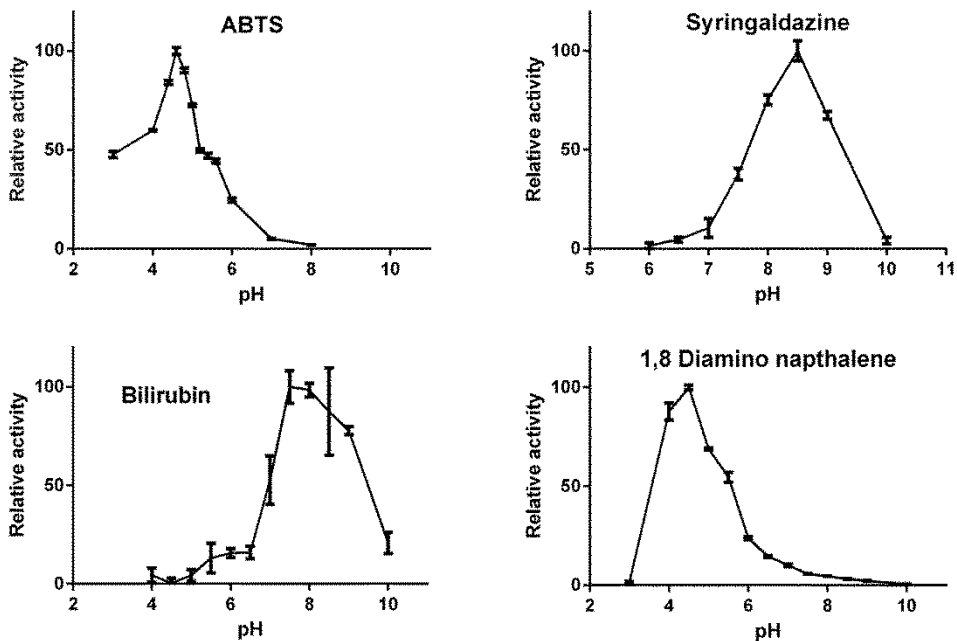


Figure S5. pH optimum for the four substrates showing highest turnover. ABTS oxidation was optimal at pH 4.5 (measured at 420 nm), SGZ was optimal at pH 8.5 (measured at 525 nm), bilirubin was optimal at pH 7.5 (measured at 440 nm), and 1,8- diaminonaphthalene was optimal at pH 4.5 (measured at 510 nm). In addition, DMP was tested but showed monotonic increase of activity with pH.

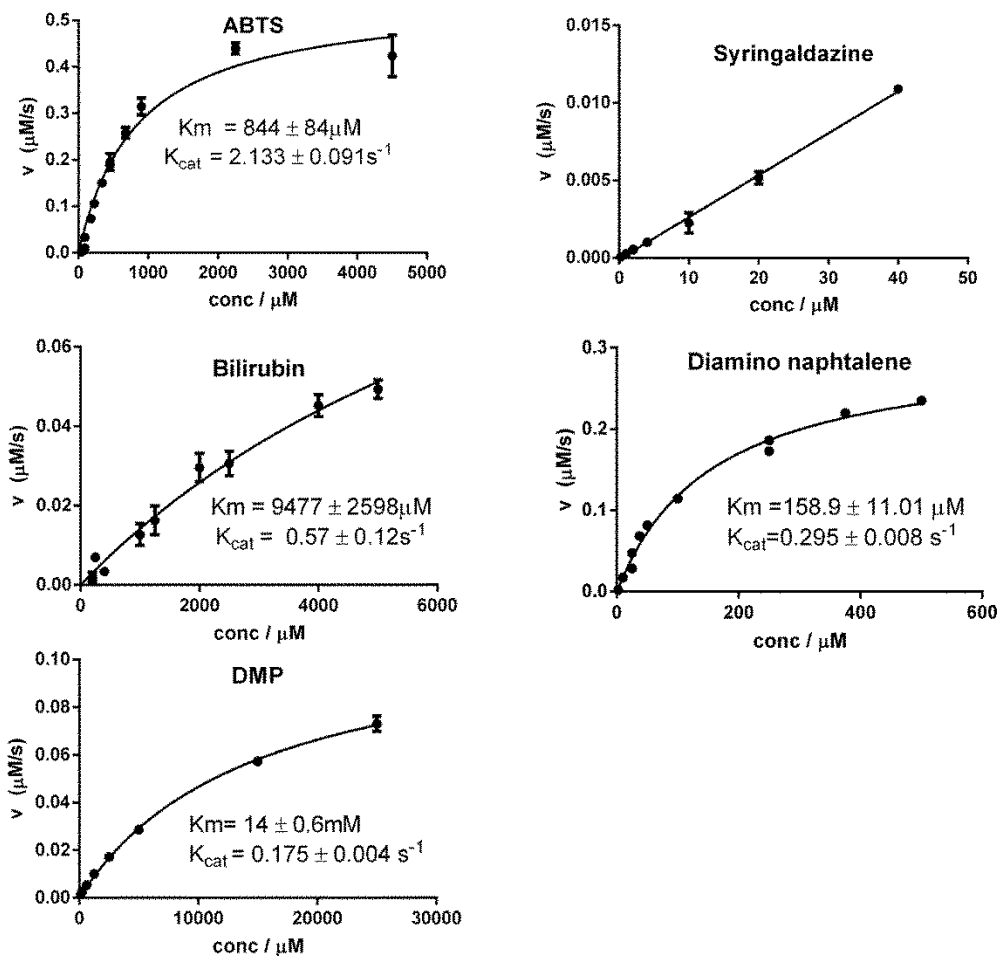


Figure S6. Michaelis Menten kinetics of TtMCO. Values are listed on the subfigures. All assays were done at the optimal pH, except for DMP which was performed at pH 8.5 because of the increasing autooxidation at higher pH. Syringaldazine did not show Michaelis-Menten kinetic behavior.

Paper III

Heat activation of the “alternative resting state” of two bacterial laccase-like multicopper oxidases

Søren Brander, Christian Kjærgaard, Susanne Mossin, Edward I Solomon*, Kasper P. Kepp*

Draft manuscript

Heat activation of the “alternative resting state” of two bacterial laccase-like multicopper oxidases

Søren Brander, Christian Kjærgaard, Susanne Mossin, Edward I Solomon*, Kasper P. Kepp*

Abstract

Heat activation of laccase-like proteins has often been reported in the literature, but despite the potential biological and industrial importance, the details and mechanism behind this activation is poorly understood. We show here that heat activation of two very different bacterial laccase-like proteins, CotA from *Bacillus subtilis* and TtMCO from *Thermobaculum terrenum*, are accompanied by the same spectral changes similarly to what has been characterized for the two resting states of bilirubin oxidases. We use this spectroscopic handle to analyze heat activation and activity increase from semi-anaerobic expression, and we systemize the active and inactive EPR data of laccase-like enzymes. Most importantly, we report a molecular mechanism for the transformation from the alternative resting state (inactive) laccase-like multicopper oxidases (MCOs) to the active state and identify a superoxide transformation. .

Introduction

The laccase-like multicopper oxidases (LMCOs)¹ constitute an inhomogeneous group of enzymes including cuprous oxidases², ferrous oxidases³, fungal laccases⁴, bilirubin oxidases⁵, spore coat proteins⁶ and actinobacterial synthases. They are characterized by having three cupredoxin domains and four catalytic copper ions that allow full reduction of molecular oxygen from one side of the protein and oxidation of various substrates on the other side¹. These coppers are categorized by their spectral properties. The type 1 (T1) copper is located in the substrate binding site, is observed by Electron Paramagnetic Resonance (EPR) spectroscopy and gives an intense UV-VIS absorption band around 600 nm which produces the characteristic blue color of these proteins. The T1 copper can make electron transfers to a coupled binuclear copper of type 3 (T3). The two T3 coppers are EPR inactive due to antiferromagnetic coupling, but gives rise to an UV-VIS absorption band around 330 nm. Next to the T3 coppers is a fourth and uncoupled copper of type 2 (T2) and together they form a trinuclear cluster that functions in binding and reduction of molecular oxygen. The T2 copper is EPR active, with typical features of square planar coordinated copper, i.e. $g_{\parallel} \sim 2.24$ and $A_{\parallel} \sim 180 \times 10^{-4} \text{ cm}^{-1}$, but showing no UV-VIS electronic absorption features.⁷ The spatial positions and common redox states of the copper in LMCOs are outlined in Figure 1A. These spectroscopic features provide an important and powerful tool in the characterization of LMCOs.

LMCOs are of general interest in industrial applications because of the broad substrate specificity, high stability and clean mechanism that does not produce toxic byproducts⁸. They have been suggested for use in applications such as delignification of biomass⁹, bioremediation¹⁰ and as catalysts in green synthetic chemistry¹¹.

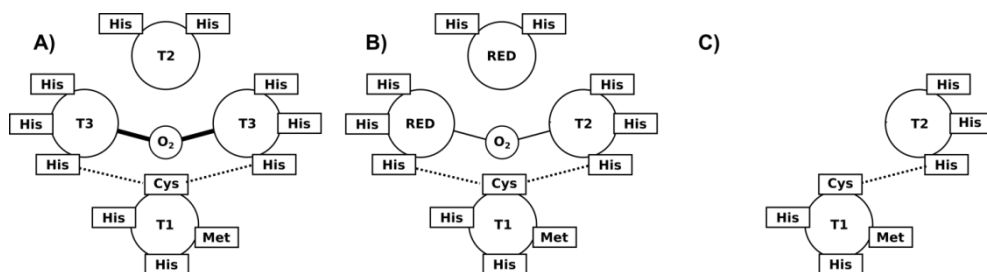


Figure 1. Copper conformations in LMCOs and in CuNIR. Thick lines represents the oxygen bridged binuclear copper bonds. Thin dashed lines indicate sequential amino acid neighbors involved in electron transfer. A) The common oxidized resting state of LMCOs with four oxidized coppers and an intact binuclear T3 pair. B) Alternative resting state in bilirubin oxidase from *Magnaporthe oryzae* where the T3 coupled binuclear copper bond is broken leaving one T2 type copper and two reduced coppers C) Copper conformation in CuNIR where the T2 copper has a similar spatial position to the LMCO T3 copper.

Recent research on laccase-like proteins has shown that at least two states persist under native conditions, and that these are critical to the spectroscopic and enzymatic properties: Bilirubin oxidase from *Myrothecium verrucaria* exists in an authentic and a recombinant form where the latter is heterologously expressed in *Aspergillus oryzae* and is of much lower activity. The recombinant form lacks the significant T3 absorption band at 330 nm and has an unusual T2 copper EPR spectrum compared to normal LMCOs, with $g_{\parallel}=2.34$ and $A_{\parallel} = 84 \times 10^{-4} \text{ cm}^{-1}$, respectively¹². The bilirubin oxidase (BO) from *Magnaporthe oryzae* has recently been expressed in *Pichia pastoris* with similar spectroscopic characteristics termed the “alternative resting state” which has been characterized as a half-reduced LMCO having only the T1 and one T3 copper being oxidized as outlined in Figure 1B. The binuclear copper bridge is broken, which explains the new T2 EPR signal¹³.

Another LMCO, *Bacillus subtilis* CotA, has been characterized twice by the same group^{14,6}, yielding two different sets of spectroscopic data. First, Martins et al. characterized CotA with spectroscopic features resembling the inactive bilirubin oxidases, i.e. a small 330-nm absorption band, $g_{\parallel}=2.344$, $g_{\perp}=2.08$, and $A_{\parallel} = 102 \times 10^{-4} \text{ cm}^{-1}$ for the T2 EPR copper⁶. Afterwards, Durao et al. reported a prominent 330-nm band and the more typical EPR parameters $g_{\parallel} = 2.25$, $g_{\perp} = 2.04$, and $A_{\parallel} = 179 \times 10^{-4} \text{ cm}^{-14}$. This reoccurrence of two sets of spectroscopic parameters similar to the bilirubin oxidases indicate similar redox states and possibly mechanism for the bilirubin oxidases and CotAs, as first suggested by Sakurai et

al.¹² The observation of two redox states may be of both biological importance and of substantial industrial importance, as the activity and stability and other properties of the proteins may be state-dependent.

A seemingly uncorrelated observation is the sometimes massive (up to 900%¹⁵) heat activation of LMCOs, which is specifically observed for the before-mentioned *Myrothecium verrucaria* Bilirubin oxidase⁴ but also for fungal *Myceliophthora thermophila* laccase⁴ and many bacterial LMCOs, including *Bacillus subtilis* CotA.^{6, 15, 16, .}

In this paper, it is shown that heat activation of two bacterial LMCOs can be monitored by EPR spectroscopy and characterized by a transformation from the “alternative resting” to the “resting oxidized” similarly to what has been seen for fungal bilirubin oxidases¹³. Furthermore, we identify a sharp signal consistent with a copper-bound superoxide intermediate during the transformation which is the first observation of a 3-electron reduced species in native LMCOs¹⁷.

Materials and methods

Cloning and expression

Cloning of pETM13 vectors and heterologous expression of *Bacillus subtilis* CotA and *Thermobaculum terrenum* TtMCO in *Escherichia coli* was previously reported¹⁶, (Brander2014b). In the previous work, expression was carried out in *Escherichia coli* BL21(DE3) grown LB medium and induced in microaerobic conditions. In this work, protein expression was carried out similarly, but using an auto-inducing medium ZYM-5052¹⁸, which was supplemented to 250 μ M CuCl₂. 600 mL auto-inducing medium in a 3L Erlenmeyer flask was inoculated by addition of 600 μ L pre-culture and shaken at 180 rpm and 20°C for 3.5 days. We found that protein expression had stalled at this point, and we left the cultures in microaerobic conditions¹⁴. The next day, the cells were harvested and protein purification followed as previously described¹⁶ (and Brander2014b). In short, CotA was purified by heating to 70°C for 30 minutes in 50-mM MOPS buffer pH 7.6, 200mM NaCl, clarified and purified to homogeneity by cation exchange

chromatography on a 5-mL HiTrap SP FF column. TtMCO was purified by heating to 90°C for 45 minutes in 50 mM MOPS buffer pH 7.0 without NaCl, clarified and purified to homogeneity by anion exchange chromatography on a HiTrap Q FF column.

Sample preparations

Purified TtMCO was changed into 50mM MOPS buffer pH 7 and divided into three portions. One portion was kept on ice and is termed “as isolated”. One was heated for one hour at 70°C and is termed “activated”, and one was supplemented with NaCl to a total concentration of 200mM and then heated one hour at 70°C and is henceforth termed “inactivated”. The three samples were characterized by EPR, UV/VIS, activity, and SDS PAGE.

Purified CotA was changed into 50mM MOPS pH 7.6 200mM NaCl buffer, split in four and diluted to 200 μ L. One portion was diluted into buffer and left at room temperature, one portion was reduced by diluting into 1mM ascorbate containing buffer, one portion was heated to 70°C for 30 minutes and one portion was diluted into 1 mM ascorbate containing buffer and immediately incubated at 70°C for 30 minutes. The four portions were characterized by EPR and catalytic activity when the faint blue color had reappeared. In addition, the heated portion was reduced by addition of 20 μ L 10-mM ascorbate and characterized by EPR after the blue color had reappeared.

CotA anaerobic transformation

200 μ L Purified CotA in 50mM MOPS pH 7.6 was purged with argon and transferred to an anaerobic glovebox. The enzyme was titrated with three times excess ascorbate as determined by EPR. The excessive reducing agent was removed by five rounds of dilutions with 0.5 mL buffer and concentration to 100 μ L using an Amicon Ultra-0.5mL centrifugal filter (10 kDa cut-off).. Still in the glovebox, the sample was heated in a water bath to approximately 65 °C for 30 minutes, and the precipitation was removed by centrifugation. All steps were followed by EPR spectroscopy while keeping the sample strictly anaerobic. The sample was removed from the glove box in a rubber-sealed EPR tube and immediately frozen in liquid nitrogen. After spectrum acquisition, the sample was thawed in the air lock chamber under vacuum and reentered into the glove box for

continued manipulation. Finally, the reduced and heat-treated sample was uncapped in normal atmosphere and left to reoxidize before measurement of a final EPR spectrum.

Characterization of activity

To measure the activity of each protein state, turnover was determined using 200 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate in 100 mM acetate buffer at pH 4.5, which is near pH_{opt} for this substrate. The rates were calculated from the linear time dependent absorbance increase of the 420 nm band arising from ABTS oxidation.

Spectroscopic characterization

TtMCO samples were characterized by their UV/VIS spectrum as measured on a Shimadzu UV-1800. As well as EPR spectrum measured on a Bruker EMX at 9.45GHz and -196 °C with microwave power 20.4 mW and modulation amplitude 32.5 Gauss. CotA samples were characterized by their EPR spectrum as measured on a Bruker EMX at 9.63GHz and -196 °C with microwave power 10.0 mW and modulation amplitude 10 Gauss.

Results and discussion

TtMCO is a hyperthermostable LMCO from *Thermobaculum terrenum* with only low affinity for phenolic substrates and a proposed function in the maturation of antibiotics. (Brander2014b) It was observed that the residual activity of TtMCO decreased rapidly when heat incubated with NaCl, but concomitant with only minor losses of protein as observed on SDS PAGE gel (Supporting Information, Figure S1). This prompted the use of a salt-free lysis buffer, which increased the yield of active protein dramatically. At the same time, heat incubating without salt led to increased apparent activity of TtMCO. Together these observations indicated that NaCl and heat both affect heat activation and provided a simple handle for characterization of this often reported but poorly understood process.

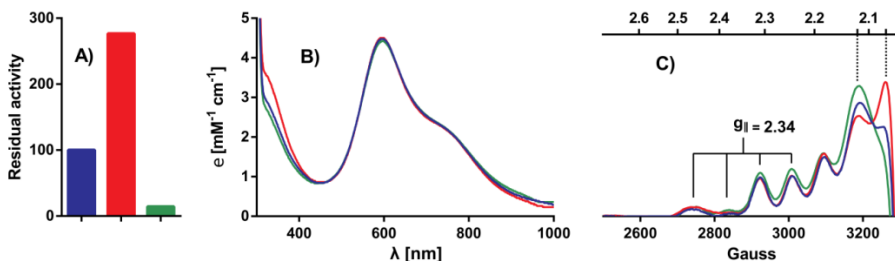


Figure 2. Spectroscopic characterization of the activated and inactivated form of TtMCO. Three samples were prepared from the same stock (Isolated, blue). One part was heat incubated without salt (Activated, red) and another part was heat incubated with 200mM NaCl (Inactivated, green). A: Activity as measured on the substrate ABTS, normalized to 100% for the isolated TtMCO stock. B: UV-VIS spectra. C: X-band EPR spectra showing the hyperfine region of the copper signals. Important features at $g=2.12$ and $g=2.07$ are marked with dashed lines. EPR conditions are microwave frequency 9.45GHz, microwave power 20.44 mW, modulation amplitude 32 Gauss. The spectrum of the activated preparation was originally reported in Brander2014b.

We compared the activated and the inactivated form of TtMCO by EPR and UV/VIS as seen in Figure 2. The samples used for the spectroscopic characterization are completely comparable, starting from the same batch of TtMCO (isolated) and prepared in parallel. The activated sample was prepared by heating for one hour at 70°C, pH 7 without NaCl, while the inactivated sample was similarly incubated, but in a buffer containing 200 mM NaCl. Heat activation of this batch increased activity to 300%, while the combined heat and salt treatment reduced activity to 15%. TtMCO is very stable at 70°C having an apparent $t_{1/2}$ of 2.12 days (Brander 2014b) and only minor heat degradation is to be expected as is also apparent by the SDS PAGE gel (Figure S1, Supporting Information). Indeed, the blue absorption band at 600 nm in Figure 2B is comparable for all three samples, showing that the difference in reactivity is not due to the T1 copper and indirectly not the protein concentration. The 330-nm band, which is attributed to the anti-ferromagnetically coupled T3 Cu(II) pair, is most intense in the activated sample and least in the inactivated suggesting that this site is perturbed.

The EPR spectra in Figure 2C reveal significant differences between the three samples. The difference is most clearly seen from the prominent feature at g

= 2.07 in the active form which is shifted to $g = 2.12$ in the inactivated. In addition, a hyperfine band is building up at $g=2.38$ in the inactivated sample. Neither of the spectra can cleanly be simulated with just two types of copper. However, the changes are significant and the feature at $g = 2.07$ and $g = 2.12$ corresponds well with two coppers signals with $g_{\perp} = 2.5$ and $g_{\perp} = 2.10$ similarly to the two forms of T2 observed in bilirubin oxidases. The feature at $g = 2.38$ is part of a hyperfine quartet $g_{\parallel} = 2.34$ and $A_{\parallel} = 100 \times 10^{-4} \text{ cm}^{-1}$ which matches the atypical T2 copper described for the alternative resting state of bilirubin oxidases. Thus, our EPR data suggest that heat activation of TtMCO is followed by a redox change similar to that in the transformation of bilirubin oxidases.

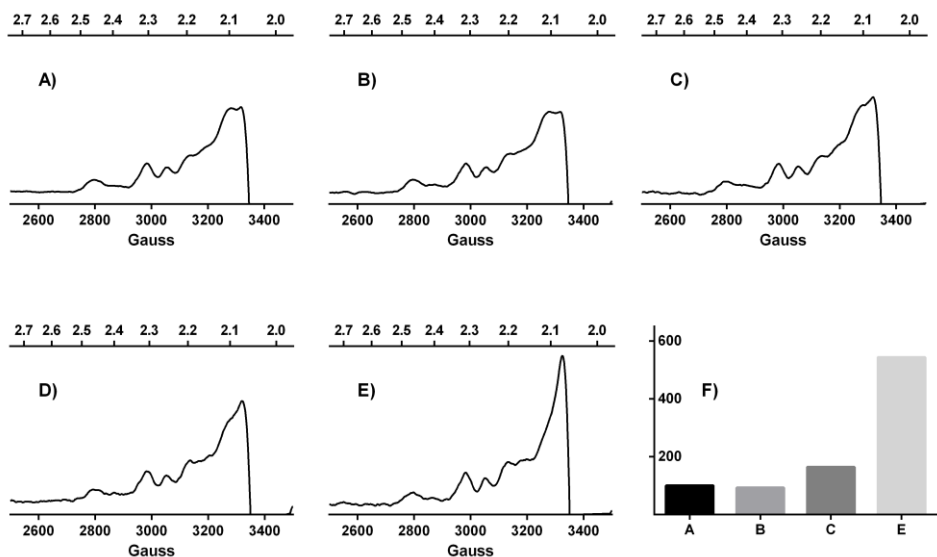


Figure 3. Spectral dependency of *B. subtilis* CotA on heat and ascorbate. A) Incubating at room temperature. B) Incubating with 1mM ascorbate. C) Incubating at 70°C for 30 minutes. D) Incubating at 70°C for 30 minutes followed by incubation with 1mM ascorbate at room temperature. E) Incubating with 1mM ascorbate at 70°C for 30 minutes. F) Activities measured on CotA samples from A, B, C, and E.

Because a similar set of spectroscopic data is available for the widely studied, first-in-class *Bacillus subtilis* CotA^{6,14}, we wanted to investigate if the link between TtMCO and the bilirubin oxidases also included *Bacillus subtilis* CotA. From the literature, CotA does not respond as easily to heat activation as does TtMCO^{6,16} Brander2014b. However, heat activation of laccase activity was previously observed for a crude extract from CotA producing *Bacillus subtilis* spores⁶, which indicates that transformation from inactivated to activated form is possible for CotA, but heat is not enough to drive the transformation. *Magnaporthe oryzae* bilirubin oxidase was activated by addition of the reducing agent dithionite¹³ and we turned to investigate CotAs behavior in a similar reducing environment.

CotA precipitated with dithionite, and instead the milder ascorbate was used as a reducing agent. The EPR spectra are shown in Figure 3. First, simply incubating in 1mM ascorbate or heating to 70 °C for half an hour only slightly changed the EPR spectrum (Figure 3B, 3C). A slight increase in the $g = 2.07$ feature is observed from first heat incubation followed by incubation with ascorbate (Figure 3D), but when the two are combined by heating CotA in the ascorbate reduced buffer, a prominent change is seen from $g = 2.12$ to $g = 2.07$ (Figure 3E). This spectroscopic observation is accompanied by a 500% increase in enzyme activity (Figure 3F) and shows that CotA is heat activated similarly to TtMCO. The observation that CotA needs to be reduced in order to fully heat activate explains why Martins et al. only observed heat activation of CotA for the crude extract⁶. In contrast to the purified state, antioxidants are expected to be present in a crude extract..

Figure 4A shows EPR spectra recorded after excessive anaerobic reduction (see Supporting Information Figure S2), subsequent heating, and finally reoxidation of the CotA enzyme. Even in fully reduced state, a distinct EPR signal was clear showing a redox inactive copper with $g_{\perp} = 2.08$ and $g_{\parallel} = 2.35$ and $A_{\parallel} = 95 \times 10^{-4} \text{ cm}^{-1}$ (Figure 4B). These parameters correlates perfectly with the first reported T2 copper by Martins et al.⁶ and the previously discussed inactive states of bilirubin oxidases^{12,13} and TtMCO. After heating and reoxidation, the EPR spectrum showed that CotA had turned completely into the active form with no trace of the redox inactive copper.

In order to shed further light on the function of the reducing agent in heat activation, we carefully removed excess ascorbate after reduction of CotA under anaerobic conditions followed by heating to 65°C. In doing this, it was possible to capture an intermediate complex before reoxidation as shown in figure 4C. This EPR spectrum is unconventional with a sharp signal with $g_{\perp} = 2.028$ suggesting a free radical. The signals in the hyperfine range is of limited resolution but two Cu(II) are suggested. The four best resolved peaks split in two pairs with $A \sim 61 \times 10^{-4} \text{ cm}^{-1}$ and a larger split of $154 \times 10^{-4} \text{ cm}^{-1}$. Kjaergaard et al. characterized the redox inactive copper in the alternative resting state to be one of the T3 coppers trapped without its partner¹³ as depicted in Figure 1B. By this model, the radical is most likely a O_2^- that has accepted an electron from one of the reduced coppers. This would explain the sharp $g_{\perp} = 2.028$ peak¹⁹ and leave two oxidized coppers in the trinuclear site which can give rise to the hyperfine signals. This is a unique spectral characterization of a native 3-electron reduced laccase intermediate which have previously only been observed for T1-silenced laccases²⁰. The latter form is found to have abridging peroxide molecule between oxidized T2 and T3 _{β} ¹⁷.

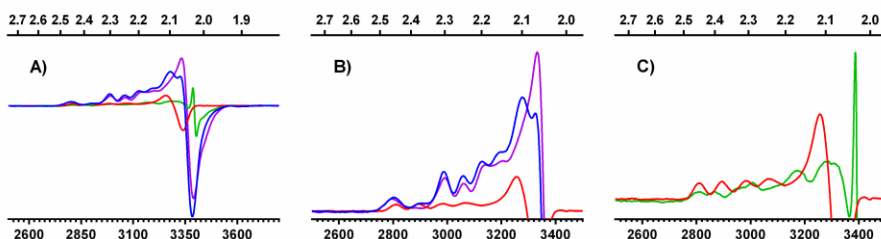


Figure 4. Anaerobic reduction and heat activation of CotA followed by EPR. The as-isolated enzyme (blue) was reduced anaerobically (red), dialyzed and heated intermediate (green) followed by oxidation (purple). A) EPR spectra of the four species. B) Hyperfine region of the isolated, reduced and oxidized spectra highlighting the disappearance of the $g_{\perp}=2.08$ signal by heating. C) Hyperfine region of the reduced and intermediate spectra.

Durao et. al.¹⁴ utilized prolonged semi-anaerobic incubation of CotA expressing *Escherichia coli* to enhance the formation of the activated form over the inactive. The hypothesis was that semi-anaerobic incubation will raise the intracellular copper levels and thus produce correctly folded and fully active CotA laccase. This hypothesis is supported by studies of copper homeostasis and

accumulation in *Escherichia coli*²¹. In consideration of the reported conversion of *Magnaporthe oryzae* bilirubin oxidase to the “resting oxidized” form after using the reducing chemical dithionite, and now the activation of subtilis cotA with ascorbate and heat we suggests that a similar mechanism is part of the activation observed under semi-anaerobic incubation with *Escherichia coli*. The long incubation (>16hours) in the reducing environment of the cytoplasm can function similarly to dithionite and ascorbate in activating the enzyme. We find that incubating unpurified CotA overnight with CuCl₂ and ascorbate gives similar activity increase compared to overnight static incubation (See Supporting Information, Figure S4). Interestingly, both proteins were found to be activated in environments that are native to their original hosts, suggesting a biological relevance of the alternative resting state. Indeed, it seems inadventagous to have a functional laccase-like protein inside the cytoplasm considering that it readily oxidizes vitamins and anti-oxidants. In lack of the organelle protein-trafficking mechanisms of eukaryotic cells, bacterial cells must have a different means to protect themselves from laccase-induced oxidative stress during protein expression and export. Producing the laccase in the “alternative resting” state for later activation seems like an ideal solution, and our findings showing how these states can be converted, support such reversible conversion mechanisms.

Similarly, it seems desirable to be able to inactivate the highly stable laccases. Formation of the alternative resting state of CotA was observed by simply incubating the enzyme solution with excessive ascorbate overnight at 4°C. The protein partitioned in an upper layer that had turned blue and a lower layer still being reduced and white and oxygen depleted. As shown in the Supporting Information Figure S3 both layers were significantly transformed towards the alternative resting state, but mostly so for the blue upper layer. The upper layer had access to oxygen and CotA here must have turned over more times than in the lower layer. This suggests that formation of the alternative resting state actively happens during normal turnover and that the protein will automatically inactivate itself if not stimulated.

Conclusion

We have reported the observation of an inactive bacterial laccase form similar to the alternative resting state of the bilirubin oxidases. The fingerprint of this state is a strong EPR feature at $g = 2.12$ and the absence of the 330 nm absorbance band. Specifically a redox inactive copper with EPR parameters: $g_{\perp} = 2.08$ and $g_{\parallel} = 2.345$ and $A_{\parallel} = 95 \times 10^{-4} \text{ cm}^{-1}$ was identified.. We show the transformation from the inactive state to the active state for two laccase-like multicopper oxidases, TtMCO from *Thermobaculum terrenum* and CotA from *Bacillus subtilis*. TtMCO is readily activated by heating to 70°C for an hour in low salt buffer while being transformed to the inactive form by a similar heat treatment in 200mM NaCl buffer.

CotA from *Bacillus subtilis* has been observed in two different forms. We show that the spectral differences of these forms resemble those of TtMCO. However, the conversion seems more complicated, as heat activation was only observed for the ascorbate-reduced enzyme. We followed the anaerobic reduction and heating of CotA by EPR spectroscopy and report a clear spectrum of the redox inactive copper with EPR parameters: $g_{\perp} = 2.08$ and $g_{\parallel} = 2.344$ and $A_{\parallel} = 93 \times 10^{-4} \text{ cm}^{-1}$ that disappear upon heating. Importantly, these findings show that the activation-inactivation process is a redox process rather than a protein conformational change, of potentially large importance to the living organisms, and of substantial relevance to engineering and use of these proteins in general. Finally, we have observed a unique 3-electron reduced protein intermediate that is interpreted as a superoxide of the trinuclear cluster, an important alternative state relative to the 3-electron reduced intermediate that has been reported for T1 silenced *Rhus Vernicifera* laccase.

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Supporting Information.

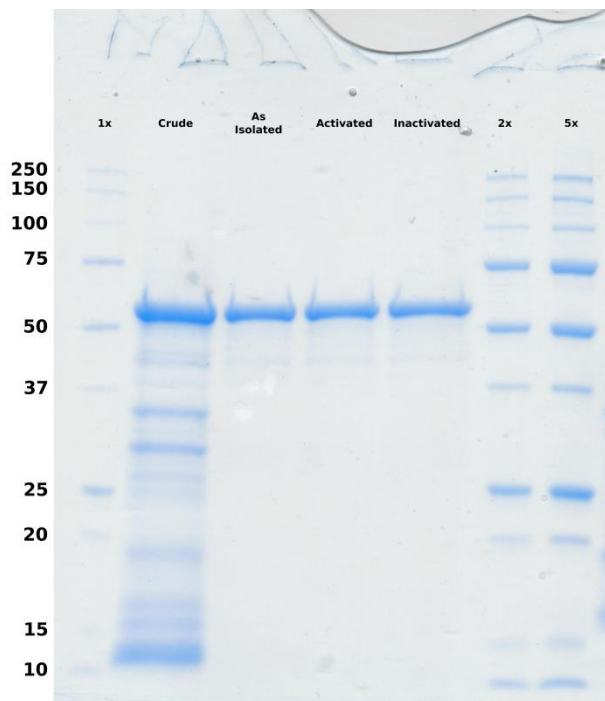


Figure S1 SDS PAGE gel of TtMCO: Lane 1,6 &7 are protein markers. Lane 2 is the crude extract after heat treatment. Lane 3 after purification on HiTrap Q column. Lane 4: Heated for 1 hour at 70°C without NaCl, Lane 5: Heated for 1 hour at 70°C with NaCl

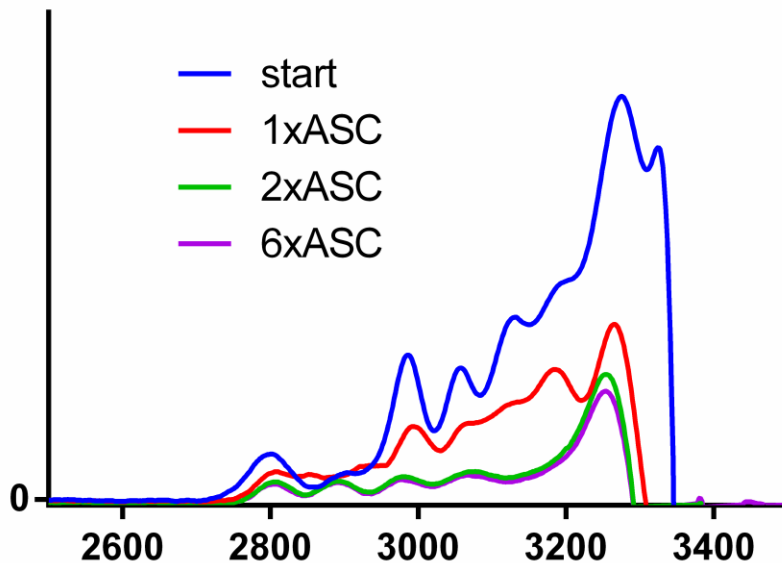


Figure S2: Anaerobic titration of CotA followed by EPR spectroscopy. Very little change in the EPR spectrum is seen between reduction of 2 and 6 equivalence of ascorbate, showing the existence of a redox inactive copper is.

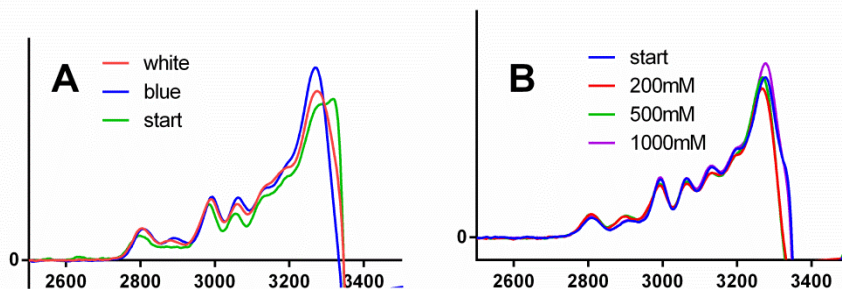


Figure S3: Inactivation of CotA: A: The CotA sample from figure 3A-D was pooled and left overnight at 4°C with 1mM ascorbate. Next day the sample was showing two colors. The upper half was blue, and the lower was clear (white). The white fraction was extracted with a needle and the EPR signal measured after full oxidation. The blue fraction was significantly more on the “alternative resting” form. B: Similar incubation of CotA overnight with 1mM ascorbate and varying concentration of NaCl. In this experiment,

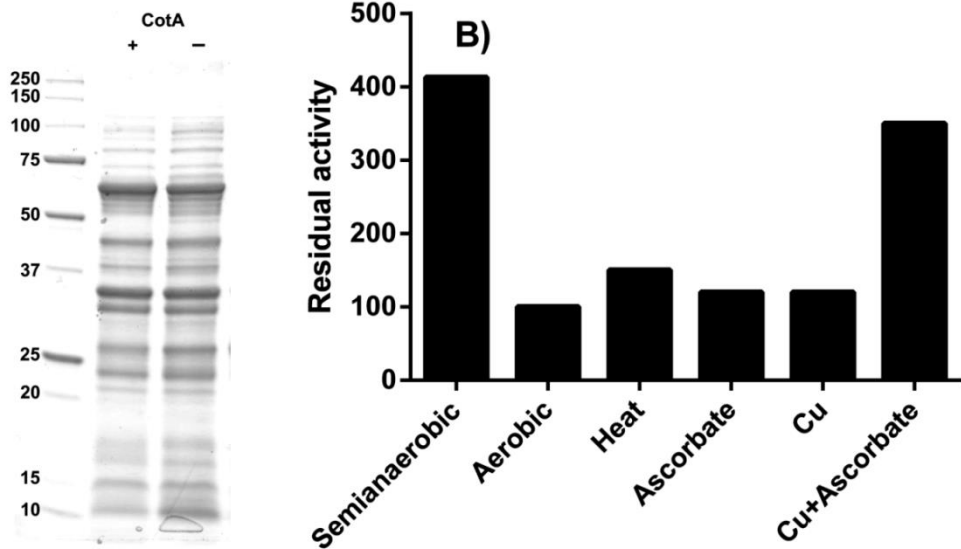


Figure S4: A) SDS PAGE gel of CotA expression. The two protein samples were extracted with BPER from the same expression culture before (-) and after (+) overnight microaerobic incubation. B) The aerobic protein sample was manipulated in various ways. Heated 60 minutes at 70°C (heat), reduced with ascorbate (ascorbate), incubated with 50µM copper (Cu) and incubated with a combination of ascorbate and copper (Cu+ascorbate). After these manipulations, the catalytic activity was measured on ABTS. Incubation with copper and ascorbate simulates the environment of microaerobic incubation within *E. coli*. Indeed, this manipulation activated the aerobically expressed CotA to the same level as the microaerobically expressed CotA.