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Two- and three-domain bacterial laccase-like genes are present in drained peat soils

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ABSTRACT

Laccases of fungal origin have been intensively studied due to their importance in various biotechnological applications. There is a constant demand for new laccases with improved properties such as stability at higher temperatures or at an alkaline pH. Growing molecular evidence suggests that laccases may also be widespread in bacteria. While only a handful of bacterial laccases have been purified and characterized, several novel traits have already been discovered (e.g. pH-stability and 2-domain organization of the enzyme as opposed to the usual 3-domain structure of fungal laccases). The aim of this study was to examine the diversity of bacterial laccase-like genes in two types of high-organic peat soil using a cloning and sequencing approach. Gene libraries prepared of small fragments (150 base pairs) revealed an amazing diversity of bacterial laccases. The fragments clustered in 11 major lineages, and one third of the 241 sequences resembled laccase-like genes of *Acidobacteria*. Additionally, a new primer was used to retrieve several larger fragments of the putative bacterial laccase genes that spanned all four copper-binding sites. Both "conventional" 3-domain laccases and the recently described 2-domain small laccases have been obtained using this approach, demonstrating the potential of the primer. The present study thus contributes to the understanding of the diversity of bacterial laccases and provides a new tool for finding laccase-like sequences in bacterial strains and soil samples.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to a group of multicopper enzymes found in all domains of life (Hoegger et al., 2006). They have low specificity and can oxidize a variety of phenolic compounds and some non-phenolic substrates, usually via mediators (Widstein and Kandelbauer, 2008), by coupling the oxidation of a substrate to the reduction of molecular oxygen to water. The reaction is catalyzed by four copper atoms that are held in place in the reaction center of the enzyme by four histidine-rich copper-binding regions (Claus, 2004). The amino acid sequences of these regions are conserved in different organisms and the respective gene regions have been utilized as primer-binding sites for PCR amplification (Luis et al., 2004; Kellner et al., 2008). Fungal laccases are most extensively studied (Sharma, et al. 2007; Baldrian, 2006) and significantly contribute to degradation of lignin, but are also associated with other processes in morphogenesis and pathogenesis of fungi (Arora and Sharma, 2010). They have already been applied in a range of biotechnological processes in the paper, textile, pharmaceutical and petrochemical industries (Rodriguez Couto and Herrera, 2006).

On the basis of molecular data, it was proposed that diverse laccase genes may also be present in bacteria (Alexandre and Zhulin, 2000; Claus, 2003). The ecological roles of bacterial laccases are not understood to the full extent, but they may also play an important role in the degradation of recalcitrant (poly)phenolic compounds (Claus, 2003; Bugg et al., 2010). Bacterial laccase are also involved in other physiological processes, such as pigment formation in spores of *Bacillus subtilis* (Martins et al., 2002). A novel lineage of two-domain bacterial laccases has been recently described (Nakamura et al., 2003) in addition to the "regular" three-domain laccases similar to those found in fungi. Representative enzymes have been found in *Streptomycetes* (Machczynski et al., 2004; Endo et al., 2003) and *Nitrosomonas europea* (Lawton et al., 2009) These enzymes are smaller than "conventional" three-domain (fungal) laccases, and in the active form assume a homotrimeric structure. Metagenomic data suggests that two-domain laccases may also be present in other bacterial groups, such as the *Bacteroidetes* (Komori et al., 2009). However, only a few bacterial laccase-like genes and/or protein products have been

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characterized (reviewed by Sharma et al., 2007). This seriously impedes our understanding of the physiological and environmental role of these enzymes as well as their introduction into biotechnological processes, which are now exclusively operated with fungal laccases.

Studies addressing the diversity and activity of bacterial laccases in soil are extremely scarce (Theuerl and Buscot, 2010; Luis et al., 2004). To our knowledge, there has been only one study describing the diversity and distribution of bacterial laccases in environmental samples. Kellner et al. (2008) devised a primer set to amplify short fragments (approximately 150 bp) of bacterial laccase-like genes to study their diversity and distribution in vertical profile of forest soil and in grassland soil. They found an unusually high diversity of putative bacterial laccases with the sequences clustering into 16 distinct groups, several of which had no closely related sequence in public databases. The highest diversity was found in the humic horizon of the forest soil, as found previously for fungal laccases (Luis et al., 2004).

A colorimetric assay for measuring phenol oxidase activity was recently developed by Floch et al. (2007) which was based on the oxidation of 2,2′-azinobis-(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). ABTS, which is one of the substrates used for testing the enzymatic activity of laccases is readily oxidized by the bacterial laccases CotA of *B. subtilis* (Martins et al., 2002) and SLAC of *Streptomyces coelicolor* (Dube et al., 2008). However, measuring laccase enzymatic activity in complex environments such as soils is not an easy task due to the lack of specific substrates or inhibitors for laccases, and due to their overlapping activities with other groups of enzymes. A modification of this assay was used in the present study to assess phenol oxidase enzymatic activities in a number of different soils as the first step to evaluate the potential of these soils to produce laccase-like enzymes.

On the basis of soil phenol oxidase activity, two soils (high-organic peat soils) with the highest relative activity were selected to investigate the diversity and composition of bacterial laccase-like genes using molecular approaches. The soils differed in pH and soil organic matter content but had been exposed to the same climatic conditions. Laccase gene libraries were constructed from both types of soil using existing and new primers, developed in this work that allow the examination of almost-complete genes of the target enzymes. Laccase diversity in both peat soils was assessed using phylogenetic methods and diversity indices. This study thus provides a novel view of laccase diversity within soil bacterial communities in two divergent peat soils.

2. Materials and methods

2.1. Sampling of soils from sites known for different land use and screening for phenol oxidase activity

Five soil samples were selected for phenol oxidase activity measurements. The top 30 cm were sampled (5 samples were obtained by inserting an auger) and homogenized through a sieve. The samples included: (I) forest soil sampled in Slovenia near Škofja Loka (46°9′, 14°17′), with organic carbon content of 12.7% and pH 4.1, (II) grassland soil sampled in Jesenice, Slovenia (46°26′, 14°0′), with organic carbon content of 7.5%, C:N ratio of 0.26, and pH 6.7, (III) agricultural soil sampled in the Netherlands near Vredepeel (51°32′, 5°52′). It is a sandy soil with the organic carbon content of 3.5% and pH 5.5, and (IV, V) two types of peat soils that were chosen for further studies are described in more detail below and in Table 1.

Phenol oxidase activity was measured in five soil samples using the modified method of Floch et al. (2007) to fit the reactions into a microtiter plate setup. 10-g samples were shaken (150 rpm) in flasks with 90 ml of sterile 0.9% NaCl solution for 1 h 10 ml were transferred to 15-ml centrifuge tubes and shaken vigorously on a horizontal shaker with glass beads (diameter 2 mm) for 15 min. This soil suspension was used for measuring the enzymatic activity. Each reaction in a microtiter plate well contained 130 μl of citrate-phosphate buffer (pH 4.0, 5.8 or 7.6), 20 μl of soil suspension and 50 μl of ABTS (stock concentration 50 mM). The microtiter plates were incubated with shaking (100 rpm) for 1 h then centrifuged at 3500 rpm for 4 min and the supernatant was transferred to a new plate. The absorbance was measured using a Multiscan Spectrum spectrophotometer (Thermo, Vantaa, Finland). The controls were prepared in the same way, with the difference that soil samples were autoclaved beforehand (20 min at 121 °C). All measurements were made in triplicates. Absorbances of the controls that were due to nonbiological oxidation were subtracted from the absorbances in the samples. Mean values and standard errors of absorbances were calculated, normalized to the dry weight of the added samples, and multiplied by a factor of 1000 – these are the arbitrary units reported in Fig. 1.

Table 1
Properties of the fen and bog soils. Values represent the mean ± standard error [$N = 3$] for the upper 30 cm of soil. *WHC – water holding capacity. **Soils have been classified under the World reference base for soil resources (2006). The table was adopted from Ausec et al. (2009).

	Fen soil	Bog soil
C_{org} content (%)	16.3 ± 0.12	45.4 ± 0.12
N_{org} content (%)	1.40 ± 0.006	2.75 ± 0.006
CN ratio	11.7 ± 0.12	16.5 ± 0.006
pH	7.55 ± 0.12	4.58 ± 0.09
WHC* (g_{H20}/g_{soil})	1.65 ± 0.12	8.14 ± 0.17
Bulk density (g cm^{-3})	0.59 ± 0.03	0.16 ± 0.03
Mean annual T (°C)	17.8 ± 7.7	12.6 ± 5.9
Mean annual water table (cm)	−53.2 ± 12.7	−24.4 ± 8.0
Vegetation	Grassland	Forest
Soil classification**	Rheic Fibric Histosol	Rheic Fibric Histosol (Dystric)

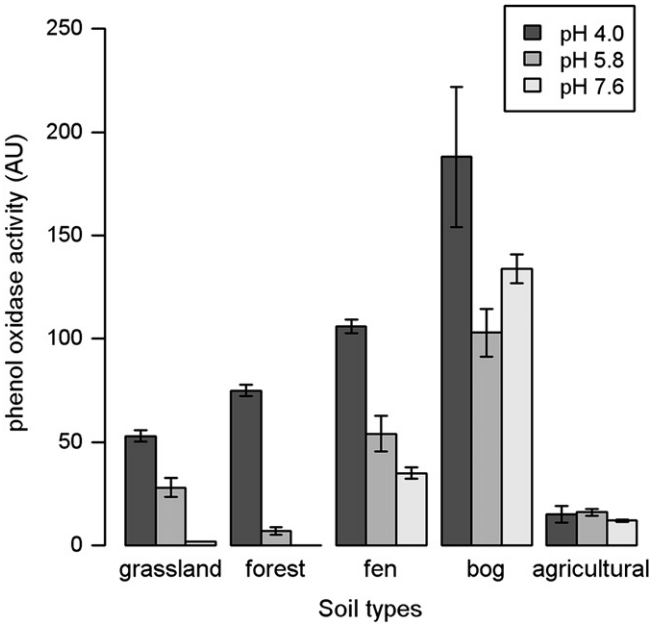

Fig. 1. Phenol oxidase activity in five different soil samples measured in a colorimetric assay using ABTS as a substrate. Measurements were made at three different pH values: 4.0 (black), 5.8 (dark gray) and 7.6 (light gray). Error bars represent standard errors of arithmetic means ($N = 3$). Activity is expressed in arbitrary units that are proportional to the measured change in absorbance.