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Characterization of a novel 2,4,6-trichlorophenol-inducible gene encoding chlorophenol *O*-methyltransferase from *Trichoderma longibrachiatum* responsible for the formation of chloroanisoles and detoxification of chlorophenols

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ABSTRACT

De novo sequencing of eight internal peptides of purified chlorophenol *O*-methyltransferase, or CMT1 (before named as CPOMT), from *Trichoderma longibrachiatum* was performed by MALDI-TOF/TOF and ESI-IT. A novel gene (*cmt1*) encoding CMT1 was cloned by using a PCR approach based on the amino acid sequence of two internal peptides. The gene (1637 bp) encoded a protein of 468 amino acids with a deduced molecular mass of 52.4 kDa, and a theoretical isoelectric point of 5.93. This gene contains four introns, whose location was confirmed by comparison of cDNA and chromosomal sequences. The expression of *cmt1* gene was induced at transcriptional level by exposure of fungal mycelia to 2,4,6-trichlorophenol (2,4,6-TCP). Putative homologous genes were detected in many different fungal strains, including other *Trichoderma* species. Partial silencing of *cmt1* gene resulted in a 48.9% (±5.2) decrease of CMT1 activity levels in a *T. longibrachiatum* At37 transformant strain by comparison with the wild type, whereas a decrease of up to 53.0% was observed in the levels of 2,4,6-TCA produced in liquid cultures. Efficient expression of *cmt1* gene in *Escherichia coli* unequivocally confirmed that it encodes a CMT1 enzyme.

1. Introduction

Chloroanisoles, specially 2,4,6-trichloroanisole (2,4,6-TCA) and pentachloroanisole (PCA) are significant environmental pollutants frequently detected in many ecosystems such as different types of soils (Palm et al., 1991; D'Angelo and Reddy, 2000), river and marine sediments (Barakat et al., 2002), lake and river waters (Nystrom et al., 1992; Jiang et al., 2000), marine troposphere (Führer and Ballschmiter, 1998), and vegetable materials like the bark of the cork-oak in Mediterranean forests (McLellan et al., 2007). Chloroanisoles arise as a consequence of a detoxification reaction of highly toxic chlorophenolic pesticides, which consists in the O-methylation of their reactive hydroxyl group (Cserjesi and Johnson, 1972; Gee and Peel, 1974). In fact, chlorophenols toxicity is mainly due to the high reactivity of their hydroxyl group that can react with proteins and nucleic acids in the cell resulting in important cellular damages. The blockade of the CPs hydroxyl group by O-methylation produces almost the total loss of its toxicity. This reaction has an important environmental significance since chlorophenols, specially 2,4,6-TCP and pentachlorophenol (PCP), are some of the contaminants more routinely found in many ecosystems, like surface and groundwaters, atmospheric air, soils and sediments (Sinkkonen and Paasivirta, 2000). In fact, chlorophenols are recognized as major pollutants in countries like China and the United States (Xia and Zhang, 1990; USEPA, 1991). O-methylation of chlorophenols seems to be very usual in filamentous fungi, as it has been documented to occur in many different fungal strains (Cserjesi and Johnson, 1972; Gee and Peel, 1974; Whitfield et al., 1991; Álvarez-Rodríguez et al., 2002; Miki et al., 2005; Maggi et al., 2008). Furthermore, the capability of several bacterial strains to carry out the O-methylation of chlorophenols has been reported (Allard et al., 1987; Neilson et al., 1988; Nystrom et al., 1992; Prat et al., 2009), although the overall contribution of these bacterial strains to the general level of chloroanisoles in the environment seems to be of minor importance. In the fungal strain *Trichoderma longibrachiatum*, O-methylation of chlorophenols is catalyzed by a novel *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase, named chlorophenol-*O*-methyltransferase (CMT1), which has been previously purified and characterized (Coque et al., 2003). This enzyme showed two remarkable traits: first, its synthesis was induced by several chlorophenols, particularly if they contained three or more chlorine atoms in their structure; and second,

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substrate specificity studies showed that the enzyme activity was specific for halogenated phenols containing fluoro-, chloro-, or bromo-substituents, whereas other hydroxylated compounds such as hydroxylated benzoic acids, hydroxybenzaldehydes, phenol, 2-metoxyphenol and dihydroxybenzene were not methylated (Coque et al., 2003).

Chloroanisoles exhibit a marked lipophylic character and have a high bioconcentration potential in biological samples and humic materials (Neilson et al., 1984). Chemically, they are very stable compounds. This is the reason why, until very recently, they were supposed to be more resistant to biodegradation than chlorophenols, being highly persistent in the ecosystems (Allard et al., 1987). Nowadays, this perspective has changed as chloroanisoles have been reported to be biodegraded by both aerobic bacteria (Goswami et al., 2007) and ligninolytic fungi (Campoy et al., 2009), although the biodegradative mechanisms employed by both types of microorganisms are different.

Besides its environmental significance, 2,4,6-TCA is the main agent responsible for musty/mouldy off-odors in wine since it is able to confer a very unpleasant fungal aroma to wine, even at concentrations as low as 2–4 ng L^{-1}. In fact, wine contamination with fungal aromas is currently recognized as one of the worse threats for the cellars all around the world. This kind of pollution is responsible for important economical losses in wine industry, which are estimated to be on the order of US $10 billion annually (Silva Pereira et al., 2000). Although the 2,4,6-TCA found in wines can have different origins, cork stoppers have been frequently blamed as the most frequent source of wine contamination by means of that chemical compound (Silva Pereira et al., 2000).

Despite all the recent information reviewed above regarding the formation and biodegradation of chloroanisoles in the environment, no genetic data were known up to date. Thus, the main aim of this work is the cloning and characterization of a novel inducible gene (named *cmt1*) from *T. longibrachiatum* that encodes an enzyme with chlorophenol *O*-methyltransferase activity. The characterization of this gene is of both environmental and applied significance since the encoded enzyme (CMT1) is responsible for the detoxification of chlorophenolic pesticides by O-methylation in many different ecosystems, which results in the formation of chloroanisoles (one of the most unpleasant contaminants for cork stoppers and wines).

2. Materials and methods

2.1. Chemicals

2,4,6-TCA and 2,3,5,6-tetrachlorophenol (2,3,5,6-TeCP) were obtained from Aldrich-Chemie (Steinheim, Germany). 2,4,6-TCP was purchased from Fluka Chemie AG (Buchs, Switzerland). DTT, PMSF, SAM, ampicillin and kanamycin were supplied by Sigma–Aldrich (St Louis, MO). Phleomycin was obtained from InvivoGen (San Diego, CA).

2.2. Microorganisms and culture conditions

T. longibrachiatum CECT 20431 is a wild type strain isolated from cork samples (Álvarez-Rodríguez et al., 2002). For short-time storage, fungal strains were maintained on Malt Extract Agar (MEA) (Sigma–Aldrich) plates at 4 °C; whereas for long-time conservation, they were preserved as spore suspensions in 40% glycerol at −20 °C or −80 °C. Cultures in liquid media were routinely grown in Malt Extract Broth (MEB) as previously reported (Álvarez-Rodríguez et al., 2002).

Recombinant bacteriophages were selected in *Escherichia coli* XL-1 Blue MRA P2 strain (Stratagene, La Jolla, CA), whereas the strain *E. coli* LE392 was used as the normal host strain for bacteriophage propagation. All plasmid subcloning experiments were performed in *E. coli* DH5α, being chemically competent cells obtained by standard protocols (Sambrook and Russell, 2001). The expression of *cmt1* gene in *E. coli* was carried out using the *E. coli* strains Mach1-T1 and BL21(DE3) (Invitrogen, Carlsbad, CA). *E. coli* strains were conserved at 4 °C on LB agar plates (Sambrook and Russell, 2001).

2.3. Purification of CMT1

Enzyme protein was purified by the protocol of Coque et al. (2003), improved with some modifications that are described below in steps 1–6. All purification steps were performed at 4 °C on a fast protein liquid chromatography (FPLC) ÄKTA system (Amersham Pharmacia Biotech).

(i) *Step 1. Preparation of cell extracts.* A 6 g sample of mycelia was used to obtain cell extracts in breaking buffer as described before (Coque et al., 2003). After passage through four PD-10 columns, the proteins (14 mL) were recovered in Tris–HCl 10 mM (pH 8.0) containing 5 mM $MgCl_2$, 0.2 mM DTT and 10% glycerol (buffer DA).

(ii) *Step 2. HiTrap DEAE column chromatography.* This chromatographic step was carried out as described previously (Coque et al., 2003) except that fractions containing enzyme activity (between 50 and 110 mM of NaCl) were pooled and buffer changed in PD-10 columns, being the activity recovered in sodium phosphate 10 mM (pH 7.0) (buffer HA).

(iii) *Step 3. Hydroxyapatite bio-scale CHT-1 column chromatography.* The enzyme preparation in buffer HA was loaded onto a 5-mL bed volume hydroxyapatite Bio-Scale CHT-1 (Bio-Rad, Richmond, CA), previously equilibrated as indicated by the manufacturer. Enzyme activity was eluted by using a 100 mL-linear gradient in buffer HB (sodium phosphate 100 mM; pH 6.5) at a flow rate of 2.5 mL min^{-1}. Fractions containing the enzyme activity (eluting in the interval 150–300 mM of sodium phosphate) were collected and pooled to achieve next purification step.

(iv) *Step 4. Resource Q column chromatography.* This step was performed as previously reported (Coque et al., 2003).

(v) *Step 5. Ultrafiltration.* The active fractions were pooled and concentrated to a final volume of 150 μL by centrifugation at 4 °C in a 10 kDa cut-off Microcon device (Millipore, Billerica; MA) as indicated by the manufacturer.

(vi) *Step 6. Superdex 200 HR 10/30 column chromatography.* The concentrated sample was finally applied to a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) as reported by Coque et al. (2003). Fractions (0.5 mL) were collected, and assayed for CMT1 activity. Those showing activity and containing the purified enzyme were combined and preserved at −20 °C for subsequent analysis.

2.4. MALDI-TOF/TOF and ESI-IT peptide sequencing and identification

Purified CMT1 was identified in SDS–PAGE by photolabelling with *S*-adenosyl-L-[*methyl*-^{3}H]methionine (84 Ci $mmol^{-1}$; purchased from Amersham Pharmacia Biotech) as previously described (Coque et al., 2003). After identification, the CMT1 band was manually excised from a Coomassie-stained gel by biopsy punches, placed in an Eppendorf tube and washed twice with ddH_2O. The protein was digested following the method of Havlis et al. (2003). After digestion, the supernatant was collected and divided into two microcentrifuge tubes for speed-vacuum drying. The first one, used for MALDI TOF/TOF analysis, was resuspended in 4 μL of 50% ACN-0.1% trifluoroacetic acid (TFA) in ddH_2O. 1 μL