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Solvent tolerance acquired by *Brevibacillus brevis* during an olive-waste vermicomposting process

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ABSTRACT

In this work, a cultivable, Gram-positive, solvent-resistant bacterium was isolated from vermicomposted olive wastes (VOW). The highest 16S rRNA sequence similarity (99%) was found in *Brevibacillus brevis*. The genome of the isolate, selected for trichloroethylene (TCE)-tolerance, contained a nucleotide sequence encoding a conserved protein domain (ACR_tran) ascribable to the HAE1-RND family. Members of this family are hydrophobic/amphiphilic efflux pumps largely restricted to Gram-negative bacteria. No DNA sequences of HAE1 transporters were detected in the genome of a reference *B. brevis* strain isolated from natural soil. Since no cultivable solvent-tolerant bacterium was detected in the unvermicomposted olive waste, a transfer of solvent-resistance genes from Gram-negative bacteria during the vermicomposting process could explain the presence of HAE1 transporters in *B. brevis* isolated from the vermicompost. Under TCE stress conditions, the acquired nucleotide sequence could be translated into proteins, and the tolerance to solvents is conferred to the bacterium. The isolate was designated as strain BEA1 (EF079071).

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1. Introduction

Trichloroethylene (TCE) is one of the most common organic pollutants found at hazardous waste sites (Cunningham et al., 1996). TCE is a synthetic chemical that does not occur naturally in the environment; it is a suspected human carcinogen and it has been known to influence the structure and the function of the soil community (Miller and Guengerich, 1983; Fuller et al., 1997).

Enhanced bioremediation is the general term used to describe the addition of microorganisms or nutrients to the subsurface environment to accelerate the natural biodegradation process. The potential of microorganisms for bioremediation of TCE has been extensively investigated over the last decades, and it has been found that methanogens, methanotrophs, and certain species of bacteria able to degrade aromatic compounds are microbes capable of removing TCE (Nelson et al., 1988). Nevertheless, there are no described organisms capable of using TCE as a sole energy source under aerobic conditions; rather they co-metabolically degrade the compound using an individual enzyme. The oxygenase enzymes involved in the breakdown of TCE, as well as substrates for co-metabolism, have been widely described (Ensley, 1991; Ensign et al., 1992).

In recent years, organic-releasing materials as biosolids from wastewater treatment plants are shown to be suitable co-substrates to support the co-metabolism of TCE (Kao et al., 2001, 2004). To have sufficient carbon bioavailability, to be readily accessible, and to be relatively inexpensive have been described as the three main characteristics that an organic material should display for its use as substrate for remediation. The presence of such organic materials of microorganisms capable of degrading or resisting high levels of solvents could actually improve the bioremediation efficacy, especially when certain environmental conditions in the contaminated soils (excessive toxic levels of contaminant, low levels of nutrients, etc.) are unfavorable for the growth and the activity of the indigenous soil microflora. To be effective in transformation or degradation of chemicals, the indigenous microorganisms should be capable of resisting chemical pollutants, above all (Filip and Demmerova, 2006).

The molecular mechanisms for the resistance of bacteria to organic solvents and some relationship to the presence in the microorganisms of resistance-nodulation-division (RND) efflux pumps have been previously studied (Rojas et al., 2001; Ramos et al., 2002). The extrusion of toxic compounds by efflux transporters is the major pathway for bacterial detoxification and such efflux is due to the activity of membrane transporter proteins and drug efflux pumps. Phylogenetic analysis of RND transporters has shown that they could be separated into seven distinct families, including three hydrophobic/amphiphilic efflux families: the largely Gram-negative bacterial efflux family (HAE1), the

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Gram-positive bacterial putative efflux family (HAE2), and the largely archaeal putative efflux family (HAE3) (Tseng et al., 1999). The HAE1 transporters comprise of all known efflux pumps identified for organic solvents.

Recently, molecular characterization of RND transporters from solvent-resistant bacteria by a polymerase chain reaction (PCR) strategy has been successfully developed in petroleum-contaminated soils by Meguro et al. (2005). Comparing the amino acid sequences of all known RND transporters, the authors found two regions that were conserved among the HAE1 transporter sequences. These conserved sequences were detected only in Gram-negative bacteria.

In the search for new bioremediation strategies, the potential for using olive-mill solid waste, previously stabilized through vermicomposting processes, as an organic amendment for environmental remediation has been explored. It has been concluded that vermicompost from olive-mill wastes may be a suitable and economical method for reclaiming polluted tailings from Pb/Zn mines in the Mediterranean region as well as the degraded soils (Benitez et al., 2004; Romero et al., 2005). Since vermicompost from olive-mill waste contains high levels of available carbon and nutrients, as well as high hydrolytic enzyme activities involved in the biodegradation of TCE (Benitez et al., 2002, Saavedra et al., 2006), it could also be a promising agent for bioremediation of solvent-contaminated soils.

The main objective of this study was to isolate from vermicomposted olive wastes (VOW), and then to characterize bacterial strains able to grow in high levels of TCE. The molecular genetic detection of solvent tolerance mechanism (RND efflux pumps) was also investigated in the isolated bacteria.

2. Materials and methods

2.1. Treatment of olive wastes with Eisenia fetida

2.1.1. Vermicomposting process

The olive waste (OW) was obtained from a commercial olive-oil manufacturer (ROMEROLIVA, Deifontes, Granada, Spain). The vermicomposting process was performed on a pilot-scale experiment. For this, 60 kg (fresh weight) of olive waste were deposited on a 1.5 m^2 vermicomposting bed composed of a wooden frame-set on a 5% slope. The bed was 30 cm deep with a layer of plastic sheeting and an outlet for leachate drainage underneath. The bed was prepared in a greenhouse without temperature control. For improved structure and C:N ratio, olive waste was mixed with manure at a ratio of 4:1 (wet weight). A belt of vermicomposted cattle manure was placed around the layer of olive waste to provide an initial habitat for *Eisenia fetida* earthworms and also to act as a source of microbial inoculum (Benitez et al., 2002). During the vermicomposting process (9 months), the moisture content of the substrate was kept at 80–85% by irrigation. One portion of vermicomposted olive waste was air-dried prior to chemical and phytotoxicity analysis. The second portion was stored at 4 °C until biological assays were performed.

2.1.2. Phytotoxicity measurement

Chemical analyses and phytotoxicity test were determined using the validated methods (Sims and Haby, 1971; M.A.P.A., 1986) in the olive waste and in the vermicomposted olive waste. The phytotoxicity bioassay was a slight modification of the method described by Zucconi et al. (1981). Water extracts (1:5) from OW and VOW were incubated (25 °C) in darkness for 24 h with cress seeds (*Lepidium sativum* L.). Distilled water was used as a control. Ten seeds were placed, by quintuplicate, in Petri dishes (7 cm diameter) lined with filter paper containing 1 ml of each extract or distilled water. Germinated seeds (G) were counted and radicle growth (L) was measured. The germination index (GI) was calculated according to the formula $GI = (G/G_o) \times (L/L_o) \times 100$, where G and L are the germination percentage and the radicle growth of the organic substrates, respectively while G_o and L_o are the germination percentage and the radicle growth of the control, respectively. Analytical results are shown in Table 1.

2.2. Trichloroethylene biodegradation/tolerance

The biodegradability was determined by measuring the ability of microorganisms from both OW and VOW to grow aerobically on TCE. Two suspensions of microorganisms were prepared, according to Berselli et al. (2004), by shaking 10 g of each organic material in 100 ml of saline for 24 h at 120 rpm in a sterile 250 ml flask; after filtration on a filter paper (porosity, 25 μm), the water phases were used to inoculate (3% v/v) all cultures. The suspensions of microorganisms were then inoculated on mineral minimal medium plates, which were thereupon arranged in closed 5 l glass boxes in an atmosphere with TCE as the sole carbon energy source. The plates were incubated at 28 °C and no growth was observed after 21 days.

The concentration of aerobic bacteria capable of growing in high concentrations of TCE occurring in OW and VOW was counted by inoculating the above-described suspensions of microorganisms on Tryptic Soy agar plates. Then, 200–1000 μl of pure TCE was added on a paper filter disk in the cover of the Petri dishes, which were then placed in closed 5 l glass boxes. The plates were incubated at 28 °C. Colonies of a single strain of bacteria appeared after 19 days on plates inoculated with a suspension of vermicompost's microorganisms and with TCE concentration $\leq$800 μl/plate. No growth was observed in plates inoculated with microorganisms from unvermicomposted olive waste.

2.3. Molecular identification of the TCE-resistant bacterial strain

DNA was extracted from the vermicompost's bacterial isolate from plates with 800 μl TCE with the genomic DNA isolation kit (MBL-dominion) according to the manufacturer's instructions. The DNA concentration was determined on a nanodrop spectrophotometer (Nanodrop Technologies, Inc., DE, USA). The DNA was characterized by sequence analysis of small ribosomal subunit (16S ribosomal DNA). PCR was carried out to amplify nearly the entire gene with the eubacterial primers 27f and 1495r (Lane, 1991). The amplified DNA was analyzed on 1.2% (w/v) agarose gel and the DNA of the resulting fragment was recovered from the gel matrix using the QiaEx II Extraction kit (Qiagen; Hilden, Germany), cloned and subsequently sequenced in both directions by using an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystems, Inc.). Sequence comparison was performed using the BLAST program and the GenBank databases (http://www.ncbi.nlm.nih.gov/).

2.4. Detection, sequence analyses, and phylogeny of HAE1 genes in the isolate

The DNA extracted was amplified using the PCR primers A24f2 (5′-CCSRTITTYGCITGGGT-3′) and A577r2 (5′-SAICCARAIRCGCATSGC-3′), which were designed by Meguro et al. (2005) using two conserved regions among the HAE1 transporter genes.

Assays were performed at the same time with a reference strain of *B. brevis* isolated from soil and not exposed to TCE stress, with a Gram-positive bacteria *Bacillus thuringiensis*, and with a *Pseudomonas putida* DOT-T1E resistant to high soil–toluene levels (Rojas et al., 2001). Bacteria were obtained from the Microbiology Department, University of Valencia, Spain (*B. brevis*, *B. thuringiensis)* and from Estacion Experimental del Zaidin culture collection, CSIC, Granada, Spain (*P. putida*).

No bands were observed when the amplified product of reference *B. brevis* and *B. thuringiensis* was run on a 2% agarose gel.

The PCR products were purified by MBL-PCR QuickClean kit (Dominion-MBL SL, Spain), cloned and analyzed by direct sequencing using an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystems, Inc., CA, USA). Sequence comparison was performed using the BLAST program and the GenBank databases (http://www.ncbi.nlm.nih.gov/).

Neighbour-joining (NJ) tree was calculated using 100 bootstrap replicates with the CONSENSE program of the PHYLIP package (Felsenstein, 1992).

Table 1
Analytical characteristics of olive waste (OW) and vermicomposted olive waste (VOW).

	TOC ($g\,kg^{-1}$)	HA ($g\,kg^{-1}$)	WSC ($g\,kg^{-1}$)	C/N	N ($g\,kg^{-1}$)	P ($g\,kg^{-1}$)	K ($g\,kg^{-1}$)	Ca ($g\,kg^{-1}$)	Fe ($mg\,kg^{-1}$)	Mn ($mg\,kg^{-1}$)	Cu ($mg\,kg^{-1}$)	Zn ($mg\,kg^{-1}$)	pH	Polyphenols ($g\,kg^{-1}$)	Germination index (%)
OW	483	118	74.0	38	12.8	1.47	21.0	15.3	1962	54.0	26.7	20.0	6.6	19.0	0
VOW	329	38.0	7.91	16	19.9	2.98	11.6	28.5	4325	112	45.7	136	8.4	3.20	89

TOC: Total Organic Carbon; HA: Humic acids; WSC: Water soluble carbon.

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