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Short communication

Enrichment of bacterial strains for the biodegradation of diclofenac and carbamazepine from activated sludge

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ABSTRACT

Carbamazepine and diclofenac have been pointed out as important markers for environmental pollution by pharmaceuticals. This study reports on the isolation of bacterial strains capable to degrade these micropollutants from activated sludge of a municipal wastewater treatment plant (WWTP). After selective enrichments, one strain able to degrade diclofenac and two strains able to degrade carbamazepine were isolated. The strains were identified by 16S rRNA gene sequencing. Strain *Brevibacterium* sp. D4 was able to biodegrade 35% of 10 mg L^{-1} of diclofenac as a sole carbon source; periodic feeding with acetate as a supplementary carbon source resulted in enhancing biodegradation to levels up to 90%, with a concomitant increase of the biodegradation rate. Strains *Starkeya* sp. C11 and *Rhizobium* sp. C12 were able to biodegrade 30% of 10 mg L^{-1} of carbamazepine as a sole carbon source; supplementation with acetate did not improve the biodegradation of carbamazepine by these strains. The activated sludge harboured bacteria capable to degrade the two top priority environmental contaminants and may be potentially useful for biotechnological applications.

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1. Introduction

The consumption of pharmaceuticals has increased worldwide and new compounds are continually being introduced in the market (Barra Caracciolo et al., 2015). Most of these compounds are not completely metabolized in the human body and their excretion, as well as excretion of their metabolites and/or conjugated compounds, is one of the main sources of pharmaceutical residues in the environment (Rivera-Utrilla et al., 2013). Other important sources are the disposal of unused or expired medicines in domestic sewage and hospital wastes (Li, 2014). A major source of contamination of natural water bodies is the effluent discharge from Wastewater Treatment Plants (WWTPs), which often are not prepared to remove these micropollutants efficiently (Onesios et al., 2009). Due to its incomplete removal in WWTPs, pharmaceuticals represent a threat to the receiving aquatic ecosystems, since most of them present high ecotoxicological risk (Pereira et al., 2015). Albeit in trace amounts, the presence of pharmaceuticals in the environment is a motif of concern due to their continuous release into aquatic systems and the simultaneous contamination with several compounds, further enhancing their chronic toxicity effect (Bouju et al., 2016). Ecotoxicity effects of pharmaceuticals have been point out by several studies at different trophic levels: human embryo cells (Pomati et al., 2006), birds (Oaks et al., 2004), fish (Hong et al., 2007), algae (Vannini et al., 2011), microorganisms (Andersson and Hughes, 2014) and microbial communities (Alvarino et al., 2013).

The antiepileptic drug carbamazepine (CBZ) and the nonsteroidal anti-inflammatory drug diclofenac (DCF) are frequently detected in water bodies, including rivers and groundwater (Barra Caracciolo et al., 2015; Madureira et al., 2010), and show high persistence to removal in WWTPs (Lindholm-Lehto et al., 2015; Patrolecco et al., 2014). CBZ and DCF were classified as medium to high-risk pollutants in WWTP effluents and surface waters

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(Patrolecco et al., 2014; Pereira et al., 2015). CBZ has been suggested as a molecular marker of wastewater contamination in surface water and groundwater (Tran et al., 2014). The field data indicate that these compounds are environmentally persistent and deep knowledge on their complete degradation is not available so far (Barra Caracciolo et al., 2015). Moreover, CBZ and DCF have been detected in drinking water (Simazaki et al., 2015), presenting a human health risk. In the latest revision of the Water Framework Directive (European Community, 2000; Directive, 2000/60/EC), The European Union has included DCF in the watch list of substances (European Parlliament, Coucil of the European Union, 2013. Directive, 2013/39/EU) to be monitored, pending a possible subsequent definition of Environmental Quality Standards.

Microorganisms are key players in the degradation of pollutants in the ecosystems through metabolic and/or co-metabolic pathways (Barra Caracciolo et al., 2015). Several biodegradation studies have been conducted for a wide range of compounds, and many authors have deemed that this is one of the most important processes for the removal of the majority of xenobiotics, including pharmaceuticals, in different environmental compartments (Bouju et al., 2016; Barra Caracciolo et al., 2015; Vieno and Sillanpää, 2014; Gauthier et al., 2010; Onesios et al., 2009).

CBZ is well documented for its persistence to biodegradation. Low removal rates (0–8%) were reported for biological treatment in conventional WWTP (Clara et al., 2004; Martin Ruel et al., 2012). Consequentially, CBZ is often found at high concentrations in treated sewage, reported to be between 1 and 10 μg L^{-1} in secondary effluents (Martin Ruel et al., 2012). Moreover, some metabolites of CBZ have been detected on the effluents of WWTP (Leclercq et al., 2009). Albeit being reported as non-biodegradable (Joss et al., 2006), studies have been carried out over the last years to evaluate CBZ biodegradation by several bacterial strains in the presence of readily degradable carbon sources, with no success (Gauthier et al., 2010; Dawas-Massalha et al., 2014; Suárez et al., 2005). Li et al. (2013) isolated a bacterial strain with the ability to degrade CBZ, *Pseudomonas* sp. CBZ-4, from activated sludge of a municipal WWTP. An average removal of approximately 50% occurred when the initial concentration of the compound varied between 10 and 160 mg L^{-1}; however, at lower concentrations *Pseudomonas* sp. CBZ-4 was not able to biodegrade CBZ. Recently, Popa Ungureanu et al. (2015) selected the strain *Streptomyces* MIUG 4.89 for its ability to degrade CBZ in submerged systems under aerobic conditions, achieving 30% of biotransformation of CBZ supplied at 0.2 mg L^{-1}).

Several studies have reported low removal rates of DCF in WWTP (Barra Caracciolo et al., 2015; Pereira et al., 2016; Zhu et al., 2014). In a review, Vieno and Sillanpää (2014) summarized mean concentrations of DCF found in municipal WWTPs, reporting levels between 0.11 and 2.3 μg L^{-1} at the inlet and between <0.002 and 2.5 μg L^{-1} at the outlet. Langenhoff et al. (2013) demonstrated the ability of cultures enriched from activated sludge to biodegrade DCF (50–300 mg L^{-1}), however the responsible strain(s) were not identified. In a study by Tiehm et al. (2011), batch assays inoculated with activated sludge have shown elimination of DCF supplied at 2–5 μg L^{-1} in a mixture of pharmaceuticals. A recent study on 15 Portuguese WWTP revealed a mean concentration of DCF of 27.4 ng L^{-1} in influent, 14.9 ng L^{-1} in effluent and a mean removal efficiency of 45.6% (Pereira et al., 2015).

The present work was undertaken with the objective to isolate through enrichment bacterial strains from activated sludge with the ability to degrade CBZ and DCF and to evaluate the degradation of these compounds as single carbon source and in co-metabolism.

2. Materials and methods

2.1. Selective enrichments

Activated sludge from a municipal WWTP was chosen as inoculum, since WWTPs are often exposed to pharmaceuticals compounds (Pereira et al., 2015), imposing a selective pressure on the bacterial community, potentially selecting for microorganisms with the ability to survive in the presence of these compounds. The WWTP is located in Northern Portugal (Ponte de Moreira, Maia – Portugal) encompassing primary and secondary treatments, with the latter being based on activated sludge. It operates at 17 °C, with a HRT of 5.6 h and an organic load of 3370 Kg COD d^{-1}. Selective enrichments were established by inoculating 250 mL flasks containing 75 mL of sterile minimal salts medium (MM) with 5 mL activated sludge from aerated tanks. Pharmaceutical compounds were supplied at a concentration of 24.0 mg L^{-1}. Cultures were incubated on a rotary shaker (150 rpm) at 25 °C. After an acclimation period of 6.5 months, half of the suspension was removed and replaced with fresh medium and cultures were supplied with the respective pharmaceutical compound, at 15 day intervals. Enrichment consortia were used to isolate degrading strains. Growth was monitored by measuring the optical density (OD) at 600 nm. Biodegradation was followed by HPLC. Controls without inoculum and with heat-inactivated sludge, which was obtained by autoclaving at 120 °C for 30 min (as described by Chen and Hu, 2010), were also monitored. All the experiments were carried out protected from light. The whole enrichment experiment lasted 304 days.

2.2. Isolation of degrading strains

After CBZ and DCF selective enrichments, culture samples were spread onto Nutrient Agar (NA) plates and onto MM agar plates containing 10 mg L^{-1} of each pharmaceutical compound, and incubated at 25 °C for 48 h in order to carry out the characterization of the isolates with the ability to grow in the presence of the respective pharmaceutical compound. Bacterial isolates were grouped according to species similarity, based on RAPD profiles, as previously described by Amorim et al. (2014b). Isolates displaying unique RAPD profile were re-inoculated into liquid MM containing 10 mg L^{-1} of CBZ or DCF as a sole carbon and energy source to evaluate their capacity to degrade the target compound, using as initial biomass concentration an OD_{600} of ca. 0.05.

2.3. Identification of the CBZ and DCF degrading strains

Isolates with the ability to biodegrade CBZ and DCF were subsequently identified by 16S rRNA gene sequencing analysis. Genomic DNA extraction and further amplification by polymerase chain reaction (PCR) was performed as described elsewhere (Amorim et al., 2014a). Sequencing was performed at Macrogen Inc. (Seoul, Republic of Korea) using universal bacterial 16S rRNA primers (f27, f518, r800 and r1492). To determine the phylogenetic affiliation, similarity searches were done using the EzTaxon-e program (http://eztaxon-e.ezbiocloud.net). Phylogenetic analysis was performed to accurately determine the taxonomic position of the strains. For that, 16S rRNA gene sequences were aligned with reference sequences available in the GenBank/EMBL/DDBJ database. The phylogenetic tree was constructed with the MEGA software (version 5.1) using the neighbour-joining method (Kimura two-parameter distance optimized criteria). The partial 16S rRNA gene sequences were submitted to the GenBank database.

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