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Bacterial degradation of naproxen – Undisclosed pollutant in the environment

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ABSTRACT

The presence of non-steroidal anti-inflammatory drugs (NSAIDs) in the environment is an emerging problem due to their potential influence on human health and biocenosis. This is the first report on the biotransformation of naproxen, a polycyclic NSAID, by a bacterial strain. *Stenotrophomonas maltophilia* KB2 transformed naproxen within 35 days with about 28% degradation efficiency. Under cometabolic conditions with glucose or phenol as a carbon source degradation efficiency was 78% and 40%, respectively. Moreover, in the presence of naproxen phenol monooxygenase, naphthalene dioxygenase, hydroxyquinol 1,2-dioxygenase and gentisate 1,2-dioxygenase were induced. This suggests that degradation of naproxen occurs by its hydroxylation to 5,7,8-trihydroxynaproxen, an intermediate that can be cleaved by hydroxyquinol 1,2-dioxygenase. The cleavage product is probably further oxidatively cleaved by gentisate 1,2-dioxygenase. The obtained results provide the basis for the use of cometabolic systems in the bioremediation of polycyclic NSAID-contaminated environments.

1. Introduction

The introduction of non-steroidal anti-inflammatory drugs (NSAIDs), also known as pain killers, into natural matrices such as soil, sediments, groundwater and even drinking water is an emerging problem due to their potential influence on human health and natural environments (Almeida et al., 2013). These pollutants enter the environment as a result of pharmaceutical industry activity, and the improper disposal of unused or expired drugs, wastes generated in hospitals and stock-raising farms (Wu et al., 2012). In many countries large amounts of non-steroidal anti-inflammatory drugs are consumed annually. For example, in Germany about 836 tonnes of acetylsalicylic acid, 622 tonnes of paracetamol, 345 tonnes of ibuprofen and 86 tonnes of diclofenac were consumed in 2001 (Nikolaou et al., 2007). In 2000, 35 tonnes of naproxen were consumed in England (Nikolaou et al., 2007), while in Poland the consumption of 58 tonnes of ibuprofen, the other important drug in this group, was recorded (Sosnowska et al., 2009). Picquet (2013) announces that Albemarle Company produces about 500 tonnes of naproxen per year.

Most of non-steroidal anti-inflammatory drugs are not fully degraded after application, and get into the environment in an unchanged or slightly modified form. NSAIDs have been detected in different environments at concentrations ranging from nanograms to micrograms per liter (Lloret et al., 2010; Wu et al., 2012). Due to their stability they are not totally eliminated by the sewage treatment plants, and can be unintentionally consumed by humans in tap water (Gentili, 2007).

Physicochemical processes are frequently used for the removal of naproxen. However, these processes require harsh reaction conditions, generate free radicals and/or secondary pollutants, and incur high operational costs (Zhang et al., 2013). Therefore, bioremediation processes are an attractive alternative to these methods. Bioremediation strategies are cost effective and enable the mineralization of NSAIDs into innocuous products (Ahmed et al., 2001). Quintana et al. (2005) tested the ability of activated sludge to degrade naproxen and observed its 49% removal with the simultaneous appearance of desmethylnaproxen. Carballa et al. (2004) showed a 40–55% removal of naproxen during biological treatment.

Although the activated sludge process is used to treat wastewaters containing non-steroidal anti-inflammatory drugs, it is not sufficient for the complete removal of these compounds and thus they are still detected in the effluents (Rodarte-Morales et al., 2011). Therefore, there is a need to isolate microorganisms with a high capacity for non-steroidal anti-inflammatory

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drug degradation, to study their metabolic pathways and enzymes, and to characterize optimal conditions for NSAIDs degradation.

Until now, only a few microorganisms, mainly fungi (*Penicillium* sp., *Trametes versicolor*, *Cunninghamella elegans*, *C. echinulata*, *C. blakesleeana*, *Beauveria bassiana*, *Phanerochaete chrysosporium*, *Ph. sordida*, *Actinoplanes* sp., *Bjerkandera* sp. R1, *Bj. adusta*, *Irpex lacteus*, *Ganoderma lucidum*) have been identified to transform or degrade non-steroidal anti-inflammatory drugs (Lloret et al., 2010; Marco-Urrea et al., 2010b; Rodarte-Morales et al., 2011, 2012). In the transformation of naproxen to 2-(6-hydroxynaphthalen-2-yl)propionic acid and 1-(6-methoxynaphthalen-2-yl)ethanone by fungus *T. versicolor*, cytochrome P-450 and laccase were probably engaged (Marco-Urrea et al., 2010a; Rodriguez-Rodriguez et al., 2010). Degradation of naproxen by commercial laccase isolated from *Myceliophthora thermophila* was also shown by Lloret et al. (2010). They observed 100% degradation of this pharmaceutical in the presence of the redox mediator (Lloret et al., 2010).

Much less is known about the degradation of naproxen by bacteria. Until now, only a few bacterial strains, mainly from genera *Pseudomonas*, *Sphingomonas*, *Patulibacter*, *Nocardia*, *Rhodococcus* and *Stenotrophomonas*, able to degrade non-steroidal anti-inflammatory drugs, have been isolated (Ahmed et al., 2001; Almeida et al., 2013; Chen and Rosazza, 1994; Gusseme et al., 2011; Ivshina et al., 2006; Murdoch and Hay, 2005; Wu et al., 2012; Zhang et al., 2013). According to our best knowledge the biodegradation of naproxen by pure bacterial strains has not been described. Therefore, the main aim of our study was to investigate the treatability of naproxen by the aromatic compound degrader *Stenotrophomonas maltophilia* KB2. It was also important to determine the enzymes engaged in naproxen degradation.

2. Material and methods

2.1. Bacterial strain and growth conditions

S. maltophilia KB2 (VTT E-113197) was routinely cultivated in BBL nutrient broth at 30 °C and 130 rpm for 24 h. Then 6 mg L^{-1} naproxen was added to the culture. After 48 h cells were harvested by centrifugation (5000×*g* at 4 °C for 15 min), washed with fresh sterile medium and used as inoculum.

Degradation of naproxen in a monosubstrate, as well as cometabolic systems, was performed in 500 mL Erlenmeyer flasks containing 250 mL of the mineral salts medium (Greń et al., 2010) inoculated with cells to a final optical density of about 1.5 and 0.1 at $\lambda = 600$ nm (OD_{600}) for the monosubstrate and cometabolic systems, respectively. For degradation experiments two control cultures were prepared. The uninoculated control (I) contained 250 mL of sterile mineral salts medium, while the heat-killed control (II) consisted of 250 mL of autoclaved culture prepared under conditions identical to those of the experimental cultures.

Naproxen was added to each flask to obtain a final concentration of 6 mg L^{-1}, and all cultures were incubated with shaking at 30 °C for 35 days.

For studies on the cometabolic transformation of naproxen, as well as the induction of enzymes, 1 mg L^{-1} glucose or 3 mM phenol were added respectively. Cultures in 250 mL of sterile mineral salt medium supplemented with appropriate growth substrates and 6 mg L^{-1} naproxen were inoculated with cells to a final optical density of about 0.1 at $\lambda = 600$ nm (OD_{600}), and incubated at 30 °C with shaking at 130 rpm. If the complete degradation of the suitable growth substrate was observed, a successive dose of phenol or glucose was introduced and the culture was left for incubation until it reached $OD_{600} = 1.0$. All cultures were grown in triplicates.

2.2. Determination of substrate concentration

To study the degradation of naproxen, 1 mL samples were taken periodically (every 7 days) from the culture medium and centrifuged (6000×*g*, 15 min). The concentration of aromatic substrate in the culture supernatant was determined by HPLC (Merck HITACHI) equipped with a LiChromospher® RP-18 column (4 × 250 mm), liChroCART® 250-4 Nucleosil 5 C18 and a DAD detector (Merck HITACHI). The mobile phase was acetonitrile and 1% acetic acid (50:50 *v*/*v*) at a flow rate of 1 mL min^{-1}. The detection wavelength was set at 260 nm. Naproxen in the supernatant was identified and quantified by comparing the HPLC retention times and UV–visible spectra with those of the external standards.

Phenol concentration in the culture supernatant was determined using a colorimetric method with *p*-nitroaniline (Lurie and Rybnikova, 1974). The concentration of glucose in the culture supernatant was determined with a colorimetric method using 3,5-dinitrosalicylic acid (Miller, 1959).

2.3. Preparation of cell extracts

After 35 days in culture, cells of *S. maltophilia* strain KB2 were harvested by centrifugation (4500×*g* for 15 min at 4 °C) and the pellet was washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cell-free extracts were prepared by sonication of the whole cell suspension (6 times for 15 s) and centrifugation at 9000×*g* for 30 min at 4 °C. Clear supernatant was used as a crude cell extract for enzyme assays.

2.4. Enzyme assays

Monooxygenase activity was determined spectrophotometrically by measuring NADH oxidation ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) (Divari et al., 2003). In order to determine the activity of dioxygenase-catalyzed dihydroxylation, the formation of *cis*,*cis*-dihydrodiol was measured at 262 nm ($\varepsilon_{262} = 8230\ M^{-1}\ cm^{-1}$) (Cidaria et al., 1994). The activity of catechol 1,2-dioxygenase was measured spectrophotometrically by the formation of *cis*,*cis*-muconic acid at 260 nm ($\varepsilon_{260} = 16{,}800\ M^{-1}\ cm^{-1}$). In order to determine catechol 2,3-dioxygenase activity, the formation of 2-hydroxymuconic semialdehyde was measured at 375 nm ($\varepsilon_{375} = 36{,}000\ M^{-1}\ cm^{-1}$) (Wojcieszyńska et al., 2011). The activity of protocatechuate 3,4-dioxygenase was assayed by measuring the oxygen consumption (Hou et al., 1976). The activity of protocatechuate 4,5-dioxygenase was measured spectrophotometrically by the formation of 2-hydroxy-4-carboxymuconic semialdehyde at 410 nm ($\varepsilon_{410} = 9700\ M^{-1}\ cm^{-1}$) (Wojcieszyńska et al., 2011). In order to determine gentisate 1,2-dioxygenase activity, the formation of maleylpyruvate was measured at 330 nm ($\varepsilon_{330} = 10{,}800\ M^{-1}\ cm^{-1}$) (Feng et al., 1999). The activity of hydroxyquinol 1,2-dioxygenase was measured spectrophotometrically by the formation of maleylacetate at 243 nm ($\varepsilon_{243} = 44{,}520\ M^{-1}\ cm^{-1}$) (Wei et al., 2010).

One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute. Protein concentration in the crude extract was determined by the Bradford method using bovine serum albumin as a standard (Wojcieszyńska et al., 2011).

2.5. Statistical analysis

All experiments were performed in three replicates. The obtained data were analyzed by one-way ANOVA using STATISTICA 10.0 PL software package.