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Quantitative proteomic analyses of the microbial degradation of estrone under various background nitrogen and carbon conditions

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ABSTRACT

Microbial degradation of estrogenic compounds can be affected by the nitrogen source and background carbon in the environment. However, the underlying mechanisms are not well understood. The objective of this study was to elucidate the molecular mechanisms of estrone (E1) biodegradation at the protein level under various background nitrogen (nitrate or ammonium) and carbon conditions (no background carbon, acetic acid, or humic acid as background carbon) by a newly isolated bacterial strain. The E1 degrading bacterial strain, *Hydrogenophaga atypica* ZD1, was isolated from river sediments and its proteome was characterized under various experimental conditions using quantitative proteomics. Results show that the E1 degradation rate was faster when ammonium was used as the nitrogen source than with nitrate. The degradation rate was also faster when either acetic acid or humic acid was present in the background. Proteomics analyses suggested that the E1 biodegradation products enter the tyrosine metabolism pathway. Compared to nitrate, ammonium likely promoted E1 degradation by increasing the activities of the branched-chain-amino-acid aminotransferase (IlvE) and enzymes involved in the glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway. The increased E1 degradation rate with acetic acid or humic acid in the background can also be attributed to the up-regulation of IlvE. Results from this study can help predict and explain E1 biodegradation kinetics under various environmental conditions.

1. Introduction

Natural and synthetic estrogens have been frequently detected in surface water, groundwater, and domestic and agricultural wastewater (Arnon et al., 2008; Swartz et al., 2006; Vajda et al., 2008). The occurrence of these steroid hormones can pose risks to the health of human beings and aquatic ecosystems at the ng/L level (Imai et al., 2005). Due to the broad occurrence and potential health effect of estrogens, US EPA added estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2) to its Contaminant Candidate List 3 (EPA, 2009). One of the major sources of estrogens in the aquatic ecosystem is wastewater effluent as biological wastewater treatment process cannot effectively remove these compounds (Gomes et al., 2003; Leech et al., 2009; Liu et al., 2009), E1 in particular (Silva et al., 2012).

Nitrogen source (Bagnall et al., 2012; Gaulke et al., 2008, 2009; Khunjar et al., 2011; Ren et al., 2007; Shi et al., 2004; Vader et al., 2000) and background carbon (Joss et al., 2004; Lee et al., 2011; Li et al., 2008; Racz et al., 2012; Stumpe and Marschner, 2009; Tan et al., 2013, 2015) in the environment may influence the estrogen biodegradation by mixed microbial communities. Some studies reported that ammonia oxidizing bacteria (AOB) were responsible for degrading estrogens in activated sludge, where the ammonia monooxygenase (AMO) produced to oxidize ammonia can co-metabolize estrogens, and hence suggested ammonium as nitrogen source could benefit estrogen biodegradation (Gaulke et al., 2009; Khunjar et al., 2011; Ren et al., 2007; Shi et al., 2004; Vader et al., 2000). In contrast, another study found that *Nitrosomonas europaea* and *Nitrosospira multiformis*, two AOB species, could not degrade 17α-ethinylestradiol and suggested that heterotrophic bacteria were responsible for estrogen degradation in wastewater (Gaulke et al., 2008). Bagnall et al. also reported that changing the nitrogen source from ammonium to nitrate reduced the relative abundance of AOB in activated sludge without affecting estrogen degradation, suggesting that heterotrophic bacteria were

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involved in estrogen degradation (Bagnall et al., 2012).

Estrogen degradation becomes more complicated when background carbon is also considered. Some studies documented that high organic loading rates inhibited estrogen biodegradation in activated sludge (Joss et al., 2004; Ren et al., 2007), and attributed the phenomenon to the general mechanism of substrate inhibition (Joss et al., 2004): bacteria preferentially utilize easily biodegradable carbon substrates before using recalcitrant substrates like estrogens. However, Tan et al. reported that the addition of a mixture of carbon substrates, including easily biodegradable organic carbons, increased E1 biodegradation rates due to biomass growth (Tan et al., 2013). Some studies also looked into specific, easily biodegradable organic carbons. When glucose was present, it could result in higher E2 and EE2 degradation in soil due to co-metabolism (Stumpe and Marschner, 2009), while it could also slow down E1 and E2 biodegradation in activated sludge reactors (Li et al., 2008). Other studies reported that microbially derived organics in the background could benefit E1 biodegradation (Tan et al., 2015), while humic acid would lower E1, E2, and EE2 biodegradation (Lee et al., 2011). These studies suggest that the quality of background organic carbon substrates could substantially affect E1 biodegradation in the environment.

Despite the documented observations, little is known about the underlying mechanisms on how different nitrogen sources and carbon substrates affect microbial degradation of estrogen compounds. The objective of this study was to elucidate the molecular mechanism of E1 biodegradation at the protein level under various background nitrogen and carbon conditions by a newly isolated bacterial strain. In this work, an E1 degrading bacterial strain was isolated from river sediment. E1 degradation experiments were conducted in batch reactors amended with different nitrogen sources (nitrate or ammonium) or with different carbon substrates (none, acetic acid, or humic acid). Quantitative proteomics was performed to investigate the proteomes of the E1 degrading bacterial strain under different experimental conditions. Outcomes from this study are expected to advance our understanding of microbial E1 degradation in diverse environments.

2. Materials and methods

2.1. Isolating estrogen degrading bacteria

Sediment samples were collected in the Plum Creek downstream from the discharge point of the City of Seward Water Treatment Facility (Seward, Nebraska, USA) and were used to isolate E1 degrading bacteria following a published protocol (Yu et al., 2007). Briefly, 1 g of sediment was suspended in 30 mL of nitrate mineral salts (NMS: 11.76 mM $NaNO_3$, 0.98 mM K_2SO_4, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.07 mM $CaSO_4 \cdot 2H_2O$, 0.08 mM $FeSO_4 \cdot 7H_2O$, 3.9 mM KH_2PO_4, 6.1 mM Na_2HPO_4, 0.1 mM H_2SO_4, 0.001 mM KI, 0.002 mM $ZnSO_4 \cdot 7H_2O$, 0.002 mM $MnSO_4 \cdot H_2O$, 0.002 mM H_3BO_3, 0.004 mM $CoMoO_4 \cdot H_2O$) media (Chu and Alvarez-Cohen, 1996) amended with 3 mg/L of E1 (i.e., 2.4 mg/L as C). An E1 stock solution was prepared by adding 10 mg E1 in 10 mL acetone. After the stock solution was added to the NMS solution and before the sediment sample was added, acetone was removed by heating and purging (Yu et al., 2007). The sediment slurry was then transferred to a 1 L amber bottle with a stir bar, and fresh NMS media containing 3 mg/L of E1 were added to make the total volume of 200 mL. The reactor was aerated with 0.2 μm filtered air at a flow rate of 100 mL/min, stirred at 200 rpm and operated at room temperature. Every 4 days, 50 mL well-mixed reactor content was replaced with fresh NMS media containing 3 mg/L of E1. After 12 days incubation, E1 degrading bacteria were isolated using Reasoner's 2A (R2A) agar plates (van der Linde et al., 1999) amended with 3 mg/L of E1 and incubated at 30°C. One isolate, strain ZD1, was selected for further analysis because it degraded E1 faster than the other isolates. DNA of strain ZD1 was extracted using FastDNA® SPIN Kit for soil (MP Biomedicals, USA), and the 16S rRNA gene was amplified using PCR primers 27F and 1492R (Xia et al., 2010) on a Mastercycler ep realplex thermocycler (Eppendorf International, Germany). The PCR product was sent to the Eurofins MWG Operon (Huntsville, AL) for sequencing, and the sequencing result showed that strain ZD1 belonged to *Hydrogenophaga atypica*.

2.2. E1 degradation experiment

Strain ZD1 was grown in R2A media, without E1, to the late exponential phase. After being washed twice in phosphate buffered saline (PBS, pH = 8.0), the cells were added to a series of 250-mL Erlenmeyer flasks covered with aluminum foils at a final concentration of 10^8 CFU/mL. Four types of media were used: NMS, ammonia mineral salts (AMS, which differs from NMS by replacing 11.76 mM $NaNO_3$ with 5.88 mM $(NH_4)_2SO_4$ on an equal N basis (Chu and Alvarez-Cohen, 1996)), NMS with 6 mL acetic acid (AA, 2.4 mg/L as C), and NMS with 4.36 mg/L humic acid (HA, 2.4 mg/L as C). The elemental composition of C in humic acid was estimated at around 55% (Rice and MacCarthy, 1991). All four media contained 3 mg/L of E1 in a volume of 100 mL. Effects of nitrogen source (NMS vs. AMS) and background substrate (NMS, NMS + AA, and NMS + HA) on microbial E1 degradation were investigated. No-cell controls and autoclaved-cell controls were also included. The degradation experiments were conducted on a shaking table with 150 rpm at 30°C for 7 days. 5 mL liquid samples were collected at 0, 24, 72, 96, 120, 144, and 168 h for E1 concentration measurement using gas chromatography–mass spectrometry (GC-MS). The degradation experiment under each condition was conducted in triplicates at three separate times. The resulting reaction rate constants from different experimental conditions were compared using two-sample t-tests with three replicates (n = 3) for each condition.

2.3. GC-MS for E1 quantification

E1 was quantified using GC-MS combined with solid phase extraction (SPE) and derivatization. Briefly, 500 μL of reaction solution and 100 μL of 10 ng/μL androsterone (surrogate) were passed through the SPE cartridges (C-18, Water, USA). After that, the absorbed E1 was eluted from cartridges using 3 mL ethyl acetate. The eluted samples were blown down to ~0.5 mL using nitrogen gas, and mixed with 100 μL of 10 ng/μL C^{13}-17β-estradiol (internal standard). The mixtures were then blown dry using nitrogen gas, reconsitituted and derivatized using 100 μL dimethylformamide and 100 μL BSTFA +1% TMCS at 75 °C for 30 min. After cooling to room temperature, the derivatized samples were transferred to autosampler vial inserts and capped using crimping tool, and then analyzed on GC-MS.

2.4. Protein extraction and digestion

Biomass samples were collected from triplicate experiments. 100 mL reaction solution was collected at 0 h of the degradation experiment, and then at 96 h for each treatment condition. After two washes using PBS (pH = 8.0), bacteria cells were re-suspended with ~600 μL autoclaved Zirconia beads (100 μm diameter) in 600 μL lysis buffer (i.e., 25 mM pH 8.0 Tris buffer containing 8M urea and 1 mM phenylmethysulfonyl fluoride). The cell samples were vortexed three times for 2-min intervals with 30 s on ice in between. After the third vortexing, cell samples were placed in ice for 2 min to allow the beads to settle. The supernatants of the crude