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Estrogen mimics induce genes encoding chemical efflux proteins in gram-negative bacteria

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HIGHLIGHTS

- Bacteria were exposed to 17α-ethynylestradiol, nonylphenol, and bisphenol-A.
- The inner membrane proteins of AcrAB-TolC and YhiUV were overexpressed in *E. coli*.
- Nonylphenol and bisphenol-A induced mexK and mexF in *Pseudomonas aeruginosa*.
- Chemical efflux may contribute to estrogen attenuation during wastewater treatment.

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ABSTRACT

Escherichia coli and *Pseudomonas aeruginosa* are gram-negative bacteria found in wastewater and biosolids. Spanning the inner and outer membrane are resistance-nodulation-cell division superfamily (RND) efflux pumps responsible for detoxification of the cell, typically in response to antibiotics and other toxicity inducing substrates. Here, we show that estrogenic endocrine disruptors, common wastewater pollutants, induce genes encoding chemical efflux proteins. Bacteria were exposed to environmental concentrations of the synthetic estrogen 17α-ethynylestradiol, the surfactant nonylphenol, and the plasticizer bisphenol-A, and analyzed for RND gene expression via q-PCR. Results showed that the genes *acrB* and *yhiV* were overexpressed in response to the three chemicals in *E. coli*, and support previous findings that these two transporters export hormones. *P. aeruginosa* contains 12 RND efflux pumps, which were differentially expressed in response to the three chemicals: 17α-ethynylestradiol, bisphenol-A, and nonylphenol up-regulated *mexD* and *mexF*, while nonylphenol and bisphenol-A positively affected transcription of *mexK*, *mexW*, and *triC*. Gene expression via q-PCR of RND genes may be used to predict the interaction of estrogen mimics with RND genes. One bacterial response to estrogen mimic exposure is to induce gene expression of chemical efflux proteins, which leads to the expulsion of the contaminant from the cell.

1. Introduction

Attenuation of estrogenic endocrine disruptors such as 17α-ethynylestradiol, nonylphenol, and bisphenol-A during wastewater treatment is problematic as chemicals are released in effluent at low but biologically active levels to surface water (Soares et al., 2008; Miege et al., 2009; Hannah et al., 2009; Deblonde et al., 2011; Staples et al., 1998). Several factors contribute to lack of biodegradation, including poor substrate specificity of enzymes and sorption onto biomass, among others. Here, a novel biochemical pathway is explored and may provide insight into estrogen persistence during biological treatment: bacterial chemical efflux. Elkins and Mullis (2006) previously reported that the estrogens 17β-estradiol and estrone, and the androgen testosterone are substrates of chemical efflux proteins typically responsible for cellular detoxification in gram-negative bacteria, specifically the AcrAB-TolC system in *E. coli*. As estrogen mimics are structurally similar to the human estrogens, they will likely interact with bacterial efflux proteins.

When toxins accumulate within a cell, response mechanisms are activated that result in production of chemical efflux proteins. These membrane-bound proteins facilitate export of toxic chemicals from the cytoplasm or periplasm of a bacterium. They are classified as ATP driven pumps, proton/sodium ion anti-porters, or multi-unit proton anti-porters, and include the ATP-binding cassette (ABC) efflux, the multi-drug and toxic compound extrusion (MATE), small multi-drug resistance (SMR), major facilitator superfamily (MFS), and resistance-nodulation-division (RND) protein

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families. As drugs are exported from the bacterium, a cation (H^+/Na^+) is taken up, or ATP is hydrolyzed (Piddock, 2006).

Multi-component RND pumps are present only in gram negative bacteria as they span the inner and outer membranes, while other efflux proteins are present in both gram-negative and gram-positive bacteria (Nikaido, 1996; Lomovskaya et al., 2006; Nikaido and Takatsuka, 2009). RND efflux pumps consist of an inner membrane protein, a membrane fusion protein, and an outer membrane channel. In *E. coli* for instance, AcrB (inner membrane protein), AcrA (membrane fusion protein), and TolC (outer membrane channel) form the complex AcrAB-TolC (Ma et al., 1993; Tikhonova et al., 2002; Tikhonova and Zgurskaya, 2004). During export, an antibiotic (or other) compound enters the protein complex and is pumped out of the bacterium. Similar RND proteins are found in the *Pseudomonas*, *Salmonella*, and *Cupriavidus* genera (Nies, 1995; Grass et al., 2000; Pontel et al., 2007; Poole, 2008).

The substrate range of RND proteins varies. RND complexes in *E. coli* (e.g. AcrAB-TolC, AcrEF-TolC) export dyes, detergents, antibiotics, bile salts, human hormones, metals, and solvents (Elkins and Nikaido, 2002; Elkins and Mullis, 2006; Pos, 2009). Substrates of pumps found in *Pseudomonas aeruginosa* (MexAB-OprM for example) are limited to antibiotics, and include macrolides, fluoroquinolones, polyketides, and β-lactams (Chuanchuen et al., 2002; Mao et al., 2002; Jeannot et al., 2005; Poole, 2008). Other *Pseudomonas* strains are resistant to solvents and polynuclear aromatic hydrocarbons (e.g. TtgGHI in *Pseudomonas putida*) (Kieboom and de Bont, 2001; Hearn et al., 2003; Hearn et al., 2006; Terán et al., 2007). Bacteria that export metals include *Salmonella enterica* (GesABC and SilABC), and *Cupriavidus metallidurans*, in addition to *E. coli* (CusCFBA), *P. putida* (CzcCBA), and *P. aeruginosa* (CzrABC) (Nies, 1995; Gupta et al., 1999; Hassan et al., 1999; Grass et al., 2000; Pontel et al., 2007; Leedjärv et al., 2008; Conroy et al., 2010). While the substrate range of certain metal-specific RND-proteins is quite specific, multi-drug efflux proteins export a broad range of chemicals (Conroy et al., 2010).

To test if environmental estrogens interact with RND-type efflux proteins, we determined if 17α-ethynylestradiol (EE2), bisphenol-A (BPA), and nonylphenol (NP) exposure induces genes encoding chemical efflux pumps found in gram-negative wastewater bacteria. *E. coli* and *P. aeruginosa* were exposed to environmentally relevant concentrations of 17α-ethynylestradiol, nonylphenol, and bisphenol-A, all estrogen mimics that are found in wastewater. Gene expression levels were evaluated by q-PCR.

2. Materials and methods

2.1. Chemicals

Endocrine disrupting chemicals (17α-ethynylestradiol, bisphenol-A, and nonylphenol) were obtained from Sigma–Aldrich. Stock solutions (100 mg L^{-1}) were prepared in ethanol and diluted in sterile Milli-Q water.

2.2. Strains and culture

Wild-type *E. coli* W3110 (Bachmann, 1972) and *P. aeruginosa* PAO1 (Poole et al., 1996) were used in gene expression experiments. Bacteria were individually pre-cultured in 5 mL of LB broth (Sigma) at 37 °C for 5–6 h until log growth. The culture was used as seed for gene expression experiments.

2.3. Gene expression

2.3.1. Total RNA isolation and reverse transcription

A 5-mL test culture was seeded with 100 μl of pre-culture and estrogen mimics (final concentrations: 10 ng L^{-1} of 17α-ethynylestradiol, 10 μg L^{-1} of nonylphenol, and 1 μg L^{-1} of bisphenol-A) and growth until log growth. RNA was extracted with Qiagen RNeasy Protect Bacteria Mini Kit (Cat. 74524). Total RNA was isolated by transferring a 0.3 ml aliquot of the culture, corresponding to 2×10^8 cells, to 1 ml of RNAprotect Bacteria Reagent. After removal of genomic DNA by RNAse-free DNAase, RNA was precipitated, resuspended in 100 μl of RNAse-free H_2O, dispensed into 20 μl aliquots and store at −20 °C.

Complementary DNA (cDNA) was synthesized according to Qiagen instructions (QuantiTect Reverse Transcription kit, Cat. 205311). Before cDNA synthesis, a reaction consisting of 2 μg of RNA and DNA elimination buffer was incubated at 42 °C for 2 min to remove residual DNA. Reverse transcription was performed with 1.5 μg of DNA elimination mixture and incubated at 42 °C for 30 min by using random hexamer primers with reverse transcriptase. The remaining 0.5 μg RNA was used as the non-reverse-transcription control, which was the negative control sample with no reverse transcription. In the non-reverse-transcription control sample, RNA was treated same as reverse transcription reaction except RNase-free water was used instead of reverse transcriptase enzyme.

2.3.2. RT-PCR

The primers used for real-time PCR were verified and optimized by PCR and RT-PCR reactions (Table 1). The reaction was performed on 384-well plates with 20 ng of cDNA, with 250 nM of primer, and 2.5 μl of 2X QuantiFast SYBR Green PCR master mix (Qiagen Cat. 204054) in a total volume of 5 μl. PCR was accomplished after a 5 min activation at 95 °C, followed by 50 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 70 °C or 68 °C for 30 s (QuantStudio™ 12K Flex Real-Time PCR System from Life Technologies). Each sample was tested in triplicate.

2.3.3. RT-PCR analysis

Gene expression data are presented as fold-difference relative to the bacterial house-keeping gene and control reactors using collected Ct values (Hellemans et al., 2007; Willems et al., 2008). Values were determined according to the following calculations:

1. Ct (threshold cycle), which is the cycle number of the fluorescent signal baseline, was collected for each PCR reaction.
2. Ct values were normalized by calculating the difference (ΔCt) between target gene and endogenous control (housekeeping gene):

$$\Delta Ct_{\text{target gene}} = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$$

3. Gene expression was compared to the control sample, which was bacteria gene expression in response to no chemical (ΔΔCt):

$$\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{control sample}}$$

4. Since PCR amplification doubles the target DNA fragment per cycle, the gene expression change (R) is:

$$R = 2^{-\Delta\Delta Ct}.$$

3. Results and discussion

3.1. Estrogenic endocrine disrupting chemicals in water

The three estrogenic endocrine disruptors were selected based on attenuation during wastewater treatment. 17α-ethynylestradiol, nonylphenol, and bisphenol-A are not completely removed during wastewater treatment and persist somewhat in the environment (Holbrook et al., 2002, 2004; Teske and Arnold, 2008).