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Organic acids enhance bioavailability of tetracycline in water to *Escherichia coli* for uptake and expression of antibiotic resistance

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ABSTRACT

Tetracyclines are a large class of antimicrobials used most extensively in livestock feeding operations. A large portion of tetracyclines administered to livestock is excreted in manure and urine which is collected in waste lagoons. Subsequent land application of these wastes introduces tetracyclines into the soil environment, where they could exert selective pressure for the development of antibiotic resistance genes in bacteria. Tetracyclines form metal-complexes in natural waters, which could reduce their bioavailability for bacterial uptake. We hypothesized that many naturally-occurring organic acids could effectively compete with tetracyclines as ligands for metal cations, hence altering the bioavailability of tetracyclines to bacteria in a manner that could enhance the selective pressure. In this study, we investigated the influence of acetic acid, succinic acid, malonic acid, oxalic acid and citric acid on tetracycline uptake from water by *Escherichia coli* bioreporter construct containing a tetracycline resistance gene which induces the emission of green fluorescence when activated. The presence of the added organic acid ligands altered tetracycline speciation in a manner that enhanced tetracycline uptake by *E. coli*. Increased bacterial uptake of tetracycline and concomitant enhanced antibiotic resistance response were quantified, and shown to be positively related to the degree of organic acid ligand complexation of metal cations in the order of citric acid > oxalic acid > malonic acid > succinic acid > acetic acid. The magnitude of the bioresponse increased with increasing aqueous organic acid concentration. Apparent positive relation between intracellular tetracycline concentration and zwitterionic tetracycline species in aqueous solution indicates that (net) neutral tetracycline is the species which most readily enters *E. coli* cells. Understanding how naturally-occurring organic acid ligands affect tetracycline speciation in solution, and how speciation influences tetracycline uptake by bacteria, allows more accurate assessment of the selective pressure from trace levels of antibiotics in the environment on microbial communities for preserving and developing antibiotic resistance.

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1. Introduction

Tetracyclines are the most commonly used antimicrobials in concentrated livestock feeding operations, and in disease control for humans and animals. In 2011, approximately 5.6 million kilograms of tetracyclines were used for livestock production and disease control in the United States, equivalent to ~42% of the total antibiotics administered to food-producing animals (Food and Drug Administration, 2011). Large fractions of tetracyclines used in animal feeding operations are excreted with livestock feces and urine either as the parent compounds or bioactive metabolites (Aga et al., 2005; Jacobsen et al., 2004). Land application of animal wastes containing relatively high levels of tetracyclines (i.e. $\mu g\ kg^{-1}$ to $mg\ kg^{-1}$) is commonplace. This practice repeatedly introduces large quantities of tetracycline antibiotics into the soils and waters of agroecosystems. Tetracyclines are frequently detected in soils (Hu et al., 2010; Jacobsen et al., 2004), surface waters (Batt and Aga, 2005; Christian et al., 2003; Kolpin et al., 2002; Luo et al., 2011; Wei et al., 2011) and even in groundwater (Chee-Sanford et al., 2001; Gottschall et al., 2012; Hu et al., 2010). Tetracyclines introduced into the environment in this fashion plausibly exert selective pressure on indigenous bacteria for the development of antibiotic resistance genes and the enrichment of antibiotic resistant bacterial populations (Chee-Sanford et al., 2001; Gao et al., 2012; Gilchrist et al., 2007; Hong et al., 2013; Looft et al., 2012; Pei et al., 2006; Rysz and Alvarez, 2004).

In order for tetracyclines to manifest selective pressure and other biological effects on bacteria, they must pass though the cytoplasmic membrane and interact with specific receptors (Chopra and Roberts, 2001). Tetracyclines contain several ionizable functional groups such as tricarbonylmethane, diketone and dimethylammonium, and form multiple species of varying net charge in aqueous solution; the fractional distribution of these species depends on solution pH, ionic strength and composition. One species versus another can plausibly be favored for bacterial uptake, and the greater bioavailability should be expected with increase in selective pressure on bacteria. The presence of inorganic divalent cations (e.g. Mg^{2+} and Ca^{2+}) in aqueous solution has been shown to reduce the bioavailability of tetracycline to *Escherichia coli* by forming metal-tetracycline complexes (Zhang, 2013). In aqueous environments tetracyclines are primarily bound to Ca^{2+} and Mg^{2+} because of relatively high natural abundance of these divalent cations in water and their ability to form stable complexes (Werner et al., 2006). Many naturally-occurring organic acids or dissolved humic substances could function as ligands and compete with tetracyclines to form complexes with the inorganic cations Ca^{2+} and Mg^{2+} (Aiken et al., 2011; Mantoura et al., 1978). Changes of tetracycline-metal cation complexation in solution could potentially influence tetracycline uptake by bacteria hence modulating the selective pressure exerted on native bacterial communities.

The objective of this study was to investigate whether, and to what extent, organic acid ligands could modulate the impacts of the divalent metal cations Ca^{2+} and Mg^{2+} on tetracycline bioavailability to *E. coli* in aqueous solution. We hypothesize that naturally-occurring small organic acids could compete with tetracycline as ligands for metal cations to form complexes in aqueous solution thereby modulating the tetracycline speciation and hence bioavailability to *E. coli*. To test this hypothesis, we selected several organic acid ligands commonly found in the aqueous environment which display varying binding affinities for Ca^{2+} and Mg^{2+}. It is expected *a priori* that the presence of these organic acid ligands in water will alter the formation of complexes between tetracycline and Ca^{2+} or Mg^{2+}, and in doing so change the degree of tetracycline uptake by bacteria and antibiotic resistance responses evoked in *E. coli* bioreporter. The bioreporter used in this study was an *E. coli* strain MC4100 containing the plasmid pTGM with transcriptional fusion between tetracycline inducible promoter and fluorescence-assisted cell sorting optimized *gfp* gene (Hansen and Sørensen, 2000). Tetracycline uptake by the bioreporter (referred as intracellular tetracycline concentration) was quantified directly using high-performance liquid chromatography integrated with tandem mass spectrometry (LC-MS/MS), and these concentrations were correlated to fluorescence emission caused by activation of the tetracycline resistance gene (i.e. promoter activity). Shifts in the fractional distribution of tetracycline species induced by the organic acid ligands were estimated using the speciation model MINEQL+ in an attempt to relate bacterial uptake of tetracycline (bioavailability) with the presence of competing organic acid ligands that can from complexes with Ca^{2+} and Mg^{2+}.

2. Materials and methods

2.1. Chemicals

Tetracycline hydrochloride (≥95%), ampicillin sodium (≥95%), methanol (HPLC grade), and MOPS buffer (pH range 6.5–7.9) were purchased from Sigma–Aldrich (St. Louis, MO). Sodium chloride, magnesium chloride, oxalic acid, citric acid, acetic acid, ethylenediaminetetraacetic acid (EDTA), formic acid, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from J.T. Baker (Philipsburg, NJ). Bacto tryptone and Bacto yeast extracts were purchased from Becton, Dickinson and Company (Sparks, MD). Malonic acid and succinic acid were purchased from MP Biomedicals (Solon, OH). Acetonitrile (HPLC grade) and hydrochloric acid (37%) were purchased from EMD Chemicals (Gibbstown, NJ).

2.2. Tetracycline exposure to E. coli

The *E. coli* strain MC4100/pTGM bacterial bioreporter was constructed by inserting *tet*(M) gene (encoding tetracycline resistance by ribosomal protection) into plasmid pTGM, which contained a transcriptional fusion between a *tetR*-regulated promoter and flow cytometry-optimized *gfp* gene (*gfp*mut3) encoding green fluorescence protein (GFP) (Bahl et al., 2005). The *E. coli* bioreporter was cultured in a low-salt LB medium in which the pH was adjusted to 7.0 using 50 mM of MOPS buffer. The *E. coli* bioreporter was inoculated in the LB media amended with 100 mg L^{-1} of ampicillin, and incubated on a horizontally-moving shaker at 150 rpm at 30.0 ± 0.2 °C. When