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Influence of tetracycline resistance on the transport of manure-derived *Escherichia coli* in saturated porous media

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ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form
9 December 2010
Accepted 10 December 2010
Available online 21 December 2010

Keywords:
Manure
Antibiotic resistant bacteria
Escherichia coli
Bacteria transport
Groundwater contamination

ABSTRACT

In this research, tetracycline resistant (tet^R) and tetracycline susceptible (tet^S) *Escherichia coli* isolates were retrieved from dairy manure and the influence of tetracycline resistance on the transport of *E. coli* in saturated porous media was investigated through laboratory column transport experiments. Experimental results showed that tet^R *E. coli* strains had higher mobility than the tet^S strains in saturated porous media. Measurements of cell surface properties suggested that tet^R *E. coli* strains exhibited lower zeta potentials than the tet^S strains. Because the surface of clean quartz sands is negatively charged, the repulsive electrostatic double layer (EDL) interaction between the tet^R cells and the surface of sands was stronger and thus facilitated the transport of the tet^R cells. Although no difference was observed in surface acidity, cell size, lipopolysaccharides (LPS) sugar content and cell-bound protein levels between the tet^R and tet^S strains, they displayed distinct outer membrane protein (OMP) profiles. It was likely that the difference in OMPs, some potentially related to drug efflux pumps, between the tet^R and tet^S strains led to alteration in cell surface properties which in turn affected cell transport in saturated porous media. Findings from this research suggested that manure-derived tet^R *E. coli* could spread more widely in the groundwater system and pose serious public health risks.

1. Introduction

The occurrence and spread of antibiotic resistant bacteria in the environment undermines our ability to prevent and control microbial infections and is becoming a growing public health challenge both within the United States and across the world. It was estimated that antibiotic resistant pathogens are responsible for more than 2 million illnesses and 14 000 deaths each year in the United States (Pruden et al., 2006; World Health Organization, 2000). The Centers for Disease Control and Prevention (CDC) reported that antibiotic resistance cost the United States more than 4.5 billion dollars in 1990 (Institute of Medicine, 1998).

For several decades, antibiotics have been commonly used in animal farms at therapeutic levels to treat diseases and at sub-therapeutic levels for growth promotion and prophylactic purposes (Institute of Medicine, 1988; Kumar et al., 2005; Mellon et al., 2001; Teuber, 2001). The widespread use of antibiotics in animal farm environments has resulted in high levels of antibiotic resistant bacteria in animal waste (Halbert et al., 2006; Hofacre et al., 2000; Parveen et al., 2006; Ray et al., 2006, 2007; Sato et al., 2004, 2005; Varela et al., 2008; Varga et al., 2008a, 2008b, 2009). Parveen et al. (2006) reported that 85%, 81%, 91% and 80% of *Escherichia coli* isolates retrieved from manures produced in swine, dairy, poultry, and beef farms respectively, were resistant to at least one antibiotic drug.

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doi:10.1016/j.watres.2010.12.014

Manure produced in animal farms is usually stored in deep pits or outdoor lagoons before being applied to agricultural fields as a source of fertilizer (Burkholder et al., 2007; Gollehon et al., 2001; Sapkota et al., 2007). Leakage from deep pits and lagoons and downward infiltration of water through manure-laden soil can lead to the pollution of groundwater by antibiotic resistant bacteria (Anderson and Sobsey, 2006; Koike et al., 2007; Mackie et al., 2006; Mckeon et al., 1995; Sapkota et al., 2007; Storteboom et al., 2007). Mckeon et al. (1995) found that 100% of the non-coliforms and 87% of the coliforms isolated from rural groundwater samples were resistant to at least one of 16 antibiotics, with resistance most commonly directed toward novobiocin, cephalothin, and ampicillin. Approximately 60% of the coliforms were resistant to multiple drugs. Sapkota et al. (2007) reported that a concentrated swine feeding operation resulted in groundwater pollution by *Enterococci* that were resistant to erythromycin, tetracycline and clindamycin. Anderson and Sobsey (2006) found that more than 80% of the *E. coli* isolates from the groundwater influenced by swine farms were resistant to tetracycline and chlortetracycline. Additionally, many of the *E. coli* isolates were also resistant to streptomycin, trimethoprim and ampicillin. Because groundwater is the primary source of drinking water, particularly in areas where animal farms are located, contamination of groundwater by antibiotic resistant bacteria poses a direct public health threat (Ellefson et al., 2002; Solley et al., 1998).

Despite the growing concerns of groundwater contamination by manure-derived antibiotic resistant bacteria, our knowledge of the transport of antibiotic resistant bacteria in the groundwater systems remains very limited. Rysz and Alvarez (2006) investigated the transport of tetracycline resistant *Burkholderia cepacia* and found that >46% of the bacterial cells were able to travel through a 15-cm column packed with sand and higher breakthrough concentrations were observed when the concentration of the bacterial cells was increased. The influence of tetracycline resistance on the transport of *B. cepacia*, however, was not specifically examined. The main goal of this research is to evaluate the impact of tetracycline resistance on the transport of manure-derived *E. coli* through column transport experiments. Such information is needed to assess the health risks associated with groundwater contamination by antibiotic resistant bacteria and to improve manure management practices that aim at mitigating this problem.

2. Materials and methods

2.1. Isolation and antimicrobial susceptibility test of E. coli

E. coli used in this research was isolated from manure collected from a family dairy farm (~50 milking cows) located in Ozaukee County, WI using standard protocols established by US EPA (2000). Briefly, the collected manure samples were suspended in sterile phosphate buffered saline (PBS) solution and filtered through sterile PVDF 0.45 μm membranes (Millipore). The membranes were flipped and placed onto modified mTEC agar plates (Becton Dickinson). The plates were incubated at 35 °C for 2 h and then 44.5 °C for 22 h. Tentative *E. coli* isolates retrieved from the mTEC agar plates were confirmed with Enterotube II (Becton Dickinson) and MacConkey II agar plates containing 4-methylumbelliferyl-D-glucuronide (MUG) (Becton Dickinson).

The isolated *E. coli* were tested for their susceptibility to 7 representative antibiotics using Mueller–Hinton agar plates amended with various antibiotics (Clinical and Laboratory Stardards Institute, 2006; Walczak and Xu, 2011). For each antibiotic reagent, two different concentrations were tested. No *E. coli* isolates were resistant to gentamicin and ciprofloxacin, while resistance to cephalothin, ampicillin, erythromycin and tetracycline was prevalent (Walczak and Xu, 2011).

E. coli isolates that differed in tetracycline resistance but had otherwise similar antibiotic susceptibility patterns were selected for the column transport study (Table 1). To minimize variations that may be caused by different growth conditions and nutrient status among different cows, the *E. coli* isolates selected for this research were all from the same cow. The tet^R strains were referred to as RES1 and RES2 and the tet^S strains were denoted by SUS1 and SUS2.

Polymerase chain reaction (PCR) assays were performed to determine a total of 20 tet^R genes (12 efflux genes and 8 ribosome protection genes) (Hu et al., 2008). The primers used for the PCR assays and the primer-dependent annealing temperatures can be found elsewhere (Aminov et al., 2002, 2001; Hu et al., 2008; Miranda et al., 2003). Plasmid DNA was isolated from each strain by alkaline lysis (Sambrook and Russell, 2001), and served as the template for the PCR reactions. The PCR mixture also contained 0.3 μM of each primer (Invitrogen), 0.4 units of Vent polymerase (New England Biolabs), 200 μM dNTPs, 3% dimethyl sulfoxide and 1 × PCR buffer. The PCR amplification was performed using a Mastercycler thermocycler (Eppendorf). The temperature program consisted of an initial denaturing step of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing for 30 s, extension at 72 °C for 30 s, and a final extension of 10 min at 72 °C. Negative control reactions were included for each set of primers. PCR products were analyzed on 1% agarose gel that was stained with ethidium bromide.

2.2. Column transport studies

Duplicate chromatography columns measuring 2.5 cm in diameter and 15 cm in length were used for the column transport experiments. The columns were packed with silica sands (US Silica) (size range: 0.707–0.841 mm) that were alternately cleaned with hot, concentrated nitric acids and diluted NaOH. The porosity of the sand was 0.356. Peristaltic pumps (Cole–Parmer) were used to maintain a constant specific discharge value of 0.31 cm/min. Packed sand columns were equilibrated with >40 pore volumes of background electrolyte solution (1–100 mM KCl) before the bacteria transport experiment.

E. coli preserved in 20% glycerol under −80 °C was streaked onto Muller-Hinton (MH) agar plates. After overnight incubation at 37 °C, cells from the freshly formed colonies were transferred to culture tubes containing 15 mL Luria-Bertani (LB) broth. The culture tubes were incubated at 37 °C for 6 h. The starter culture was used to inoculate LB broth (1:500 dilution ratio), which was then incubated at 37 °C for 18 h. The bacterial cells were harvested using centrifuge (4000× *g*, 10 min, 4 °C). To

Table 1 – Antibiotic resistance pattern of the *E. coli* isolates used for the transport experiments.

E. coli isolate	Tetracycline		Ampicillin		Cephalothin		Gentamicin		Ciprofloxacin		Erythromycin		Sulfomethoxazole	
	4[a]	16	8	32	8	32	4	16	1	4	3	15	38	152
RES1	+	+	+	+	+	+	–	–	–	–	+	+	–	–
RES2	+	+	+	+	+	+	–	–	–	–	+	+	–	–
SUS1	–	–	+	–	+	+	–	–	–	–	+	+	–	–
SUS2	–	–	–	–	+	–	–	–	–	–	+	+	–	–

a Unit is mg/L.

remove the growth medium, the bacterial pellet was rinsed 4 times with the appropriate electrolyte solution. The concentration of cells was then adjusted to ~4 × 10^7 cell/ml for the column transport experiments. The pH of the background electrolyte solutions and the cell suspension was around 5.7. The effluents of the columns were connected to flow-through quartz cuvettes (NSG Precision) and the cell concentration was determined at a wavelength of 220 nm at 30-s intervals using a spectrophotometer (Shimadzu UV-1700). After 60 min of injection (~3.5 pore volumes), the columns were flushed with background electrolyte solution until the absorbance of effluent returned to the background values.

The deposition kinetics of bacterial cells in saturated porous media under clean-bed conditions is commonly quantified by the deposition rate coefficient (K), which is determined using the following equation (Kretzschmar et al., 1999)

$$K = -\frac{\nu}{\theta L}\ln\left(\frac{C}{C_0}\right) \quad (1)$$

where ν is approach velocity (cm/min), L is the length of the packed bed (cm), θ is the porosity of the porous medium (cm^3/cm^3), C is concentration of bacteria cell in the effluent under clean-bed conditions (cell/mL) and C_0 is the bacteria concentration in the influent (cell/mL). The values of C/C_0 were determined for each column experiment by calculating the average normalized breakthrough concentrations (i.e., C/C_0) measured between 1.8 and 2 pore volumes (Walker et al., 2005).

2.3. Determination of cell size and surface properties

The transport of bacteria cells in saturated porous media is influenced by a variety of cell surface properties such as zeta potential, surface charge and hydrophobicity which in turn were related to factors such as cell-bound proteins and LPS (Foppen and Schijven, 2006). In this research, a range of cell surface properties were determined and related to the transport of tet^R and tet^S *E. coli* cells.

Freshly harvested bacterial cells were suspended in appropriate KCl solutions (1–100 mM) at a concentration of ~10^7 cells/mL. The zeta potential of the bacterial cells was then measured with a Brookhaven ZetaPALS analyzer utilizing phase analysis light scattering. The hydrophobicity of the cells was determined through microbial adhesion to hydrocarbon (MATH) test (Pembrey et al., 1999). The MATH test involved the mixing of 1 mL of *n*-dodecane and 4 mL of cell suspension prepared using KCl solutions. The mixture was vortexed for 2 min and then allowed to stand still for 15 min. Cell concentration in the aqueous phase was determined at a wavelength of 546 nm. The fraction of bacterial cells that partitioned into the hydrocarbon phase was calculated based on mass balance and expressed cell hydrophobicity.

To quantify EDTA-extractable cell-bound proteins, fresh cell suspensions (~3 × 10^8 cell/ml) were prepared and mixed with 2.5% EDTA solution on a 2:3 (v/v) basis (Zhang et al., 1999). Following a 30-min incubation period at 4 °C, the mixture was centrifuged at 10,400 *g* (4 °C) for 50 min. The supernatant was then decanted and filtered through 0.45 μm filters. Protein contents in the filtrates were quantified using the Coomassie brilliant blue method developed by Bradford (1976). Standard solutions prepared with human serum albumin were used to calibrate this method. Additionally, the outer membrane proteins (OMPs) of the *E. coli* cells were extracted and profiled using sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) (Ben Abdallah et al., 2009; Gatewood et al., 1994; Xu et al., 2006). The bacterial cells were harvested at 4000× *g* for 10 min at 4 °C and rinsed twice with sterile 0.15 M NaCl. The cells were suspended in 5 mL sterile 0.15 M NaCl and subsequently disrupted by intermittent sonic oscillation (50 W, 8 cycles of 15 s of sonication, VirTis Virsonic). Following centrifugation at 5000× g for 40 min to remove cellular debris, the supernatant was transferred and centrifuged at 100 000× *g* for 40 min at 4 °C. The pellets were resuspended in 2% sodium lauryl sarcosinate (Sigma), incubated at room temperature for 1 h and then centrifuged at 100,000× *g* (40 min, 4 °C). The pelleted OMPs were suspended in 0.25 mL sterile 0.15 M NaCl solution and resolved on 10% sodium dodecyl-sulfate polyacrylamide gel (Laemmli). The gel was fixed in 4% perchloric acid and strained using 0.1% Coomassie G250 in hot 4% perchloric acid (Faguy et al., 1996).

To extract cell-bound LPS, 5 mL cell suspension was placed into 50 mL centrifuge tubes and subjected to sonic disruption (50 W, 20 s) (Liu et al., 2007). The resulting suspension was centrifuged (10 000× *g*, 40 min, 4 °C) and filtered through 0.2 μm cellulose acetate filters. The contents of LPS in the filtrates were measured using the phenol-sulfuric acid method and xanthan gum was used as the calibration standard (Du Bois et al., 1956).

Acid-base titration of cell suspensions was performed to determine the surface acidity of bacterial cells. Cell suspensions were prepared in the same fashion as those used in the column transport experiments. Two hundred milliliter of the suspension was added to a medium bottle and purged with high purity nitrogen gas for >60 min to remove CO_2. After the purging step, running nitrogen gas flow was maintained right above the surface of the suspension to maintain a CO_2 free environment. Sulfuric acid solution (0.1600 N, Hach Company) was then introduced into the suspension using Hach digital

titrator. Once the pH of the suspension dropped to ~4, NaOH (0.1600 N, Hach Company) was added at small volume increments to raise the pH to ~10. The pH of the suspension was continuously monitored and recorded using a pH probe (AccupH, Fisher Scientific). The acidity of bacterial cells was calculated based on the amount of NaOH that was consumed to raise the pH of the cell suspension from 4 to 10.

The size of the bacterial cells suspended in the KCl solutions were measured by taking photos using a Nikon Eclipse 50i microscope, which was equipped with a Photometric coolsnap ES digital camera and MetaMorph software. The length and width of a minimum of 50 cells were determined using the ImageJ software and the equivalent radii of the cells were then calculated.

3. Results and discussion

3.1. *Characterization of tet^R genes*

PCR assays showed that none of the tet^R genes were present in the tet^S strains while the efflux gene, *tetB*, was present in both tet^R strains (Fig. 1). This observation is consistent to previously reported results which suggested that the *tetB* gene was among the most commonly detected tet^R genes in *E. coli* and coliforms (Hu et al., 2008; Marshall et al., 1983). In Hu et al. (2008), the *tetB* gene was found in 41% of tetracycline resistant *E. coli* strains isolated from a river basin impaired by both human and animal farm waste.

3.2. *Transport of the bacterial cells in the sand packs*

The two tet^S *E. coli* strains differ in ampicillin and cephalothin susceptibility but the close match in their breakthrough concentrations suggested that the difference in ampicillin and cephalothin resistance had minimal impact on their mobility (Figs. 2 and 3). The breakthrough concentrations of the tet^R *E. coli* strains, however, were significantly higher than those of the tet^S strains under all ionic strength conditions, suggesting that tetracycline resistance can enhance the mobility of manure-derived *E. coli* (Figs. 2 and 3). In 1 mM KCl, for instance, the breakthrough concentrations of the tet^S were ~15% lower than the breakthrough concentrations of the tet^R strains. Accordingly, the values of the deposition rate coefficients (i.e., k) for the tet^S and tet^R strains were 0.0325(±0.0012) (SUS1), 0.0306(±0.0018) (SUS2), 0.0172(±0.000005) (RES1) and 0.0169 (±0.0009) (RES2) min^{-1}, respectively. When the ionic strength was increased to 3 mM, there was a significant drop in the breakthrough concentrations for all 4 *E. coli* isolates, suggesting that within this range, higher ionic strength facilitated their deposition at the surface of quartz sand (Figs. 2 and 3). While a further increase in ionic strength led to slightly lower breakthrough concentrations for the tet^S strains, the transport of the tet^R strains remained virtually unchanged (Figs. 2 and 3). Overall, the tet^R *E. coli* isolates displayed significantly higher mobility under the ionic strength conditions tested in this research (Fig. 3).

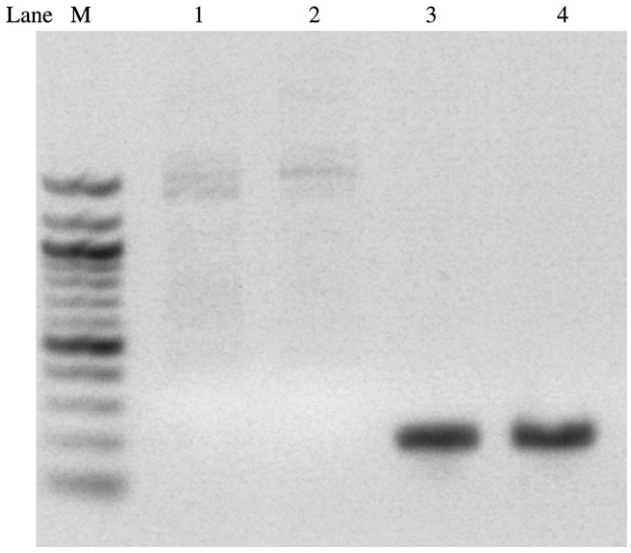

Fig. 1 – PCR detection of *tetB* in the *E. coli* isolates. Lane M: 100 bp ladder (New England Biolabs). Lanes 1–4: SUS1, SUS2, RES1, and RES2. The size of the amplicon for the *tetB* gene was 206 bp.

Our results suggested that environmental *E. coli* isolates could display marked variability in mobility, which was reported in several recent publications (Bolster et al., 2010, 2009; Foppen et al., 2010; Lutterodt et al., 2009). Bolster et al. (2009) and Bolster et al. (2010) compared the transport of 12 and 8 *E. coli* strains isolated from different animal sources (poultry, horse, beef and dairy cattle, human and wildlife) in saturated sands, respectively, and observed large variability in their mobility. Lutterodt et al. (2009) investigated the movement of 6 *E. coli* strains obtained from a soil used for cattle grazing in columns packed with sands and reported that the sticking efficiencies varied by a factor of 4–10. Foppen et al. (2010) examined the transport behavior of 54 *E. coli* strains and found that the attachment efficiency varied by a factor of ~6. The observed variability in *E. coli* transport behavior was found to be related to a range of factors such as cell surface autotransporter proteins (e.g., Ag43 protein) (Lutterodt et al., 2009), cell width (Bolster et al., 2010; Bolster et al., 2009), cell surface/zeta potential (Bolster et al., 2010), cell LPS structure (Foppen et al., 2010) as well as cell fimbriae (Foppen et al., 2010).

3.3. *Size and surface properties of bacterial cells*

The transport behavior of bacterial cells in the porous media is governed by the energy interactions between the cells and the surface of the solid matrix, which depends on a range of factors such as the Lifshitz–van der Waals force, electrostatic double layer (EDL) interactions, acid-base forces, hydrophobicity interactions and steric effects, which in turn are affected by cell size as well as cell-bound LPS and proteins and so on (Lindqvist and Bengtsson, 1991; Ong et al., 1999). In this research, various cell surface properties were measured and related to the observed difference in the transport behavior of the tet^R and tet^S *E. coli* strains.

The EDL interactions between bacterial cells and sand surface were closely related to their zeta potentials. The measured zeta potential values of the bacterial cells and the

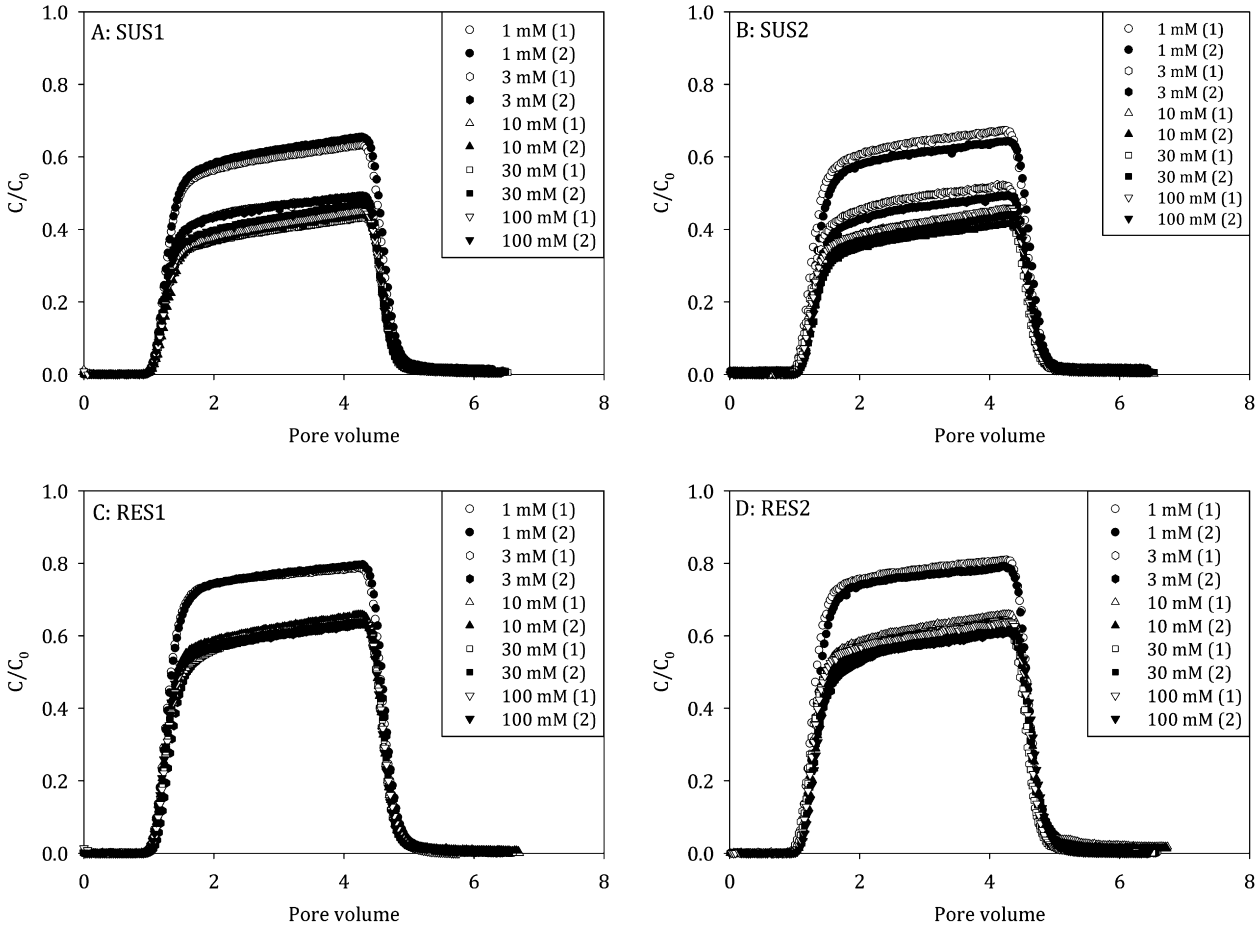

Fig. 2 – Breakthrough concentrations of the tetracycline susceptible (SUS1 and SUS2) and tetracycline resistant (RES1 and RES2) *E. coli* strains in saturated porous media under ionic strength conditions of 1, 3, 10, 30 and 100 mM KCl.

sands were negative (Fig. 4A), suggesting repulsive EDL interactions. In general, the zeta potentials of the tet^R strains were more negative than those of the tet^S strains. As a result, the repulsive interaction between the surface of quartz sands and the tet^R *E. coli* cells were stronger and the deposition rates

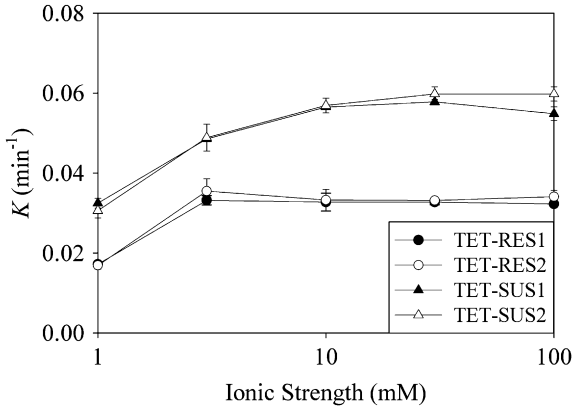

Fig. 3 – Clean-bed deposition rate coefficients (K, min^{-1}) for the 4 *E. coli* strains under ionic strength conditions of 1, 3, 10, 30 and 100 mM KCl. The values of K were calculated using Eq. (1). Error bars represent standard deviation of duplicate experiments.

should thus be lower. It is noteworthy that the zeta potentials of the tet^R strains remained virtually unchanged with ionic strength, while the zeta potentials of the tet^S strains increased slightly with ionic strength. This is consistent with the observation that the transport of the tet^R strains was less sensitive to changes in ionic strength.

The tet^R strains were more hydrophobic than the tet^S ones (Fig. 4B). On average, slightly over 90% of the tet^R *E. coli* cells partitioned into the hydrocarbon phase in the MATH tests, while less than 85% of the tet^S *E. coli* cells migrated from the aqueous phase into the hydrocarbon phase. In this research we observed higher mobility for the more hydrophobic tet^R strains. It was previously suggested, however, that cell hydrophobicity could enhance the attachment of bacterial cells to clean quartz sands (Bolster et al., 2006; Mccaulou et al., 1994). It thus seemed that the EDL interactions between *E. coli* cells and quartz sands were more significant than the hydrophobic interactions. This is consistent to recent findings which suggested that while cell zeta potential was significantly related to the transport of manure-derived *E. coli* strains, the relationship between cell hydrophobicity and *E. coli* mobility was statistically insignificant (Bolster et al., 2010).

Size is another factor that could influence the transport of colloid-sized particles in saturated porous media (Bolster et al., 2010; Yao et al., 1971). In this research, the size of the

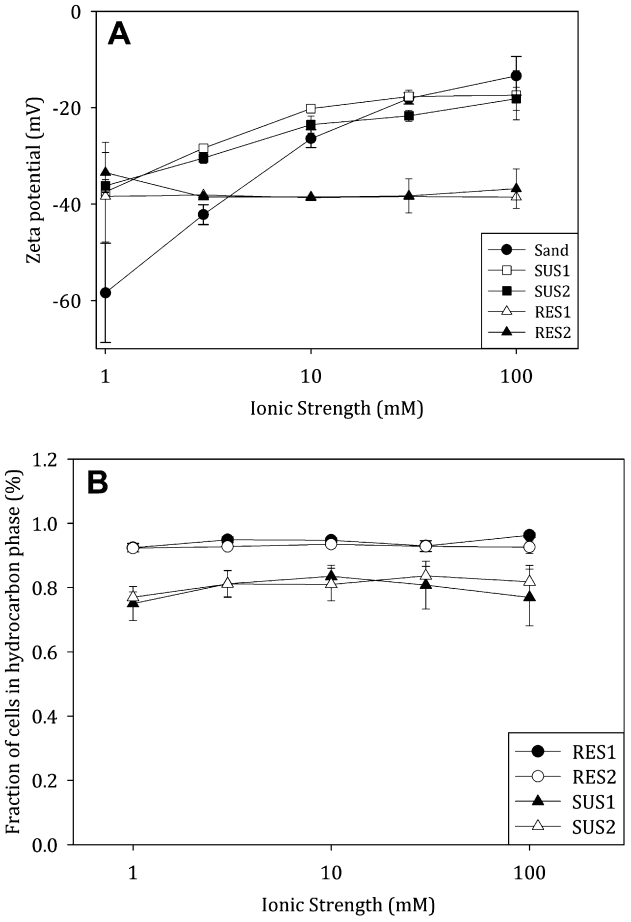

Fig. 4 – Zeta potential (A) and MATH test results (B). The MATH test results were expressed as the fraction of bacterial cells partitioned into the hydrocarbon phase. Error bars represent the standard deviation of triplicate measurements. For zeta potential, one measurement contained a minimum of 5 runs.

bacterial cells suspended in 5 different types of electrolyte solutions was measured. Our results showed that the size of the bacterial cells was not sensitive to ionic strength and the tet^R and tet^S cells have practically similar sizes (Fig. 5).

Through titration of cells suspended in 3 mM KCl, the acidity of the bacterial cells were measured as 6.10 (±0.16), 6.54 (±0.60), 7.07 (±0.83) and 6.28 (±0.24) × 10^{-4} meq/10^8 cells for SUS1, SUS2, RES1 and RES2, respectively, suggesting no significant difference between the susceptible and resistant isolates. The potentiometric titration curves showed that the pKa values of the acid-base functional groups on the surfaces of both tetracycline resistant and tetracycline susceptible cells were between 4–5 as well as 9–10. These pKa values correspond to the carboxylic/phosphoric and hydroxyl/amine groups, all of which are important components of LPS, proteins and phospholipids located on the surface of bacterial cells (Hong and Brown, 2006). Under the experimental pH conditions (~5.7), the carboxylic/phosphoric groups were deprotonated and contributed to cell surface charges.

Cell-bound LPS and protein contents varied within the range of 17.4–33.1 and 4.7–13 μg/10^8 cells, respectively (Fig. 6).

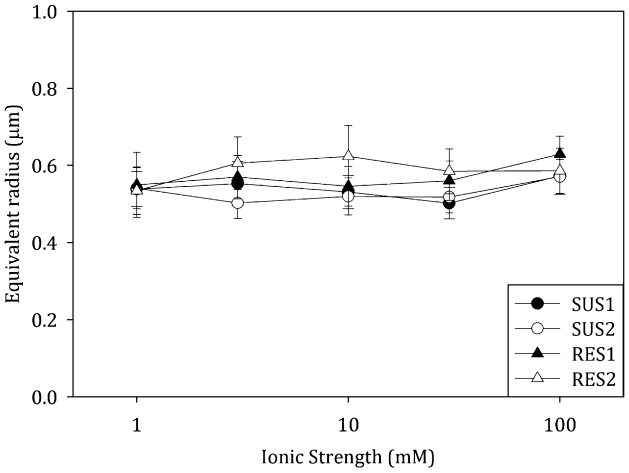

Fig. 5 – Size (equivalent radius) of the bacterial cells suspended in 1, 3, 10, 30 and 100 mM of KCl. The equivalent size was calculated as $\sqrt{\frac{L_C \times W_C}{\pi}}$, where L_C and W_C represent the length and width of the cell, respectively (Haznedaroglu et al., 2008). Error bars represent the standard deviation of a minimum of 50 measurements.

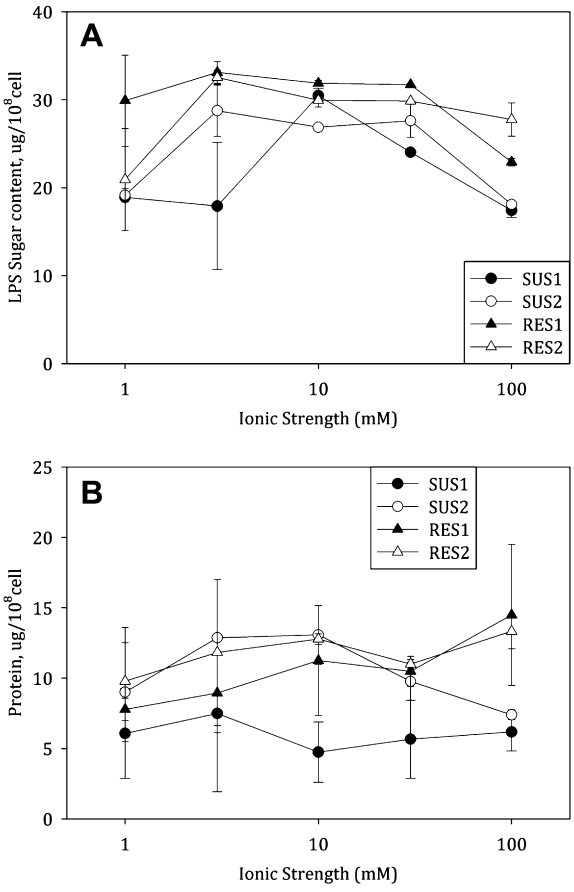

Fig. 6 – Comparison of the LPS sugar (A) and protein contents (B) of the tetracycline resistant and susceptible cells suspended in 1, 3, 10, 30 and 100 mM KCl. The error bars represent standard deviation of triplicate extraction attempts.

These values were consistent with previously reported measurements using manure-derived *E. coli* (Haznedaroglu et al., 2008). There were significant variations among the extraction attempts of LPS and protein, and among the electrolyte solutions used to prepare cell suspensions. Overall, we did not observe a clear pattern between the tet^R and tet^S strains with regard to LPS sugar and protein contents.

Although no difference was identified in the cell-bound protein contents, results of SDS-PAGE analysis of OMPs suggested that different proteins existed on the outer membrane of tet^R and tet^S strains (Fig. 7). Specifically, there were at least four proteins present in the outer membrane of the tet^R strains that were absent in the tet^S strains (indicated by arrows in Fig. 7). These proteins had approximate molecular masses of 54, 47, 44 and 40 kDa. Additionally, there were three proteins (indicated with arrow heads in Fig. 7) that were present in the tet^S strains but were absent in the tet^R strains.

As an antimicrobial agent, tetracycline has an intracellular target, inhibiting bacterial protein synthesis by disrupting the interaction of aminoacyl-tRNA with the ribosome (Walsh, 2003). Mechanisms specific to tetracycline resistance include efflux pump, ribosomal protection and modification of the antibiotic (Wax, 2008). The ribosomal protection mechanisms involve soluble structural homologues (e.g., TetM proteins) of elongation factors, which can bind to the ribosome and destabilize the interaction between tetracycline and their cellular target (Burdett, 1996; Dantley et al., 1998). Resistance to tetracycline through drug destruction was relatively rare and it was recently reported that TetX, a flavin-dependent monooxygenase, could hydroxylate the tetracycline substrate into an unstable compound which subsequently underwent non-enzymatic decomposition (Yang et al., 2004). The efflux pumps that are encoded by the tet^R genes (e.g., *tetB*) involve the transport of tetracycline from the cytoplasm to the periplasm through proteins inserted in the cytoplasmic membrane (Walsh, 2003). Out of the 20 tet^R genes examined in this research, only the *tetB* gene was detected in the tet^R strains (Fig. 1). Because the protein involved in the efflux pump is not exposed to the outside of the bacterial cells, it was unlikely that the presence of *TetB* could impact cell mobility.

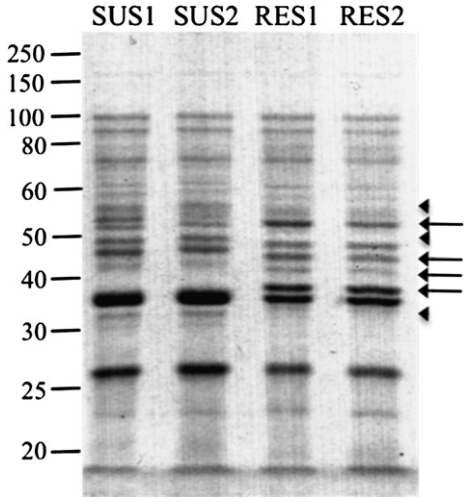

Fig. 7 – Outer membrane protein profiles of SUS1, SUS2, RES1 and RES2 using SDS-PAGE. Molecular masses (kDa) are indicated on the left. Proteins that are present in the tetracycline resistant strains, but absent in the tetracycline susceptible strains, are indicated with an arrow. Proteins that are present in the tetracycline susceptible strains, but absent in the tetracycline resistant strains are indicated with arrow heads.

The tet^R gene family, however, does not represent all the mechanisms responsible for tetracycline resistance in *E. coli*. Additional efflux pumps that involve multi-protein assemblies that often traverse both the inner and outer membranes of *E. coli* (e.g., the AcrAB-TolC pump) could lead to tetracycline resistance (Alekshun and Levy, 2007; de Cristobal et al., 2006; Xu et al., 2006). Increased expression of proteins such as TolC, OmpC, OmpW, along with decreased amounts of LamB and NlpB proteins have been observed in tet^R *E. coli* (Xu et al., 2006; Zhang et al., 2008). Additionally, it was observed that deletion of TolC led to increased sensitivity of *E. coli* to tetracycline (Zhang et al., 2008). Because processed TolC has a molecular mass of 52 kDa, it is likely that the protein extracted from the tet^R strains that migrated approximately at 54 kDa was TolC (Fig. 7). The crystal structure of the TolC protein was recently resolved and it was shown that it is a trimeric, 471-residue protein that contains an α-helical barrel and a β-barrel (Koronakis et al., 2000). The α-helical domain, which forms a tunnel through the periplasm and measure 10 nm in length, is anchored to bacterial wall by the contiguous β-barrel, which has a length of 4 nm and extends to the outside of the outer membrane. In addition to the approximately 54 kDa protein enriched in the tet^R cells, there were at least three other proteins that are differentially enriched in the tet^R cells. It is likely that the presence of these proteins, in combination with the absence of other proteins (indicated by arrow heads, Fig. 7), contributed to alterations in cell surface properties (e.g., zeta potential), which in turn impacted the mobility of the *E. coli* strains.

Recently, it was reported that Ag43, an outer membrane protein of *E. coli*, could enhance the attachment of *E. coli* cells to the surface of quartz sands (Lutterodt et al., 2009). It was proposed that the positive charges of the α-domain of Ag43, which extends from the cell surface, facilitated the attachment of *E. coli* cells to the negatively-charged quartz surfaces (Lutterodt et al., 2009). While the Ag43 and TolC proteins are structurally different and have different impacts on *E. coli* transport in saturated porous media (Koronakis et al., 2000; van der Woude and Henderson, 2008), the results of Lutterodt et al. (2009) and this research suggest that cell surface proteins can have strong influences on *E. coli* transport and more studies will be needed to elucidate the relationship between the abundance, structure and properties of cell surface proteins and bacterial transport in porous media.

3.4. *Environmental implications*

As a result of the widespread use of antibiotics in the animal farm environment, high frequencies of antibiotic resistant bacteria have been detected in animal waste (Halbert et al., 2006; Parveen et al., 2006; Ray et al., 2006; Sapkota et al., 2007; Sato et al., 2004; Varga et al., 2008a; Walczak and Xu, 2011). On the dairy farm from which the *E. coli* strains used in this research were isolated, 13.1%, 72.7%, 80.5% of the *E. coli* isolates were resistant to tetracycline, cephalothin and

erythromycin, respectively (Walczak and Xu, 2011). Additionally, 100% of the tet^R *E. coli* isolates were multi-drug resistant. The observed high mobility of the tet^R *E. coli* strains indicated that leakage from manure storage structures and application of manure as fertilizers in agricultural fields could potentially lead to the contamination of groundwater by antibiotic resistant bacteria, which in turn could pose serious public health risks when the groundwater, often untreated, is used as a source of drinking water.

Drug efflux pumps that involve outer membrane proteins like TolC are also seen in pathogenic bacteria such as *Salmonella* to gain antibiotic resistance (Ricci et al., 2006; Virlogeux-Payant et al., 2008). It is likely that the surface properties of these pathogens can also be altered in a similar fashion. As a result, the mobility of these pathogens in the subsurface system can be enhanced. Furthermore, it has long been observed that antibiotic resistance genes, which confer bacterial antibiotic resistance, can be transferred among a diverse group of microorganisms through conjugation, transduction and transformation (Levy et al., 1976; Lorenz et al., 1992; Mckeon et al., 1995; Nikolich et al., 1994). The antibiotic resistant genes harbored by antibiotic resistant *E. coli*, therefore, could potentially be horizontally transferred to bacterial pathogens such as *Samonella* in the subsurface environment and cause additional public health risks (Hunter et al., 1992; van Essen-Zandbergen et al., 2007).

4. Conclusion

- In this research, we observed that manure-derived, tet^R *E. coli* strains had higher mobility than tet^S *E. coli* strains within saturated porous media.
- The tet^R *E. coli* strains had more negative zeta potentials than the tet^S strains. This led to increased repulsive EDL interaction between the tet^R *E. coli* cells and the surface of quartz sands and could explain the observed higher mobility of the tet^R strains.
- The tet^R and tet^S *E. coli* strains had distinct outer membrane proteins profiles. It is likely that such difference led to alterations in cell surface properties (such as zeta potential), which in turn affected the transport of the tet^R and tet^S *E. coli* strains.

Acknowledgement

SX was supported by University of Wisconsin Milwaukee and University of Wisconsin Groundwater Research Program (WR10R007). SLB is the recipient of an NIH grant (5R00GM083147-04). We thank Dr. Douglas A. Steeber, Dr. Heather A. Owen and Steven E. Hardcastle for their assistance. We thank two reviewers whose comments led to improvements of our manuscript.

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