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Bacterial, particulate, and environmental factors driving E. coli attachment

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Bacterial, particulate, and environmental factors driving *E. coli* attachment

by

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ABSTRACT

Currently, 178,048 miles of impaired streams are contaminated due to elevated levels of pathogens or pathogen indicators (USEPA, 2014). While attachment of bacteria to particulates is an important transport mode, understanding of the factors driving these interactions is lacking. Previous studies have indicated bacteria attachment to particulates is influenced by bacterial surface properties, particulate properties, and environmental conditions. The goal of this study was to explore bacterial, particulate, and environmental factors driving *E. coli* attachment to particulates in the aquatic environment. Specific objectives were: 1) to determine if differences in environmental *E. coli* cell surface properties are due to extrinsic (environmental) or intrinsic (genomic) properties, or an interaction of the two; 2) to identify the impacts from bacterial and particulate properties on *E. coli* attachment fractions by constructing statistical models; 3) to elucidate mechanisms of *E. coli* attachment to particulates in livestock manure.

Cell properties including hydrophobicity, zeta potential, net charge, total acidity, and EPS (extracellular polymeric substances) composition were measured for 77 genomically distinct *E. coli* strains collected from two environmental habitats (stream sediments and water). Meanwhile, attachment assays were constructed using a single *E. coli* strain and one model particulate (ferrihydrite, Ca-Montmorillonite, or corn stover) with environmentally relevant concentrations. Our results indicated variations between stream sediment *E. coli* and water *E. coli* in hydrophobicity, EPS protein and sugar content, net charge, and point of zero charge. The diversity of cell properties was due to interactions of extrinsic and intrinsic properties. Moreover, Generalized Additive Model (GAM) successfully predicted the attachment fractions to Ca-Montmorillonite and corn stover using cell characteristics as predictor variables and net charge had a linear impact on the attachment fractions.

Three genomically different beef manure *E. coli* strains (A, B and C) and one *E. coli* O157:H7 (ATCC 43888) were analyzed for their attachment to two types of beef manure particles with size smaller than 53 μm: methylene chloride insoluble and soluble. Flow cytometry was employed to measure attachment fractions for 6 different *E. coli* concentrations and the Freundlich isotherm successfully fitted the attachment data. The

results indicated a more heterogeneous mechanism for *E. coli* attachment to methylene chloride manure particulates.

CHAPTER 1: GENERAL INTRODUCTION

Currently, 178,048 miles of impaired streams are contaminated due to elevated levels of pathogens or pathogen indicators (USEPA, 2014), and pathogens are the leading cause of water quality impairments in rivers and streams in the United States. Pathogenic organisms cause illnesses including but not limited to common gastroenteritis, diarrhea, typhoid fever, and dermatitis (Rosen, 2001; Pond, 2005).

Pathogens are present in the environment due to a variety of point and nonpoint sources of pollution, and it is crucial to unveil mechanisms of their transportation for watershed assessments and prediction of hazardous conditions to human health. Monitoring bacteria is essential for environmental protection, but analysis of all pathogens is difficult, time-consuming and potentially hazardous to laboratory personnel (Myers et al., 2007). In the past fecal coliforms were the most common fecal indicator organism used to evaluate water quality for potential pathogens. *E. coli,* however, is currently accepted more extensively. In 1986, *E. coli* was recommended by the U.S. EPA as the primary organism used to indicate the presence of fecal contamination in fresh waters in the United States (USEPA, 1986). Therefore, an improved mechanistic understanding of environmental *E. coli* transport is needed for modeling bacteria fate and transport and is essential to develop meaningful plans to reduce bacterial contamination of waters.

Agriculture is a significant contributor of bacteria in the environment. Two major sources of bacteria in streams are from land application of manure from confined animal systems and direct deposit by grazing animals (Soupir et al., 2006). After entering waters, microorganisms can move freely in water in a planktonic state or attach to suspended soil and organic particles (Jeng et al., 2005; Hipsey et al., 2006; Pachepsky et al., 2008). While attachment of bacteria to particulates is an important mode of environmental transport, understanding of the factors driving these interactions is lacking. Previous studies have indicated that bacteria attachment to particles in the aquatic environment is influenced by temperature, bacterial genotype, particle size, organic matter content, particle moisture content, pH, and dissolved nutrient concentrations (Schallenberg and

Kalff, 1993; Jiang et al., 2002; Muirhead et al., 2006; Pachepsky et al., 2006; Bolster et al., 2009). However, there are conflicts among previous findings, perhaps because of the small isolates collections utilized in those studies. Moreover, although a variety of separation techniques have been used by researchers for more than a decade, a standard method to partition between freely suspended *E. coli* and *E. coli* attach to particulates is currently nonexistent. This lack of a procedure and information has resulted in a standard assumption in water quality models that bacteria are freely suspended.

The goal of this research is to improve understanding of *E. coli* attachment in the aquatic environment. The objectives are to 1) evaluate the differences in cell properties (hydrophobicity, excess acidity, zeta potential, size, and extracellular polymeric substance composition) of stream water *E. coli* and stream sediment *E. coli* and determining the correlation between the cell properties, 2) investigate factors such as cell properties including hydrophobicity, surface charge, zeta potential, and extracellular polymeric substance composition; particulate properties including size, surface area, and organic carbon content of *E. coli* attachment behavior to model stream particles (one organic, one layer silicate, and one iron oxide), 3) assess the mechanisms of *E. coli* attachment to manure particles by developing chemical adsorption models.

CHAPTER 2. LITERATURE REVIEW

Pathogens and Water Quality

Waterborne Disease and Water Impairment

Ingestion or recreational exposures with pathogens in the aquatic environment may result in disease. Pathogenic organisms cause illnesses including but not limited to common gastroenteritis, diarrhea, typhoid fever, and dermatitis (Rosen, 2001; Pond, 2005). Waterborne disease outbreaks are often caused by ingestion or dermal contact with water contaminated by pathogenic microorganisms such as bacteria, protozoan, or virus, often present in water from human or animal excrement. According to World Health Organization (WHO), diarrheal disease alone accounts for an estimated 4.1% of the total disability-adjusted life year (DALY) global burden of disease and is responsible for the death of 1.8 million people per year (WHO, 2004). In developing countries, diarrhea is one of the leading causes of childhood death and is attributed to unsafe drinking water, lack of sanitation, and insufficient clean water for hygiene (WHO, 2008). Waterborne diseases also occur in developed countries with modern water and sanitation systems. In the United States, between 2007 and 2008, there were 36 documented outbreaks of waterborne disease associated with drinking water and 134 outbreaks due to water-related recreational activity (CDC, 2011).

Water pollution is a global problem which requires ongoing evaluation and revision of water resource policies from international to national and local levels. For over 120 years, water pollution has been regulated in the United States. The River and Harbors Appropriation Act of 1889 first made it a misdemeanor to discharge refuse matter of any kind into navigable waters. In 1948, the Federal Water Pollution Control Act was the first major law in the U.S. to address water pollution. It was amended in 1972, to what is now known as the Clean Water Act (CWA), which established the basic structure for regulation of pollutant discharges into the waters of the United States. The CWA established the goals of eliminating releases of high amounts of toxic substances into water, eliminating additional water pollution by 1985, and ensuring that surface water would meet standards necessary for sport and recreational activities by 1983

(USEPA, 1972). However, the goals were not met. The CWA regulates both point and nonpoint sources of pollution, including chemical, physical, and biological pollutants, but it does not directly address groundwater contamination. Major impairment causes such as metals, pathogens, and sediment are reported to Congress and the public every 2 years through the National Water Quality Inventory Report (305(b) report). The information in the report is useful for summarizing the nature of the water quality problems and setting priorities for restoration (USEPA, 2004).

It is required for states, tribes and other jurisdictions to identify the sources of pollutants associated with water quality impairments. Point source (PS) pollution is that which originates at a single identifiable source. Point sources include industries, municipal wastewater treatment plants, and some agricultural facilities such as concentrated animal feedlots. Among these sources, wastewater treatment effluent or concentrated animal feedlots could be considered as a source of bacteria. The National Pollutant Discharge Elimination System (NPDES, Clean Water Act Section 402) is a permit-based system for regulating the discharge from point sources into navigable waters. Nonpoint source (NPS) pollution originates from diffuse sources often generated in the upland areas of a watershed and transported to water systems during hydrologic events. Two major sources of bacteria in surface water are land application of manure from confined animal systems and direct deposit by grazing animals (Soupir et al., 2006). Both are considered NPS pollution. Significant relationship has been found between precipitation events and stream water pathogen contaminations (Kistemann et al., 2002; Chin, 2010).

Fecal Indicator Organisms and Water Quality Standards

Until recently, the U.S. EPA's recommended water quality testing strategies were dependent upon culture-based methods, which are time-consuming and may pose potential health hazards to researchers (Myers et al., 2007). Based on these difficulties, pathogen indicators are used to set water quality standards and determine if a waterbody is impaired due to elevated pathogen levels (USEPA, 2006). To be an ideal fecal indicator bacteria, an indicator should meet certain criteria: 1) a strong correlation exists between the presence/absence of the indicator and the presence/absence of fecal

contamination; 2) a relatively rapid, accurate, and cost-effective analytical method for detecting the indicator exists; 3)the indicator should have a longer survival time than the hardiest fecal pathogen (NHDES, 2003; USEPA, 2006; Myers et al., 2007; Payment, 2011). Based on the above criteria, although not all *E. coli* and enterococcus strains are pathogenic, *E. coli* and enterococci are used as fecal indicator bacteria to predict when a risk to human health is present. Commonly found in the lower intestine of warm-blooded animals, *E. coli* is a large and diverse group of Gram-negative rod-shaped bacteria. It was recommended as the indicator of fresh water fecal contamination by the U.S. EPA in 1986 (USEPA, 1986). Exposure limits for *E. coli* have been established to protect human health: the U.S. EPA recommends that the geometric mean concentration for *E. coli* should not exceed 126 colony-forming unit (CFU) 100 mL^{-1} and the single sample maximum should not exceed 235 CFU 100 mL^{-1} for designated beach areas. Enterococci are a subgroup within the fecal streptococcus and has been used as the indicator for marine water and the U.S. EPA recommended criteria is 33 CFU 100 mL^{-1} for geometric mean concentration and 61 CFU 100 mL^{-1} for single sample at designated beach areas (USEPA, 1986).

As the concentrations of fecal indicator bacteria increase, it is likely that pathogenic organisms present in fecal material will also be in the water body, threatening human health (USEPA, 2000). For example, the EPA defines acceptable recreational limits as those that will result in eight or fewer swimming-related gastrointestinal illnesses out of every 1,000 swimmers, with illness being defined as vomiting, diarrhea with a fever or disabling condition, or stomachache or nausea in addition to a fever. Others have also identified a correlation between indicator organisms in water and gastrointestinal illness in humans (Cabelli, 1983). For example, one study observed a statistically significant correlation between increased gastrointestinal illness and Enterococcus at Lake Michigan beach, and a positive correlation for indicator organisms at Lake Erie beach (Wade et al., 2006). A work by Edge et al. (2012) showed that waterborne pathogens, such as *Campylobacter*, *Salmonella* and *E. coli* H7:O157 were detected in 80% of water samples with *E. coli* concentration lower than 100 CFU $100mL^{-1}$ (Edge et al., 2012).

The Ambient Water quality criteria developed by the U.S. EPA in 1986 were recently revisited, and in 2012 the U.S. EPA established the new Recreational Water Quality Criteria (RWQC). Culturable *E. coli* (for freshwaters) and culturable enterococci (for marine waters) remain the two measures of recreational water quality, but enterococcus as measured by quantitative polymerase chain reaction (qPCR) was added (EPA Method 1609 and 1611) (USEPA, 2012b). The method measures large subunit ribosomal RNA (lsrRNA, 23S rRNA) target gene sequences from all know species of enterococci bacteria in water by using TaqMan® Environmental master mix PRC reagent and TaqMan® probe system. While detection of pathogens and pathogen indicators via qPCR is promising due to the rapid turnaround time, analytical costs are high and specialized laboratory equipment is required. Furthermore, qPCR is unable to differentiate between viable and non-culturable organisms.

TMDLs and Modeling of Bacterial Transport

Although pollution control technologies have been implemented by many pollution sources, 478,654 mile out of 1,041,527 miles of assessed waters in the U.S. still do not meet the national goal of "fishable and swimmable" (USEPA, 2012a).

Under Sections 303 (d) and 305 (b) of the Clean Water Act, states, territories, and authorized tribes are required to identify lists of impaired waters and develop total maximum daily loads (TMDL) for these waters. A TMDL is a calculation of the maximum amount of a pollutant that a waterbody can receive while still meeting water quality standards. Once established, the TMDL is used to set limits on allowable discharges to meet water quality standards by identifying and quantifying both point and nonpoint sources contributing to the problem. The calculation of a TMDL is as follows:

$$TMDL = WLA + LA + MOS$$

where WLA is the wasteload allocation from point sources of pollution, LA is the load allocation from nonpoint sources of pollution, and MOS is the margin of safety which accounts for the uncertainty in the response of the waterbody to loading reductions and it is typically set as 10% of the total load allocation.

One way to set TMDL load allocations among point and nonpoint sources is through the use of watershed-scale water quality models. A TMDL plan may contain WLAs only, LAs only, or a combination of both, including bacteria, sediment, nitrogen, and phosphorus. In developing TMDLs, public involvement is also required: the state circulates a draft for TMDL and allow 30 to 60 days for public comments. Following development of a TMDL plan, implementation plans including best management practices (BMP) and other management strategy recommendations (USEPA, 2008) are selected to target the pollution control.

Modeling the fate and transport of bacterial attachment in aquatic environments is critical to successful TMDL implementation plans. But most models simulate bacterial transport as unattached cells, mainly due to insufficient data on bacteria partitioning fractions (Paul et al., 2004; Jamieson et al., 2005; Soupir et al., 2008). Models such as the Soil and Water Assessment Tool (SWAT) may be used to predict bacterial transport (Borah and Bera, 2003; Borah et al., 2006; Gassman et al., 2007) and is allowed to partitioning bacteria. However, microorganisms can move through the environment in a planktonic state or attached to suspended soil and organic particles (Jeng et al., 2005; Hipsey et al., 2006; Pachepsky et al., 2008; Liu et al., 2011). For example, studies reported that bacteria counts are consistently higher in stream sediments, which suggests that the understanding of interaction between pathogens and sediment must be improved to properly estimate risks to public health (Droppo et al., 2009). Another study reported that sediment disturbance can account for the majority of total bacterial contamination in overlying waters (Nagels et al., 2002). The lack of understanding of the mechanisms of bacterial attachment to particles in eroded soil, stream-bottom sediments, and suspended sediments leads to inaccuracy in modeling environmental bacterial transport.

Bacterial Attachment

Definition of Bacterial Attachment

Bacteria generally exist in one of two types of population: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface or within the confines of a biofilm (Garrett et al., 2008). Bacteria are typically found attached to and living in

close association with other cells or surfaces (Hipsey et al., 2006; Pachepsky et al., 2008). To establish contact with the surface, bacteria first must overcome the hydrodynamic boundary layer and repulsive forces as they approach the surface. At distance >50 nm, there is mainly Van der Waals interactions between the bacterium and surface. These interactions are weak, but as the bacterium moves closer to the surface (10-20 nm), repulsive electrostatic interactions occur if both the bacterium and surface carries net negative charge. When the bacterium is 2-10 nm from the surface, both repulsive and attractive electrostatic interactions occur since the bacterium can carry both positive and negative charge at local sites. At shorter distances, water adsorbed at surface can act as a potential barrier for attachment, while hydrophobic function groups from cells may help redeem the barrier by removing the layer of water. Once the bacterium overcomes the hydrodynamic boundary layer and repulsive forces as they approach the surface (<1 nm), more specific chemical interactions play an important role in attachment and the chemical interactions would vary depending on different live cells and solid faces. The interactions involved in cell-surface interaction are shown in Figure 2.1.

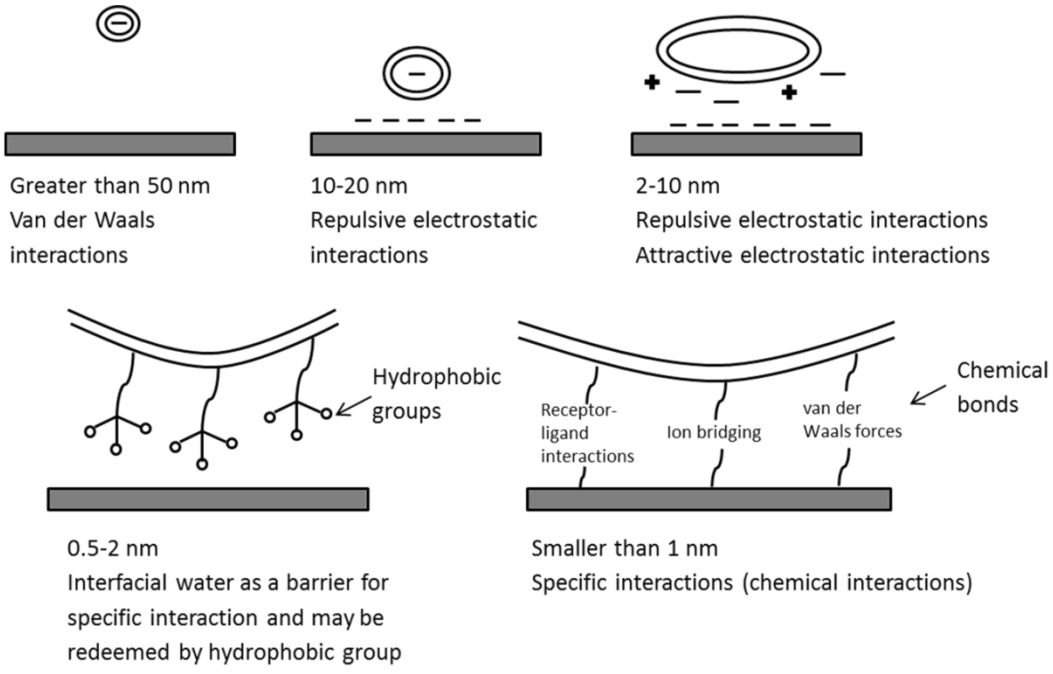

Fig. 2.1. Types of Types of force involved in interaction between cell and a negatively-charged surface: 1) when the distance is greater than 50 nm, there is only van der Waals existing; 2) as the bacterium moves to the surface (10-20 nm), mainly repulsive electrostatic interactions; 3) when the distance is 2-10 nm, both repulsive and attractive

electrostatic forces may occur since both positive and negative charge can be carried at local sites of surfaces; 4) as the distance becomes closer, interfacial water barrier and hydrophobic group can act simultaneously; 5) when the distance is smaller than 1 nm, chemical bonds such as receptor-ligand interactions, ion bridging, and van der Waals forces can promote attachment [modified from (Fletcher, 1996; Ojeda, 2012)]

If the bacterium is accepted by the surface, then bacterial attachment can be divided into two categories: reversible attachment and irreversible attachment. Reversible attachment is characterized by cells loosely attached via a single pole (flagella or pili) that may readily detach and return to the planktonic phase. Rotation of the single pole results in spinning motion of the bacteria and the bacterium may readily detach and return to the planktonic phase. If the bacteria attach to the surface along their axis, the spinning motion decreases and polysaccharide expression increases, making the attachment irreversible (Petrova and Sauer, 2012). However, the irreversibility is conditional. For example, in the development of a biofilm, bacteria reproduce after attaching to a surface and some of the cells can then detach from the cluster (Esty, 2010). Furthermore, based upon the attaching position and the mobility, bacteria can attach to surface: 1) at one end by their polar flagella, 2) along their length but still having slight Brownian movement, or 3) along their length but with no mobility (Meadows, 1971).

Bacterial Attachment in the Environment

Previous research has demonstrated that microorganisms can move freely in water in a planktonic state or attach to suspended soil and organic particles (Jeng et al., 2005; Hipsey et al., 2006; Pachepsky et al., 2008). A previous study determined that 10-20% of fecal coliform cells are adsorbed to suspended particles in untreated stormwater runoff (Schillinger and Gannon, 1985).

Bacterial attachment to soil particles results in an increased settling velocity of the cell, and sedimentation of attached bacteria is often a critical removal mechanism in surface waters (Schillinger and Gannon, 1985). The importance of this process is emphasized by the many recent studies citing concentrations of fecal bacteria in stream sediment that are 10-10,000 times higher than the concentrations in the overlying water column (Song et al., 1994; Davies and Bavor, 2000; Bai and Lung, 2005). Bacteria in bottom sediments are protected from ultraviolet radiation (Bitton et al., 1972; Schillinger and Gannon, 1985), resulting in an extended life expectancy and increasing the likelihood

of resuspension back into the water column when stream bottom sediments are disturbed during changes in flows (Song et al., 1994; Davies and Bavor, 2000; Jamieson et al., 2005). For instance, microorganisms could be stored and then survive, even multiply in riverbed fine sediments during medium and low flow may be resuspended during storms, increasing concentrations of fecal indicators and possibly pathogenic organisms in the water column (Laliberte and Grimes, 1982; Rehmann and Soupir, 2009). Moreover, it has been reported that bacteria attached to surfaces are more resistant to environmental stresses such as ultraviolet radiation and antibiotics, influencing their survival and ultimate fate in surface water systems (Meadows, 1971; Gerba and McLeod, 1976; Laliberte and Grimes, 1982; Burton et al., 1987; Davies et al., 1995; Russo et al., 2011). As an example, in a study held in Southern Ontario, Canada, the amount of tracking *E. coli* did not decrease in the stream bed for the first 5 days in alluvial streams (Jamieson et al., 2004).

Partitioning Methods for Bacterial Attachment

Despite the need to quantify attached and unattached cells, a standard protocol to partition between unattached and attached *E. coli* is currently nonexistent. This lack of a procedure has resulted in a standard assumption in water quality models that bacteria are freely suspended. A variety of separation techniques have been proposed to quantify the attached and unattached fractions of bacteria, including settling, filtration, and centrifugation (Schillinger and Gannon, 1985; Henry, 2004; Characklis et al., 2005; Jeng et al., 2005; Muirhead et al., 2005; Krometis et al., 2009). After attaching to particles, bacteria would have higher density and settle more quickly than freely suspended bacteria with lower densities. A study by Liu et al. (2011) used Stoke's Law to determine that 5 min is enough quartz-attached *E. coli* settle out and separate from freely-suspended *E. coli* from. Filtration retains particles via a sieving mechanism based on the size of the membrane pores relative to that of the particles (USEPA, 2005). Qualls et al. (1983) defined the unattached bacteria as those able to pass through an eight-micron screen (Qualls et al., 1983), and that approach has been applied by others (Krometis et al., 2009). Multiple-screen filtration is another approach to separate suspended solids into different particle sizes. In 2008, Soupir et al. utilized a series of screens and filters (35 mesh screen, 230 mesh screen, 8 μm and 3 μm polycarbonate filters) to quantify the *E. coli* attachment

percentage in surface runoff samples. Partitioning techniques involving filtration have limitations: as a typical fecal indicator bacteria, *E. coli* cell is 1.1 to 1.5 μm in width and 2 to 6 μm in length (Grismer, 2006), therefore, filtration methods might include not only free bacteria but also those sorbed to very small particles or even small bioflocculated clumps (Soupir et al., 2008).

Centrifugation is another method for determining the sediment-attached fraction of bacteria (Faegri et al., 1977; Schillinger and Gannon, 1985). In this approach, *E. coli* in the supernatant are considered suspended, and the difference between this concentration and the total concentration is assumed to be the attached bacteria fraction (Soupir et al., 2008). However, centrifugation methods also have drawbacks. Since unattached bacteria have a diameter similar to small-sized particles, determining proper centrifuge speed and time to separate attached and unattached bacteria can be challenging (Henry, 2004).

Although the above separation techniques have been used by researchers for more than a decade, they are time-consuming and labor-intensive. Furthermore, the attached and unattached fractions are typically assessed via plate counting techniques, which involves serial dilutions and colony counting. A new method was recently developed using flow cytometry to partition between unattached bacterial cells and those that are attached to small particles (Liang, in press). Flow cytometry is a technique used for measuring and analyzing multiple parameters of individual particles (Vital et al., 2010), including microorganisms, nuclei, and latex beads (Brown and Wittwer, 2000). *E. coli* treated with fluorescent nucleic acid stain can be distinguished from the particles. Meanwhile, after attaching to particles, *E. coli* would have larger side scatter signal, which could be distinguished from free *E. coli*. The limitation for this method include environmental strain diversity for application to environmental samples and the diameter of particles suitable for testing in flow cytometry.

Furthermore, in order to disperse bacteria from sediment and organic matter particles, different approaches have been applied to detach bacteria from particles. Chemical agents such as 1000mg/L Tween 85 and 1% sodium pyrophosphate have been used to loose the strong hydrogen binding, van der Waals, electrostatic and chemical

forces that tie cells and particles together (Amalfitano and Fazi, 2008; Soupir et al., 2008). Physical dispersion techniques are another method which mechanically disrupt the physical entrapment of bacteria in small pores on particles, and include blending, hand or orbital shaking, sonication probing, or submerging the sample in an ultrasonic bath. The traditional separation methods mentioned above have limitations in that they can cause cell damage and are labor insensitive due to diluting, plating and colony counting.

Properties Impacting Bacterial Attachment

While attachment of bacteria to particulates is an important mode of environmental transport, understanding of the factors driving these interactions is lacking. Previous studies have indicated that bacteria attachment in the aquatic environment is influenced by temperature, bacterial genotype, soil particle size, organic matter, water content, pH, and dissolved nutrient concentrations (Dickson and Koohmaraie, 1989; Schallenberg and Kalff, 1993; Jiang et al., 2002; Muirhead et al., 2006; Pachepsky et al., 2006; Bolster et al., 2009). The following section will discuss each of these properties in detail.

Bacterial properties

As mentioned in section 2.2.1, when the distance between bacterium and surface is smaller than 1 μm, more specific interactions may come into play and the interactions would vary depending on different surface characterization and properties of cells. Genes that encode various types of pili, fimbriae, and other surface structure are responsible for bacterial attachment to other bacteria, host tissues, or other surfaces (Garcia and LeBouguenec, 1996). For example, Cook et al. (2011) evaluated the presence of 15 genes in 17 *E. coli* isolates and found that highest attachment efficiency after transport through saturated porous media was correlated with the surface exclusion gene (*traT*) and the siderophore $iroN_{E.coli}$ as well as adhesion genes which control the expression of autotransporter proteins (Foppen et al., 2010; Cook et al., 2011). Lutterodt et al. (2009) found that the expression of *flu*, one of the adhesion genes, significantly impacted *E. coli* attachment to quartz particles.

Extracellular polymeric substances (EPS) are high-molecular-mass compounds secreted by microorganisms at the outer cell surface. They are mainly composed of

polysaccharides and proteins, but they may also include other macro-molecules such as DNA, lipids, and humic-like substances. EPS plays an important role in cell aggregation, cell adhesion, biofilm formation, and protection of cells from hostile environments (Dogsa et al., 2005). Researchers have previously found no correlation between the presence of three EPS-associated genes (*ompC, slp, and surA*) and quartz attachment (Foppen et al., 2010). Moreover, as indicated in a study, EPS sugar/protein ratio was positively correlated with cell surface charge (Shin et al., 2001). But, there was no correlation between EPS sugar/protein ratio and the transport of 12 *E. coli* strains through quartz sand. Still the role of EPS in particle attachment cannot be overlooked because EPS production most happened at the late growth stage of bacteria (Bolster et al., 2009) and the EPS sugar/protein ratio changes dramatically when culturing time increases (Shin et al., 2001).

The hydrophobicity of a bacterial cell differs between strains because it is determined by functional groups of both residues and structures on the surface of the cell, and these can be either hydrophilic or hydrophobic (Vandermei et al., 1991). Some studies have found a positive correlation between attachment ability and hydrophobicity (Lindahl et al., 1981; Hartley et al., 1993; Zita and Hermansson, 1997; Rad et al., 1998), while Bolster et al. (2009) reported no correlation between hydrophobicity and transport of 12 different *E. coli* strains through packed quartz beds.

The majority of bacterial cells are negatively charged due to the carboxyl and phosphate groups of the peptidoglycan and lipopolysaccharide that compose the cell walls (Goulter et al., 2009). The surface charge can impact attachment to particles by repulsion of similarly charged particles and by attraction of particles with an opposite charge. Studies discovered that the relative negative charge correlated well with attachment to surfaces (Dickson and Koohmaraie, 1989; Bolster et al., 2009).

Surface energy is the excess energy per unit surface area and larger-sized or high spherical bacterium has lower surface energy. Attachment decreases the total surface energy of the cell-particle system in the aquatic environment by forming contact regions with lower surface energy. Based on these and minimum total potential energy principle, smaller-sized or non-spherical bacteria are expected to have higher preference of

attaching with particles than larger-sized or higher spherical bacteria (Miller, 2010). In 2009, Bolster et al. found that *E. coli* attachment to quartz sand was strongly negatively correlated with *E. coli* width and sphericity (width/length). Previously, no relation between attachment to quartz sand and cell sphericity was indicated in a study of 54 *E. coli* isolates (Foppen et al., 2010). Lutterodt et al. (2009) studied cell sphericity of 6 *E. coli* strains obtained from a soil used for cattle grazing and found cell sphericity did not significantly correlate with attaching efficiency (Lutterodt et al., 2009).

Particle properties

Previous research has demonstrated that the particle size plays an important role in bacterial attachment. The percent of attached bacteria to particles is typically negatively correlated with particle size (Fontes et al., 1991; Fuller et al., 2000; Bolster et al., 2001; Dong et al., 2002; Levy et al., 2007). Bacteria are more likely to attach to small particles, partially because they have greater surface area available for attachment than larger particles with same mass and density (Oliver et al., 2007). In urban storm water runoff, fecal indicator bacteria were adsorbed predominantly to fine clay particles (<2 μm) (Muirhead et al., 2006).

Particle surface charge and hydrophobicity are often observed as the dominant factors impacting the attachment of bacteria to sand (Bolster et al., 2006; Jacobs et al., 2007), while in some studies they are not significantly correlated with bacterial attachment (Bolster et al., 2009). Over a wide range of pH, both bacteria and clean sediment particles have low net negative charge and so charge-based attachment is likely to behindered. However, there are also some neutral and positively charged sites on the surface of bacteria and sediment due to the surface charge heterogeneities (Pachepsky et al., 2006). Bacteria with a net negative charge may thereby be attached to positively-charge sites (Bolster et al., 2009). Since the total area of positively charged sites is much smaller than the overall surface area, charge-based attachment will likely occur only at small surface coverage (Song et al., 1994). It is possible that a combination of electrostatic force combined with hydrophobic effects can overcome the natural repulsion of bacteria and negative-charged particles (Mills, 1981). A study by Scholl, et al., (1990) found that negatively charged bacteria were more likely to attach to positively charged

surfaces, such as calcite, than to negatively charged surfaces, such as clean quartz. Bacterial attachment was enhanced significantly in the presence of Fe-oxyhydroxide and metal-oxyhydroxide coating due to increased abundance of positively charged sites. Besides, the bacterial attachment is also enhanced by increasing surface hydrophobicity of the particles: the attractive force for the hydrophobic bacteria increases with respect to hydrophobicity of the particle surface (Lindqvist and Bengtsson, 1991; Johnson and Logan, 1996; Fein et al., 1997; Ong et al., 1999; Yee and Fein, 2001; Ams et al., 2004; Foppen and Schijven, 2005; Bolster et al., 2010).

Organic particles can impact bacterial attachment differently from mineral particles. Both dissolved natural organic matter (DOM) and sediment organic matter (SOM) can impact bacterial attachment: it has been shown that *E. coli* survival in water bodies can increase after attaching to mineral aggregates with high SOM concentration (Sherer et al., 1992); under the presence of DOM, the attachment of bacteria to Fe-quartz and SOM-Fe-quartz increased, but the attachment of bacteria to clean quartz decreased (Johnson and Logan, 1996).

Environmental properties

Previous research found that environmental factors such as ionic strength, temperature, pH, and nutrient condition could impact bacterial attachment (Mafu et al., 2011; Tsuji and Yokoigawa, 2012). Bacteria attach to particle surfaces that are bathed by solutions with a wide range of ionic strengths. Under typical conditions (e.g., pH 7), both bacteria and the substratum surface are negatively charged (Pachepsky et al., 2006), and there are two opposite interactions: electrostatic repulsion and van der Waals attraction. Whether or not bacteria attach to a surface depends on which interaction dominates (Morisaki and Tabuchi, 2009). According to the size and surface polymer, bacteria can be treated as soft colloidal particles, which have much lower surface electric potential than smooth colloidal particles (Marshall, 1976). The low surface potential can reduce electrostatic repulsion between bacteria and particle surfaces, and the reduction is significant at high ionic strength (>100 mM). According to Derjaguin-Landaul-Verwey-Overbeek (DLVO) theory, the thickness of the electrical double layer of both particles and bacteria should decrease as the ionic strength increases, promoting bacterial

attachment to particles (Lee et al., 2010). Consequently, as ionic strength increases, the rate of attachment increases, which can be explained by interactions between the bacteria and surface. *E. coli* S17 showed less negative electrophoretic mobility as environmental ionic strength increased from 1 to 1000 mM (Janjaroen et al., 2013).

Temperature also impacts bacterial attachment by impacting cell properties and surface structure. *E. coli* O157:H7 grown in rich medium at 15˚C to the stationary phase exhibited lower attachment to abiotic surfaces such as stainless steel, pure titanium, glass, and plastic than those grown at 25˚C and 37˚C (Tsuji and Yokoigawa, 2012). The possible reason could be *E. coli* grown at lower temperatures tend to have high motility which may be suppressive for attachment (Turner et al., 1996). Moreover, the temperature shift from 25 ˚C to 4 ˚C during growth was found to have a negative impact on attachment to polystyrene surfaces because temperature shifts could induce a stress in the strains which could impact the attachment (Zeraik and Nitschke, 2012).

Other than ionic strength and temperature, bacterial attachment can also be influenced by the environment pH and nutrients condition. Environmental pH would influence cell surface charge as well as surface structure such as polymeric material. It was shown that *E. coli* would have higher attachment to either hydrophobic or hydrophilic surface at condition of pH 7, than conditions of pH 6 and 8 (Mafu et al., 2011). Moreover, nutrients can also impact bacterial attachment and in previous research bacteria demonstrated higher attachment when cultured in rich nutrient medium (Zeraik and Nitschke, 2012).

Bacterial attachment to sediment particles is a dynamic process affected by the bacterial properties, particle properties, and environmental factors that regulate those properties (Goulter et al., 2009). However, there are conflicts among previous research findings, and understanding of the factors driving these interactions is incomplete. In this study, we will evaluate genetic, particle, and environmental factors of *E .coli* attachment behavior to model stream particles.

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CHAPTER 3. DIVERSITY IN PROPERTIES OF *E. COLI* DERIVED FROM STREAM WATER AND SEDIMENT

A paper submitted to *Applied and Environmental Microbiology*

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Abstract

We investigated the variation in characteristics of *E. coli* obtained from stream water and stream bottom sediments. Cell properties were measured for each of selected 44 sediment and 33 water genomically different *E. coli* strains under common stream conditions in the Upper Midwestern United States: pH 8, ionic strength 10mM and 22˚C. Measured cell properties include hydrophobicity, zeta potential, net charge, total acidity and extracellular polymeric substance (EPS) composition. Our results indicate that stream sediment *E. coli* had significantly greater hydrophobicity, greater EPS protein content and EPS sugar content, less negative net charge, and higher point of zero charge than stream water *E. coli*. A significant positive correlation was observed between hydrophobicity and EPS protein for stream sediment *E. coli* but not for stream water *E. coli*. Additionally *E. coli* surviving in the same habitat tended to have significantly larger $(GTG)_5$ genome similarity. When eliminating the intrinsic impact from the genome, environmental habitat was determined to be a regulating factor in some cell properties such as hydrophobicity. The diversity of cell properties and resulting impact on particle interactions should be considered for environmental fate and transport modeling of aquatic indicator organisms such as *E. coli*.

Introduction

Currently, elevated levels of *E. coli* are the leading cause of water quality impairments in rivers and streams in the United States (1). Therefore, improved understanding of *E. coli* properties in the environment is needed for predicting its fate and transport and to support the plan development to reduce bacterial contamination of waters. Recent studies have indicated that there is high diversity of *E. coli* isolates in the environment (2-4). This strain level diversity could be attributed to differences in both genotype and phenotype, and therefore likely impacts fate and transport of *E. coli*. Moreover, bacterial survival and growth is a dynamic process affected by bacterial surface properties, such as EPS, hydrophobicity, and net charge; both genomic and environmental factors regulate those properties (5).

Under typical stream pH in the Upper Midwestern United States (6-8), *E. coli* surfaces are negatively charged due to the dissociation of carboxyl and phosphate groups in the peptidoglycan and lipopolysaccharides of cell walls (5, 6). While the surface charge of bacteria is highly environment-dependent (7), it can impact the bacterial state by repulsion of similarly charged particulates and by attraction of opposite charged particulates (3, 8). The hydrophobicity of a bacterial cell is determined by functional groups of both residues and structures on the surface of the cell, which can be either hydrophilic or hydrophobic (9). Increased culturing time to the stationary phase may reduce hydrophobicity, while the carbon concentration of the growth medium could positively impact hydrophobicity. Such impacts are partially due to the effects on lipid composition (10). The presence of divalent cations, such as Ca^{2+} and Mg^{2+}, could increase bacterial hydrophobicity (11), since the cations principally attach to the hydrophobic fraction of proteins on the bacterial surface and decrease the hydrophilicity (12-14). EPS are high-molecular-mass compounds secreted by microorganisms at the outer cell surface (15). They are mainly composed of polysaccharides and proteins, but they may also include other macromolecules such as DNA, lipids, and humic-like substances. EPS contributes to the overall heterogeneity of bacterial surface (16, 17) and plays an important role in cell aggregation, cell adhesion, and protection of cells from hostile environments (18-20). For example, the formation of biofilms in stream bottom

sediments requires involvement of EPS (21). The sugar/protein ratio of EPS has been positively correlated with the cell surface charge (22). Bolster et al. (3) reported that EPS production mostly occurred in the late growth phase of bacteria, and Shin et al. (22) showed that the EPS sugar/protein ratio may change dramatically as culturing time increases. Moreover, EPS structure has been found to become more compact as environmental pH decreases (18).

Current water quality assessment techniques are based on environmental sampling, for which only the freely suspended populations of fecal indicator bacteria are collected (23, 24); this procedure does not assess microbial contamination of stream-bottom sediments. However, previous research has indicated that after entering surface waters, microorganisms often partition into either the planktonic state or they attach to suspended soil and organic particles (25-28). The populations of bacteria surviving in bottom sediments are protected from ultraviolet radiation (29, 30), resulting in an extended survival time. When stream bottom sediments are disturbed during changes in flow, there is increasing likelihood of resuspension back into the water column (31-33). Therefore, improved understanding of the properties of sediment-associated *E. coli* is critically important for understanding bacterial fate in the environment.

An assessment of the variation of *E. coli* cell properties in different environments (stream bottom sediments versus the overlying water column) is needed to better understand environmental fate and transport. In this study, we divided the potential impacts on bacterial surface properties into two parts: genomic impact (intrinsic) and environmental impact (extrinsic). The overall goal was to determine if differences in environmental *E. coli* cell surface properties are due to extrinsic or intrinsic properties, or an interaction of these two. Assessed cell properties of 77 genomically distinct *E. coli* strains included hydrophobicity, zeta potential, net charge, total acidity, and EPS composition (protein and sugar). The objectives of our study were: 1) to compare bacterial properties between *E. coli* isolated from two environmental habitats, stream sediments and stream water; 2) to determine the correlations among bacterial surface properties within each environmental habitat and compare the correlations obtained from different environmental habitat; 3) to explore the relationship between genomic similarity with environmental habitat.

Materials and Methods

To investigate the potential impacts from intrinsic genomic and extrinsic environmental aspects, *E. coli* strains collected from two environmental habitats were studied. For each *E. coli* strain, selected bacterial surface properties were measured: hydrophobicity, zeta potential, net charge, total acidity, and EPS composition by extraction and colorimetric techniques. Genome similarities were analyzed for each pair of *E. coli* strains.

E. coli Sampling and Analysis

Stream sediment and water were collected 6 times from two locations along Squaw Creek in Ames, IA, in 2012 and 2013: Cameron School Road (latitude 42.0707, longitude -93.6728), and Brookside Park (latitude 42.0290, longitude -93.6288). Water samples were collected by lowering a horizontal polycarbonate water bottle sampler (2.2 L, Forestry Suppliers Inc., Mississippi, U.S.) from a bridge into the center of the creek at both of the locations. Sediment samples were collected from the top 2-3 cm of the streambed using a shallow water bottom dredge sampler (15 cm×15 cm opening, Forestry Suppliers Inc., Mississippi, U.S.) at the same location as the water samples were collected. Immediately after collection, samples were placed on ice. The sediment-associated *E. coli* were detached by stirring a mixture of sediment and deionized water (ratio 1:1) for 15 min at approximately 200 rpm using a magnetic stir bar. One mL of the resulting sediment solution was filtered through a 0.45-μm cellulose filter paper (EMD Millipore; Pittsburg, PA). *E. coli* strains were incubated on the filter paper using modified mTEC agar plates (34). One single colony was selected from each agar plate and the plate-sticking method was applied to ensure that the selected colony was formed by only one *E. coli* strain. Two hundred strains were isolated from the stream sediment. Each 100-mL water sample was filtered through a 0.45-μm filter paper, and another 200 strains were obtained from the water samples. After isolation, the strains were inoculated in Luria-Bertani liquid media (BD Biosciences; San Jose, CA), grown to the stationary phase, and stored at -80˚C in 15% glycerol.

Computer-assisted rep-PCR DNA Fingerprint aAnalysis

Rep-PCR was performed as described by Rademaker and de Bruijn (35) with $(GTG)_5$ (the sequence of 5'-GTGGTGGTGGTGGTG-3') as primer (36-38). Briefly, the PCR reaction contained 12.5 µL PCR-master- mix (2X, Qiagen), 10 µL primer (50 pmol) and 2.5 µL water for a total volume of 25 µL. A small fraction of a fresh *E. coli* colony was transferred to the PCR mixture as the template by using a 1-µL loop. PCR was conducted in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The thermo cycler program was set for an initial denaturation at 95°C for 2 min, 32 cycles of denaturation (94°C for 3 sec and 92°C for 30 sec), annealing (40°C for 1 min) and extension (65°C for 8 min), then a final extension at 65 °C for 8 min. Then 10 µl of resulting PCR products and 2 µL of 6X loading dye mixture (Life Technology, Grand Island, NY) were loaded onto 1.5% agarose gel, electrophoresis was applied at 4°C and 80 V for 13.5 hours, and the sample was stained for 20 minutes in TAE solution containing 0.5 µg mL^{-1} ethidium bromide. Gel pictures were captured with a Molecular Imager ChemiDoc (Bio-Rad, Hercules, CA).

The resulting gel image files were imported into Bionumerics (version 7.1, Applied Maths, Kortrijk, Belgium) for normalization, band identification, and cluster analysis. The 1Kb Plus DNA ladder (Life Technology, Grand Island, NY) was loaded into every tenth well and was used as an external control for normalization. Bands of more than 5000 bp and less than 300 bp were eliminated from analysis to avoid false clustering. Similarity coefficients for each strain pair were generated for Bionumerics programming by Pearson's correlation method with a band matching tolerance of 0.5%, and an optimization value of 0.5% (39). The technique of unweighted pair groups with mathematical averages (UPGMA) was used for clustering and generating the dendrogram. Strains with similarity less than 90% were considered genomically different. Meanwhile *E. coli*- $(GTG)_5$ genomic similarity matrix was also obtained from Bionumerics.

Genomically different *E. coli* strains were selected and inoculated in M9 broth (minimal media) with 0.4% (w/w) glucose at 37˚C and incubated to early stationary phase (OD600=1.0-1.5). To harvest the cells, *E. coli* were centrifuged for 15 min at 4,000

rpm/1878 ×*g* (Centrifuge 5430R with Rotor F-35-6-30, Eppendorf, Hauppauge, NY) at 4˚C. The supernatant was discarded, and the cell pellet was used for property analysis.

Cell Properties

E. coli outer membrane has several components that contribute to cell surface properties, such as hydrophobicity, surface charge, zeta potential, and components of extracellular polymeric substances. Fig. 1 shows a schematic summary of *E. coli* surface properties measured in this study. The microbial adhesion to hydrocarbon (MATH) method was employed to estimate the hydrophobicity of the *E. coli* strains (40, 41). Briefly, the cell pellet was resuspended in 4 mL of deionized water. The OD546 of the cell suspension (initial OD546) was measured by spectrophotometer (HACH, Loveland, CO). Then the cell suspension was transferred to individual glass test tubes (1.7 cm in diameter, 15 cm in length), each of which contained 1 mL of dodecane (99%, Fisher Scientific, Fair Lawn, NJ). The test tubes were vortexed (Fisher Scientific, Fair Lawn, NJ) at full speed for 2 min and then left vertically for 15 min for phase separation. The OD546 of the aqueous phase was determined and hydrophobic partitioning of the bacterial suspension was calculated by using this equation from Pembrey et al. 1999: hydrophobic partitioning = (initial OD546 - OD546 of aqueous phase)/ initial OD546. The analysis was performed in triplicate.

Zeta potential measurements were performed at room temperature using a Zetasizer Nano-ZS. To mimic typical stream environments of the Upper Midwestern United States, a solution of $CaCO_3$ was prepared by diluting saturated $CaCO_3$ solution to pH 8 and an ionic strength of 10 mmol L^{-1}. The ionic strength of the $CaCO_3$ solution was estimated by electrical conductivity (Eq. A1), which was measured by conductivity meter (Fisher Scientific, Fair Lawn, NJ). The *E. coli* cell pellet was washed twice with $CaCO_3$ solution then suspended in $CaCO_3$ solution to OD600=0.1. The resulting suspension was poured into a disposable capillary cell (DTS1070). Each measurement had 12 runs; a delay time of 10 seconds was used between each run to avoid Joule heating of the sample. Between each measurement, disposable capillary cells were rinsed by $CaCO_3$ buffer, followed by the test bacterial suspension. The average and standard deviation of 12 runs were given by the software output.

Potentiometric titration of *E. coli* cells was conducted to measure the acidity of the bacterial surface. The harvested cell pellet was suspended in $CaCO_3$ solution (pH=8, ionic strength of 10 mmol L^{-1}) to obtain an approximate concentration of 10^9 cells mL^{-1}. The concentration of *E. coli* cells in the suspension was determined by cellometer (Auto M10, Nexcelom Bioscience LLC, Lawrence, MA). Then the solution pH was adjusted to 4 by addition of 0.01 mol L^{-1} HCl. Next, the *E. coli* suspension was purged with nitrogen gas for 1 hour to remove dissolved carbon dioxide (17), and then it was titrated with NaOH (0.01 mol L^{-1}) from pH 4 to 10 using a titrator (Multitasking titration system, Lab synergy, Goshen, NY). A blank titration with $CaCO_3$ solution without *E. coli* was run separately. The number of moles of deprotonated sites was calculated as described by Fein et al. (2005):

$$[H^+]_{\text{net charge per cell}} = ((C_A - C_B - [H^+] + [OH^-])_{\text{sample}} - (C_A - C_B - [H^+] + [OH^-])_{\text{blank}})/N_{\text{bact}}$$

where N_{bact} is the total number of bacterial cells per mL of solution obtained by the cellometer; C_A and C_B are the concentrations (in mol L^{-1}) of acid and base (including initial amounts of acid or base added to the suspension prior to the titration); $[H^+]$ and $[OH^-]$ are the concentrations of H^+ and OH^-, calculated from the measured pH. The net charge was determined as the difference of charge between the *E. coli* suspension sample and the blank. The total acidity was obtained by subtracting the net charge at pH 10 from the net charge at pH 4. The surface charge at pH 8 and the point of zero charge (PZC) were also points of interest. The sample analyses were performed in duplicate while the blank solutions were titrated in triplicate and averaged. Fig. 2 shows an example of the potentiometric titration curve to demonstrate the useful information which can be obtained from this measurement.

The EPS, specifically the total protein and the polysaccharide content, was determined by an extraction method (42). Briefly, *E. coli* cells were incubated on a 0.45-μm filter membrane on multiple mTEC agars overnight at 37˚C to obtain the total amount of *E. coli* cell within the range of 3×10^{10} cells/ml to 6×10^{10} cells, and then the membrane was placed in 30 mL of 0.85% (w/v) NaCl solution. The *E. coli* concentration was measured by cellometer. After centrifugation at 16,300×*g* for 30 min at 4˚C, the supernatant was filtered through a 0.45-μm filter. The filtrate was then added to 90 mL of

ice-cold 100% ethanol and incubated at -20˚C for 24 h. Finally, the EPS pellet was harvested by centrifugation at 16,300×g for 30 min at 4˚C and air-dried in a fume hood. The analysis of EPS protein was conducted using the Lowry method (43), which is a spectrometric method based on measurement at a wavelength of 500 nm using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as the standard. The EPS sugar was analyzed by the phenol-sulfuric acid method, which is based on measurement at a wavelength of 488 nm using xanthan gum as the standard (44, 45).

Data Analyses

Statistical analysis of data was performed using R project software (ver. 3.1.3, Institute from Statistics and Mathematics, Vienna University of Economics and Business, Vienna, Austria). The nonparametric Wilcoxon signed-rank test was used to determine if any of the properties varied between sediment *E. coli* strains and water *E. coli* strains. To investigate the correlation between any two *E. coli* properties, the Kendall-tau correlation method and the LOESS-smoothing method were applied. The Mantel test was conducted to determine the correlation between *E. coli*- $(GTG)_5$ genomic similarity and environmental habitat (stream bottom sediments or overlying water). In this method, the statistic indicates the correlation between two matrices: one matrix contained estimates of genomic similarity between all possible pairs of strains, while the other contains the habitat similarity between all possible pairs (0 for different environmental habitats, 1 for the same environmental habitat). Moreover, the method of phylogenetic generalized least squares was employed to explore the environmental habitat impact on bacterial properties, by excluding the potential impact from genomic similarity. Phylogenetic methods are used in the analysis of interspecies data because species are non-independent for the purposes of statistical analysis (46). When applying the method of phylogenetic generalized least squares analysis (R package ‘pGLS’), one bacterial property was considered as the response (***Y***), environmental habitat was considered as the binary predictor (***X***), and similarity matrix was considered as the variance-covariance matrix. The method uses a variance-covariance matrix to weight the predictors (47).

Results and Discussion

E. coli Strain Selection and Dendrogram

By computer-assisted rep-PCR DNA fingerprint analysis, 45 sediment strains (22.5% of 200 strains) and 33 water strains (16.5% of 200 strains) were considered genomically distinct on the basis of the 90% similarity criterion. Fig. 3 shows the dendrogram of these 78 selected strains with similarities of rep-PCR fingerprint, while Fig. A1 shows the dendrogram of the electrophoresis image. There was no obvious cluster pattern for *E. coli*-$(GTG)_5$ genomic profiles of strains from the same environmental habitat (stream water or sediment). Note that one sediment strain (strain No. 122) had insufficient growth in M9 broth to proceed, so further analyses were based on 44 sediment strains and 33 water strains.

E. coli Property Comparison between Two Environmental Habitats

Although each *E. coli* strain was subjected to the same storage and growth conditions, diversities in properties of *E. coli* derived from stream water and sediment were observed. Hydrophobicity, as measured by the MATH assay, ranged from 0.01 to 0.90. Zeta potential ranged from -6.76 mV to -39.87 mV. Total EPS protein content ranged from 0.30 to 0.86 $\mu g/10^8$ cells, while total sugar content of EPS ranged from 0.80 to 1.74 $\mu g/10^8$ cells. The EPS protein/sugar ratio ranged from 0.07 to 8.78. Net charge at pH 8 varied from -2.48×10^{-4} to 1.60×10^{-5} meq/10^8 cells. Variation was also observed in the total acidity and point of zero charge. Fig. 4 shows the boxplots of property results analyzed for sediment *E. coli* strains and water *E. coli* considered separately.

Statistically significant differences in cell properties were observed between stream sediment *E. coli* and water *E. coli* in many cases (Fig. 4). For example, according to the Wilcoxon test, hydrophobicity of sediment *E. coli* was significantly greater than hydrophobicity of water *E. coli* (p-value=0.005). Similarly, sediment *E. coli* had greater EPS protein content (p-value=0.006) and sugar content (p-value=0.036), less negative net charge (p-value=0.026), and higher point of zero charge (p-value=0.009) than stream water *E. coli*. Cell surface hydrophobicity of sediment *E. coli* strains was also shown to be greater than that of water *E. coli*, consistent with the report of Stenstrom (48) and Zita and Hermansson (49), who reported that higher hydrophobicity coincided with greater

adhesion to mineral particles in water and sludge flocs in sludge liquor from wastewater treatment plan .

The zeta potential of suspended *E. coli* cells reflects the electrokinectic potential of the cells. Colloid stability (i. e, the likelihood that cells will not coagulate with one another) will increase as the absolute value of the zeta potential increases. Previous studies have indicated that zeta potential ranged from -4.9 to -29 mV for 280 different *E. coli* strains (50), which is consistent with the range of our results. In our study, there was no significant difference between zeta potentials measured for sediment *E. coli* strains and water *E. coli* strains. Previous research has found no clear correlation between the electrophoretic mobility of bacterial cells and their adhesion to negatively charged polystyrene surface (51) and quartz particles (3).

Our results also indicated that stream sediment *E. coli* strains had significantly higher EPS protein and sugar content than stream water strains, perhaps because cell adhesion and biofilm formation require EPS (18, 19). The EPS protein/sugar ratio can be dramatically impacted by environmental pH (18), culturing time (22), culturing medium and extraction method (21, 52). However, while the absolute values of EPS protein and sugar contents differed between the two habitats, no significant difference in the EPS protein/sugar ratio was observed in our study.

Moreover, the net charge at pH 8 of stream sediment *E. coli* was significantly less negative than the net charge of water *E. coli*. While *E. coli* cells in both water and sediment carry an overall negative surface charge at pH 8, those strains living in water are likely to have a greater repulsion from negatively charged sediment surfaces (3, 8). The more negatively charged cell surfaces would also have a lower point of zero charge, as was indicated in our results.

E. coli Property Correlations

Pair-wise correlations between the different cell properties measured in this study were generally low and not statistically significant when analyzed for all 77 *E. coli* strains (Table 1). Of the statistically significant correlations observed in this study, some were between properties which had shared parameters or measurements so the correlations were artificially inflated. Such correlations include: the EPS protein/sugar

ratio with EPS protein and EPS sugar, net charge with acidity, and net charge with point of zero charge. One meaningful correlation between cell properties is the strong positive correlation between hydrophobicity and EPS protein content (r (correlation coefficient) =0.283; p-value=3.914×10^{-4}). However, the correlation between hydrophobicity and the EPS protein was not the same for stream sediment *E. coli* and water *E. coli*. Using scatterplots with smoothing curves, histograms of each property, and the results from the Kendall-tau correlation method, Fig. 5 shows the correlations between *E. coli* hydrophobicity and EPS protein content for stream sediment *E. coli* and water *E. coli*, respectively. For sediment *E. coli*, there was significant positive correlation (r=0.407, p-value=1.274×10^{-4}) between hydrophobicity and the EPS protein; while for water *E. coli*, no significant correlation was observed (r=-0.103 with p-value=0.416). Previous research has found that hydrophobic components of EPS are mainly comprised of proteins (12, 53). Additional studies to characterize EPS proteins associated with the two groups of *E. coli* would be helpful to determine the mechanisms behind these observations.

By definition, surface charge and zeta potential are related. However, our results indicate only a very weak correlation between net charge and zeta potential with r=0.008 with p-value=0.316. Thus our results may temper the conclusions of some previous research in which zeta potential has been used to estimate surface charge (54, 55).

Correlation between Genomic Similarity with Environmental Habitat

To explore possible relationships between *E. coli*-$(GTG)_5$ genomic similarity and environmental habitat (stream sediment or water), the Mantel test was applied. The results showed a significantly positive correlation between genomic similarity and environmental habitat (r = 0.063; p-value = 0.002). This finding indicates that genomic similarity was larger for any two *E. coli* strains derived from the same environmental habitats.

Previous studies have been unable to determine if differences in environmental *E. coli* cell surface properties and genomic variation residing in different environmental habitats (stream bottom sediments versus overlying water) are due primarily to environmental habitat (extrinsic), genomic similarity (intrinsic), or an interaction of these two. Below is a summary of the major conclusions:

- Statistically significant differences in cell properties were observed between stream sediment *E. coli* and water *E. coli*; most notably, sediment *E. coli* had significantly greater hydrophobicity, EPS protein content, and EPS sugar content; less negative net charge; and higher point of zero charge when compared to water *E. coli*.
- Hydrophobicity and the EPS protein were positively correlated for stream sediment *E. coli* but not for water *E. coli*.
- Genomic similarity was greater for any two *E. coli* strains derived from the same environmental habitat.

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Tables

Table 3.1. Correlation coefficient matrix for cell properties for all *E. coli* strains (n=77) obtained from Kendall-tau correlation method. Statistically significant ($p \leq 0.001$) are in italic and bold.

	Hydrophobicity	Zeta potential	EPS protein	EPS sugar	Ratio (EPS protein/sugar)	Net charge at pH 8	Acidity
Zeta potential	-0.047						
EPS protein	***0.283***	-0.179					
EPS sugar	-0.060	-0.177	0.095				
Ratio (EPS protein/sugar)	0.203	-0.016	***0.403***	***-0.522***			
Net charge at pH 8	0.131	0.078	0.035	-0.141	0.066		
Acidity	0.016	-0.085	0.148	0.064	0.078	***-0.540***	
Point of Zero Charge	0.237	0.075	0.149	-0.121	0.136	***0.403***	-0.170

Figures

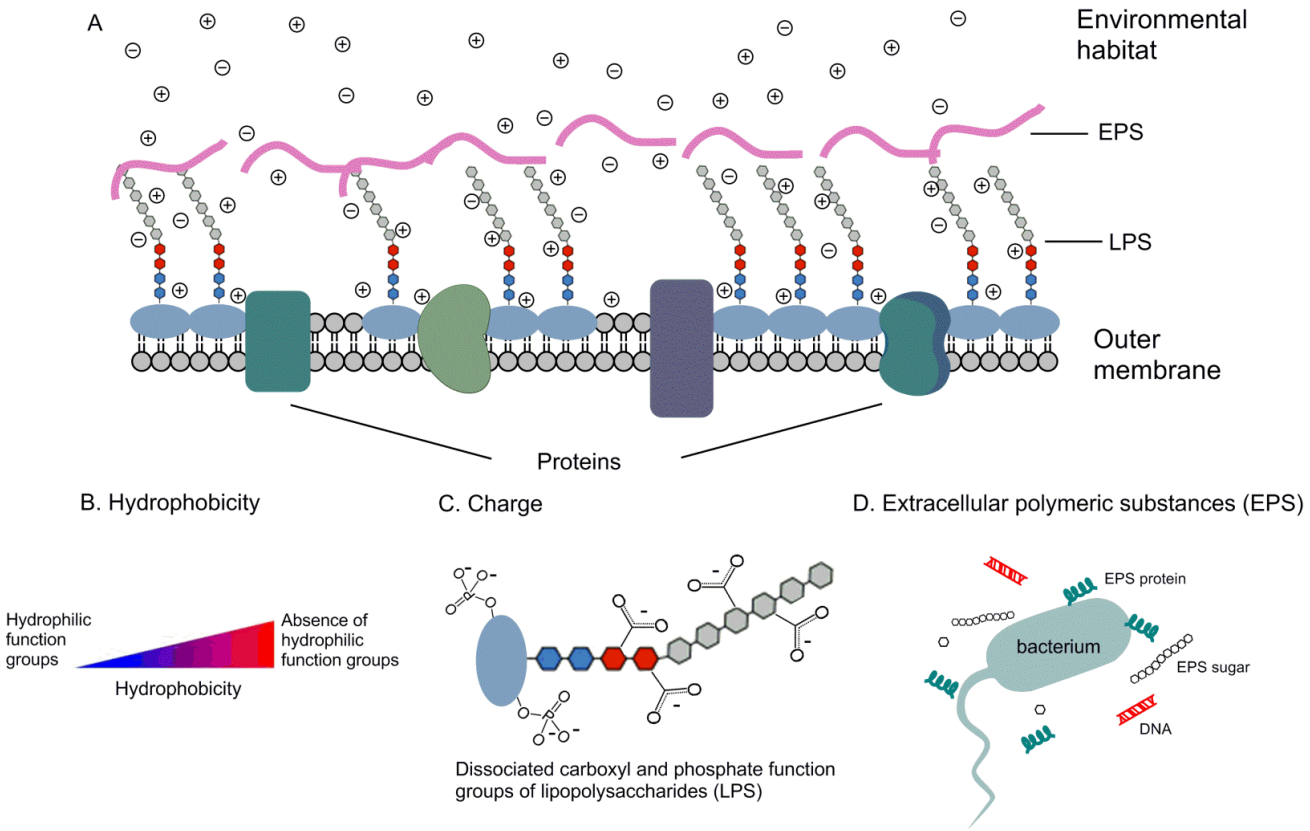

Fig. 3.1. Schematic depiction of *E. coli* surface properties. ⊕, cation in solution; ⊖ anion in solution; -, negative charge due to dissociation. A. *E. coli* outer membrane has several components that contribute to cell surface properties, such as hydrophobicity, surface charge, zeta potential, and components of extracellular polymeric substances. B. Cells that are absence of hydrophilic functions groups are more hydrophobic than the cells with hydrophilic functions groups. C. Surface is negatively-charged due to the dissociation of carboxyl and phosphate function groups, and the dissociation can also occur on the EPS and phospholipids of the membrane. Therefore, more cations than anions exist in solution, and that separation of charge is measured by the zeta potential. D. EPS is mainly composed of sugars and proteins, but they may also include other macromolecules such as DNA.

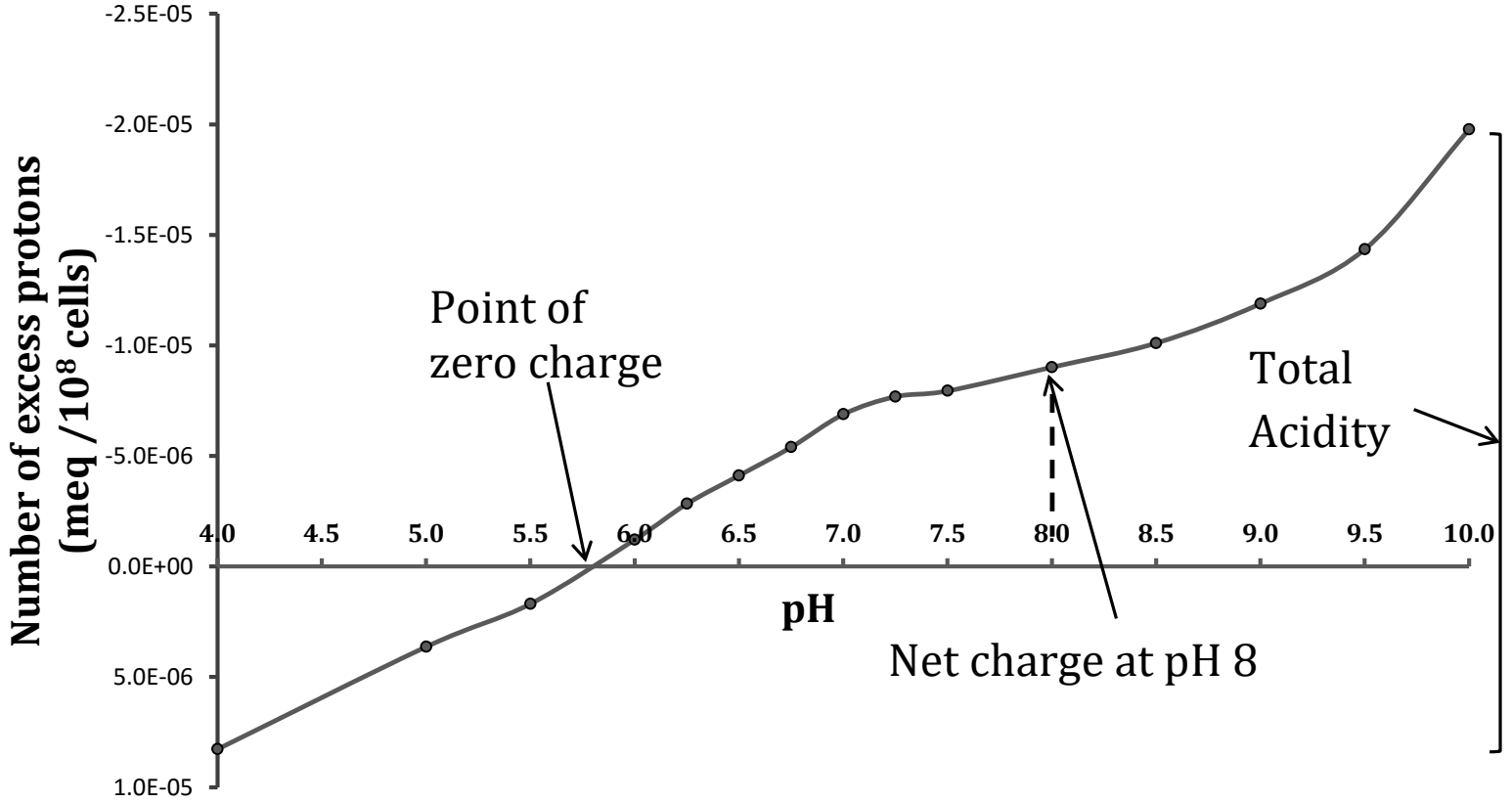

Fig. 3.2. Potentiometric titration curve example from strain number 53. Three pieces of information can be obtained from the curve: 1. Point of zero charge of *E. coli* strain; 2. Net charge at pH 8 carried by *E. coli*; 3. Total acidity of *E. coli* strain.

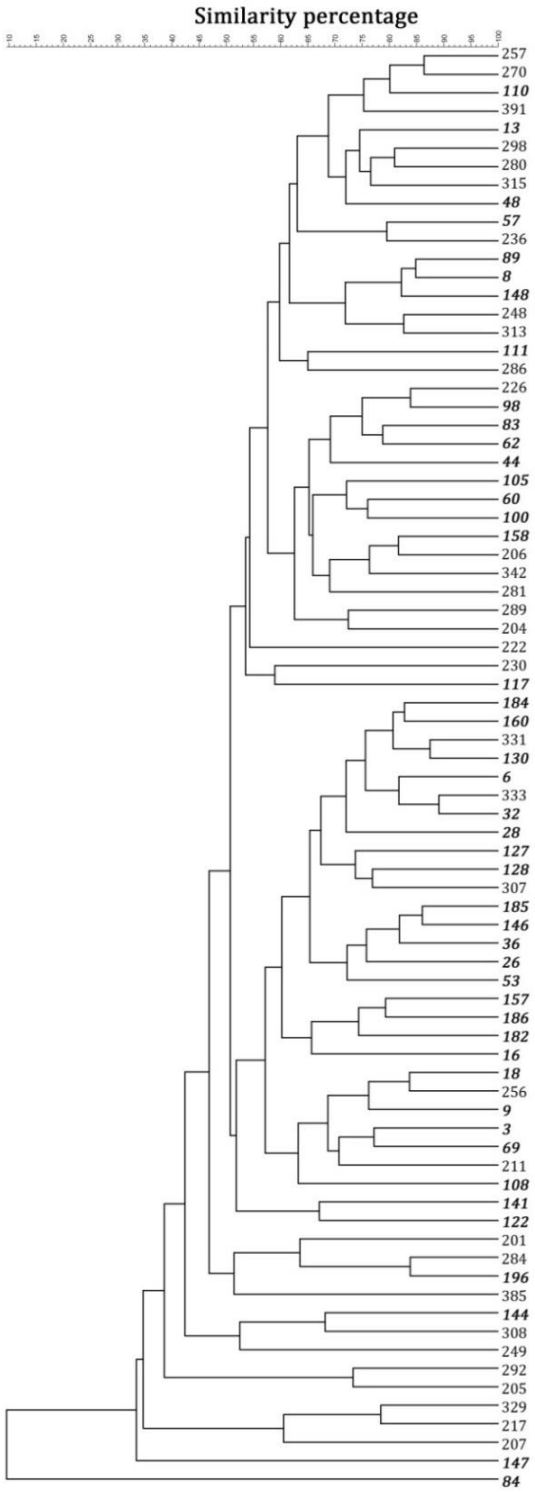

Fig. 3.3. The dendrogram shows the percent similarity for 78 selected strains with similarity less than 90%, based on a UPGMA cluster analysis. Strains 1-200 (italic and bold) were collected from stream sediment; while 201-400 were collected from stream water.

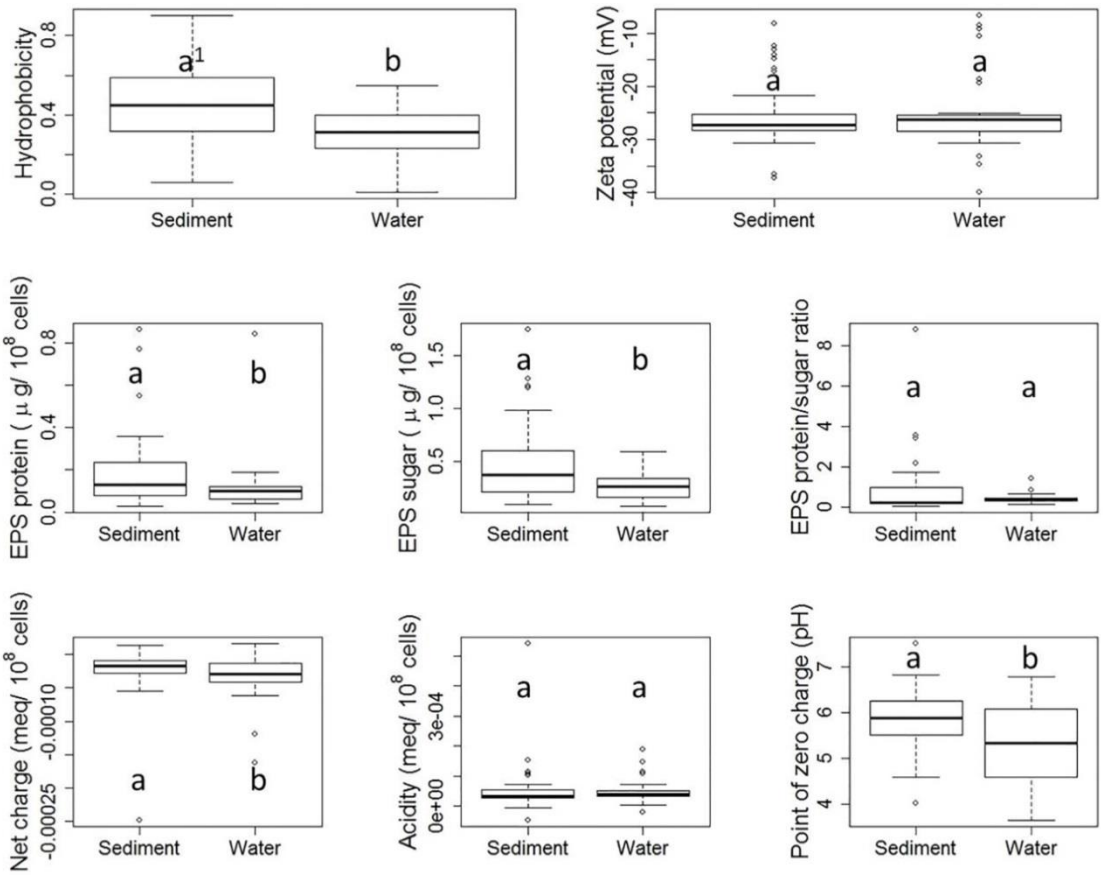

Fig. 3.4. Boxplots of property results from stream sediment *E. coli* and stream water *E. coli*.

1. Within each subplot, values with the same letter are not different at the significant level. The difference is determined by a Wilcoxon test with significance level set at α=0.05.

2. Each plot show five numerical values: the smallest observation (Q1=Q2-1.5(Q4-Q1), the low end of the whisker), 25% quartile (Q2, low boundary of box), median (Q3, the band near the middle of box), 75% quartile (Q4, high boundary of box), and largest observation (Q5=Q4+1.5(Q4-Q1), the high end of the whisker). Outliers, if any, are indicated by dots.

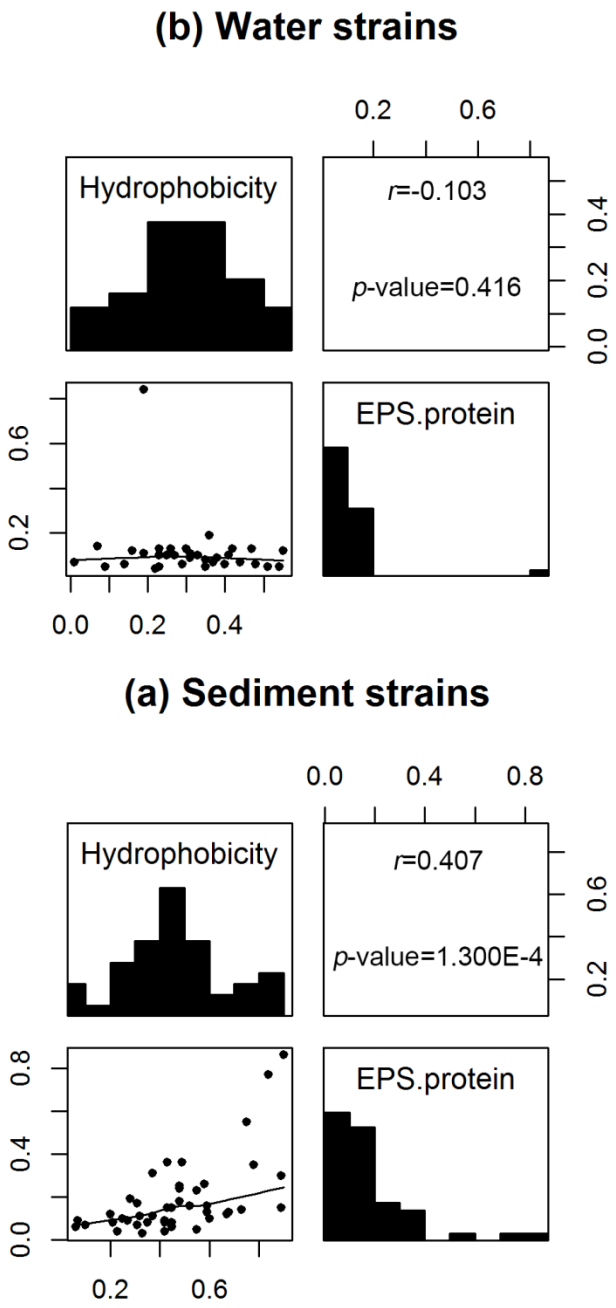

Fig. 3.5. Correlation between *E. coli* hydrophobicity and EPS protein content for (a) sediment *E. coli* strains and (b) water *E. coli* strains. The upper panels show the correlation coefficient *r* with associated *p*-value analyzed by Kendall-tau method; the diagonal panels show histograms of each property; the lower panels are scatterplots with the smoothing curves using the Lowess method.

Appendixes

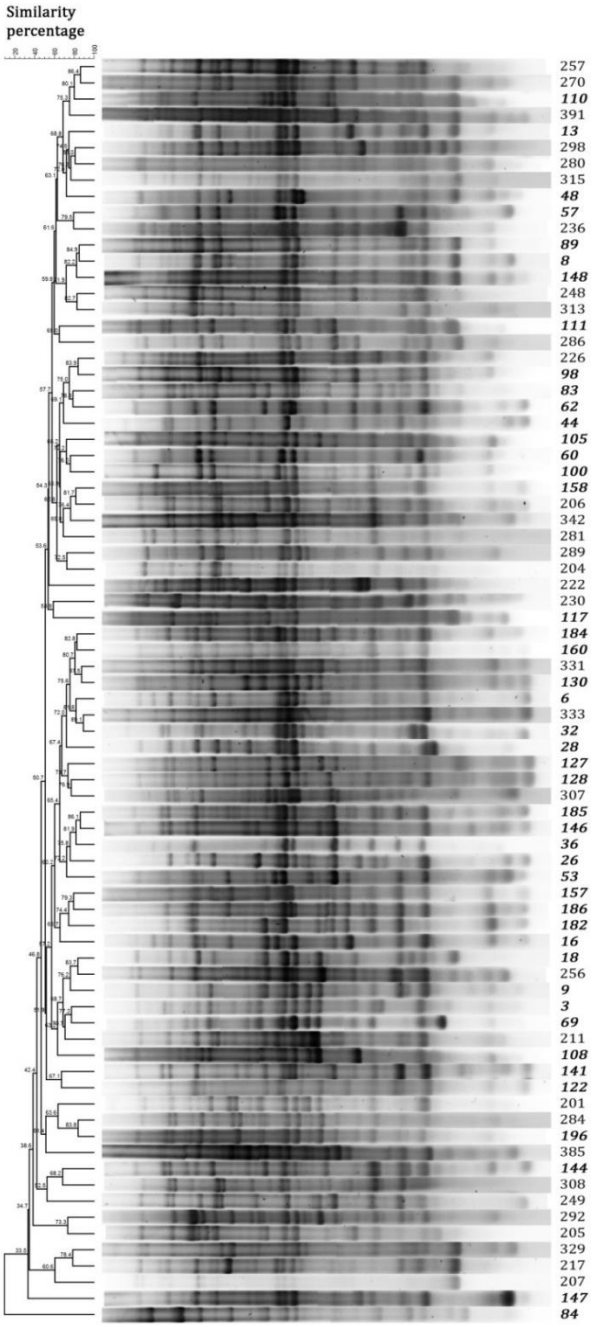

Fig. 3.A1. The dendrogram shows the percent similarity of rep-PCR fingerprint banding patterns for 78 strains with similarity smaller than 90%, based on UPGMA cluster analysis. Strains 1-200 (italic and bold) were collected from stream sediment; while 201 - 400 were collected from stream water.

Eq. 3.A1:

Ionic strength (in mmol L^{-1}) ≈0.015× Electrical conductivity (in µs/cm)

CHAPTER 4. *E. COLI* ATTACHMENT TO MODEL PARTICULATES: THE EFFECTS OF BACTERIAL CELL CHARACTERISTICS AND PARTICULATE PROPERTIES

A paper to be submitted to *Applied and Environmental Microbiology*

Xiao Liang, Chunyu Liao, Michelle L. Soupir, Laura R. Jarboe, Michael L. Thompson, Philip M. Dixon

Abstract

E. coli move freely in streams in a planktonic state or attach to suspended particulates. Attachment is a dynamic process and the fraction of attached microorganisms is affected by bacterial characteristics and particulate properties. In this study, we investigated correlations of cell and particulate properties with attachment fraction. Cell properties (hydrophobicity, zeta potential, net charge, total acidity and extracellular polymeric substance (EPS) composition) were measured for each of selected 44 sediment and 33 water genomically different *E. coli* strains. Attachment assays were conducted for each *E. coli* strain and model particulate (ferrihydrite, Ca-Montmorillonite, or corn stover) with environmentally relevant concentrations. Surface area, particle size distribution and total carbon content were analyzed for each type of particulate. Among three particulates, attachment fractions to corn stover were significantly larger than the attachments to 2-line ferrihydrite (p-value=5.5×10^{-4}) and Ca-Montmorillonite (p-value=0.0020). Furthermore, Generalized Additive Model (GAM) was used to fit the attachment fraction using cell characteristics as predictor variables. Natural logarithm absolute value of net charge showed a significant positive linear impact on the attachment fractions to Ca-Montmorillonite (p-value=0.013) but no significant impact on the attachment to corn stover (p-value=0.36). In this study, the large diversities in cell characteristics, particulate properties and attachment fractions among different *E. coli* strains and particulates clearly demonstrated the inadequacy of using a static parameter or

linear coefficient to assess the general attachment behavior. The results from this study would help with improve bacterial transport modeling in streams.

Introduction

Currently, 178,048 miles of assessed rivers and streams are contaminated due to elevated levels of pathogens or pathogen indicators, and pathogens are the leading cause of water quality impairments in rivers and streams in the United States (1). Although not all *E. coli* and enterococcus strains are pathogenic, *E. coli* and enterococci are used as indicators to predict when a risk to human health is present in fresh or marine water, respectively (2). Therefore, improved understanding of *E. coli* fate and transport in the environment is needed to identify when a risk to human health is present. In streams microorganisms move freely in water in a planktonic state or attached to suspended soil and organic particles (3-8). Therefore, to model the fate and transport, the fraction of *E. coli* that are attached to suspended particles needs to be estimated (9). Previously, the fraction of attached *E. coli* was estimated as a static parameter (10), or predicted based on a linear correlation with planktonic *E. coli*, in which the coefficient is proportional to suspended clay content (11, 12). However, bacterial attachment to particulates in aquatic environments is a dynamic process, affected by bacterial characteristics, particulate properties, and environmental factors.

Bacterial surface characteristics, such as net charge, hydrophobicity, and extracellular polymeric substances (EPS) can impact bacterial attachment to particulates (13, 14). *E. coli* cells are typically negatively charged due to the carboxyl and phosphate groups present in peptidoglycan and lipopolysaccharide that compose the cell walls. The surface charge can impact attachment to particles by repulsion of similarly charged particles and by attraction of particulates with an opposite charge (15, 16). The hydrophobicity of a bacterial strains differ because hydrophobicity is determined by functional groups of both residues and structures on the surface of the cell, and these can be either hydrophilic or hydrophobic (17). Some studies have found a positive correlation between bacterial attachment and hydrophobicity (18-22). However, Bolster et al. (2009)

reported no correlation between hydrophobicity and transport of 12 different *E. coli* strains through packed quartz beds.

Extracellular polymeric substances (EPS) are mainly composed of polysaccharides and proteins, but they may also include other macro-molecules such as DNA, lipids, and humic-like substances (23). It plays an important role in cell aggregation, cell adhesion, biofilm formation, and protection of cells from hostile environments (24). Polysaccharides cellulose in EPS is essential for *E. coli* attachment to plastic surfaces (25), and the EPS polysaccharides /protein ratio is positively correlated with cell surface charge (26). But researchers found no correlation between the presence of three EPS-associated genes (*ompC, slp, and surA*) and quartz attachment (27).

Bacterial attachment can also be affected by properties of the particulates. Size and shape of particulates can impact *E. coli* attachment. Surface energy is the excess energy per unit surface area and larger-sized or high spherical bacterium have lower surface energy. Attachment decreases the total surface energy of the cell-particle system in the aquatic environment by forming contact regions with lower surface energy. Therefore, smaller-sized or non-spherical bacteria have a higher preference of attaching to particles than larger-sized or higher-spherical bacteria (28). Consequently, the fraction of attached bacteria to particles is typically negatively correlated with particle size (29-35). Moreover, *E. coli* survival in water bodies can increase after attaching to mineral aggregates with high sediment organic matter (SOM) concentration (36).

Given the importance of understanding *E. coli* transport in the environment, we explored correlations of cell and particulate properties with attachment fraction. The objectives of this study were to: 1) identify the impact of particulate property on *E. coli* attachment fractions; 2) construct and evaluate statistical models for predicting *E. coli* particulate attachment percentage; and 3) identify cell characteristic variables important for defining *E. coli* particulate attachment. Properties measured for each strain included hydrophobicity, excess acidity, zeta potential, size, and extracellular polymeric substance composition. Particulate properties measured included size, surface area, and organic carbon content. The results of this study will be helpful in understanding *E. coli* transport and fate in the environment.

Materials and Methods

E. coli Strain Selection and Preparation

Squaw Creek is a tributary of the South Skunk River in central Iowa, U.S.A. Stream sediment and water were collected from two locations along Squaw Creek in Ames, IA: Cameron School Road (latitude 42.0707, longitude -93.6728), and Brookside Park (latitude 42.0290, longitude -93.6288). A Horizontal Polycarbonate Water Bottle Sampler (2.2 L, Forestry Suppliers Inc., Mississippi, U.S.) was lowered from a bridge into the center of the creek at both of the locations to collect stream water samples. Sediment samples were collected from the top 2-3 cm of the streambed using a Shallow Water Bottom Dredge Sampler (15 cm opening, Forestry Suppliers Inc., Mississippi, U.S.) at the same locations as water samples. To maximum potential strain diversity, samples were collected 6 times from 10/26/2012 to 6/18/2013. After each collection, samples were transferred to the lab immediately by placing on ice in coolers. The sediment-associated *E. coli* were detached by stirring the mixture of 100 g sediment and 100 g deionized water for 15 min at approximately 200 rpm using a magnetic stir bar(9). One ml resulting sediment suspension was filtered with a 0.45-μm cellulose filter according to EPA Method 1603 and incubated on modified TEC agar (BD Biosciences; San Jose, CA) plate for 24 hours (37). One single intact colony was selected from each plate and plate-streaking method was applied to ensure the selected colony was formed by only one *E. coli* strain (38). Two hundred strains were isolated from stream sediment and water samples, respectively. A pathogenic strain was also considered, ATCC™ 43888, a genetically modified version of *E. coli* O157:H7, with the genes that produce Shiga-like toxins I and II removed. After isolation, the strains were inoculated in Luria-Bertani liquid media (BD Biosciences; San Jose, CA), grown to the stationary phase, and stored at -80˚C in 15% glycerol.

Using primer $(GTG)_5$ with the sequence of 5'-GTGGTGGTGGTGGTG-3'(39) and a similarity threshold 90%, we differentiated the 400 *E. coli* strains by rep-PCR. As a result, 45 sediment strains (22.5% of 200 strains) and 33 water strains (16.5% of 200 strains) were considered genomically distinct on the basis of a 90% similarity criterion. One sediment strain (No. 122) did not grow sufficiently in M9 minimal broth (Sigma-

Aldrich, St. Louis, MO), so further analyses were based on 44 sediment strains and 33 water strains.

Time to early stationary phase of the isolates was determined by absorbance at 600 nm (spectrophotometer, HACH, Loveland, CO). *E. coli* isolates were inoculated in 10 ml M9 broth and incubated to early stationary phase (1.0<OD600<1.5). To harvest the cells, *E. coli* suspension was centrifuged for 15 min at 4,000 rpm (Eppendorf, Hauppauge, NY) at 4˚C. The supernatant was discarded, and the cells were resuspended in $CaCO_3$ solution of pH 8 and ionic strength 10mM which was prepared from diluting saturated $CaCO_3$ solution with deionized water. The $CaCO_3$ solution was used to simulate typical aquatic conditions of the Upper Midwestern United States(40). Electrical conductivity was measured by conductivity meter (Fisher Scientific, Asheville, NC) and ionic strength was estimated by:

$$\text{Ionic strength (in mmol } L^{-1}) \approx 0.015\times \text{ Electrical conductivity (in } \mu s/cm) \quad [1]$$

Model Particulates

We conducted attachment assays with three model particulates, chosen to represent common particulates in Upper Midwestern United States stream: one organic, one layer silicate, and one iron oxide. Poorly crystalline iron oxides are common in stream sediment and can potentially attach to bacteria cells because of charge interactions. At typical stream pHs, iron oxide such as 2-line ferrihydrite will carry a net positive charge to which negative charged bacterial cells can be attracted. To prepare 2-line ferrihydrite, $Fe(NO3)_3{\cdot}9H_2O$ was added to 75˚C deionized water in a water bath, and temperature was kept as 75˚C for 10-12 minutes. After the color changed from gold to dark brown which is the color of ferrihydrite, the container was cooled in ice water and the solution was transferred into dialysis bags, followed by dialysis and freeze-drying of the resultant solid (41). Smectite is the most ubiquitous clay–size layer silicate mineral in stream sediments of the upper Midwest. We used Ca-saturated montmorillonite (The Clay Mineral Society, Chantilly, Virginia) as a model smectite. Under typical conditions of pH and ionic strength, Ca-Montmorillonite occurs as low-density, stacked-layer quasicrystals, with large, low-charge surfaces and spaces between substacks where bacterial cells can attach (42). Finally, we simulated the interactions of *E. coli* cells with a

common constituent of stream particles, particulate organic matter (POM), by conducting experiments with ground corn stover particles. Corn stover particles are dominated by primary and secondary cell walls materials that contain cellulose (a beta-linked glucose polymer) and lignin (a biopolymer composed of irregularly linked phenol monomers). These surfaces may be recognized by *E. coli* attachment factors that bind to carbohydrate receptors. Corn stover was ground, pass through a 53 μm sieve and stored at room temperature.

Analysis of Particulate Properties

Surface area of the dry particles was determined by the Brunauer-Emmett-Teller (BET) method using a NOVA Surface Area Analyzer-4200 (Quantachrome Instruments, Boynton Beach, Florida). The particle size distribution was analyzed by a Mastersizer 3000 laser particle size analyzer (Malvern Instruments Ltd, Worcestershire, UK). The total carbon content of the particulate samples was determined by dry combustion using a LECO elemental analyzer (LECO Corp., St. Joseph, Michigan).

Analysis of *E. coli* Properties

We tested the hydrophobicity, zeta potential, extracellular polymeric substance (EPS) components, net charge at pH 8, and cell acidity for each *E. coli* strain. Detailed measuring procedures were included in previous work (43). Briefly, microbial adhesion to hydrocarbon (MATH) method was employed to analyze the hydrophobicity of *E. coli* (44, 45) using dodecane as the hydrocarbon. The zeta potential (in mV) of an *E. coli* suspension with OD600=0.1 was measured at room temperature using a Zetasizer Nano-ZS (Malvern instruments Inc., Westborough MA). The total protein and the polysaccharide content (in μg $(10^8$ cells$)^{-1}$) of extracellular polymeric substance (EPS) was –determined by using an ethanol-extraction method followed by spectrometric measurements (46-48). Potentiometric titration of *E. coli* suspensions was conducted to measure the net charge (in meq $(10^8$ cells$)^{-1}$) of the bacterial surfaces (49) at pH 8 and total cell acidity (in meq $(10^8$ cells$)^{-1}$).

Attachment Assays

Attachment assays of *E. coli* cells to model particulates were investigated under conditions that represent typical stream environments of the upper Midwest: pH of 8, ionic strength of 10 mM, and 22 ˚C. The concentration of *E. coli* was set at 10^3 cells/ml, a high but reasonable value based on the past record of *E. coli* concentrations in Squaw Creek, Ames, IA(9). Moreover, previously reported values of particle concentrations instreams are: 1.06 mg/L for corn stover, 1.06 mg/L for 2-line ferrihydrite, and 108 mg/L for Ca-Montmorillonite (50-53). The chosen *E. coli* and particle concentrations resulted in particle: *E. coli* surface area ratios of 600 for samples with corn stover, 2.5×10^4 for samples with ferrihydrite, and 2×10^6 for samples with Ca-Montmorillonite.

After the *E. coli* cells attached to particles, they settled out of suspension more quickly than freely suspended *E. coli* cells. For the 2-line ferrihydrite and corn stover particles, 1 min was sufficient for all particles to settle by observation. Ca-Montmorillonite settled after 180 min, as reported previously (54). Fig. 1 shows a flow chart of the experimental procedure, which was conducted in triplicate for each strain-particle interaction. Calculated volumes of *E. coli* suspensions (1.0×10^3 CFU mL^{-1}), particle suspensions (concentration listed above), and $CaCO_3$ solution (pH of 8, ionic strength of 10 mM) were made up to 15 ml in 50-ml centrifuge tubes and the samples were shaken at 80 rpm for 10 min on an orbital shaker to enhance bacteria - particle interactions and attachment (55). After shaking, the samples were transferred to a 15-ml centrifuge tube and the tubes were placed vertically in racks to allow attached *E. coli* and particles to settle via gravity for the specified settling times as described above. For samples with Ca-Montmorillonite, the samples were placed at 4°C to reduce the possibility of *E. coli* regrowth during settling. After settling, 7.5 ml of supernatant was extracted and placed in a new conical tube. Then 1ml of supernatant was removed and serially diluted in 9 mL $CaCO_3$ solution twice. The final concentration was within a countable range (20 to 80 colony forming unit per plate (56)) recommended for the membrane filtration technique. Next one drop of Tween 85 (Fisher Scientific, Fair Lawn, New Jersey) was added to the remaining 7.5 mL and shaken at 300 rpm for 10 min with a handshaker (Fisher Scientific, Asheville, NC) (57). The serial dilution procedure was the same as described for the supernatant. *E. coli* concentrations (in CFU mL^{-1}) in the

supernatant and in the remainder were enumerated by membrane filtration in duplicate on Luria-Bertani agar. The concentration difference between the remainder and the supernatant was considered the attached *E. coli* concentration. The attachment fraction was calculated as

$$\frac{C_r - C_s}{(7.5C_r + 7.5C_s)/15} = \frac{2(C_r - C_s)}{C_r + C_s} \quad [2]$$

, where C_s is the supernatant concentration while C_r is the concentration of remainder.

Data Analysis

Wilcoxon-test was employed to compare the attachment fractions to different particulate types and Analysis of Variance (ANOVA) was used to explore the impact from particulate type on attachment fractions. For each particulate type, to investigate the correlation between each *E. coli* property and attachment fraction, the Kendall-tau correlation and the LOESS-smoothing methods were applied. To model attachment fraction using multiple *E. coli* properties, a generalized additive mode was used. Generalized additive model (GAM) is semi-parametric extension of generalized linear model: the response variable depends linearly on unknown smooth functions of predictor variables, and interest focuses on inference about these smooth functions. In general, the mean μ of a response y is related to an additive function of the predictors (x_i) via a link function g (58):

$$g(\mu) = \alpha + f_1(x_1) + \ldots + f_m(x_m) \quad [3]$$

For each particulate type, models with different subsets of *E. coli* characteristics and their interactions were generated to fit attachment fractions. Several criterions were used to evaluate model performance. The coefficient of determination (R^2) estimates the collinearity between observed and predicted values, and ranges between 0 (no linear relationship) and 1 (perfect linear relationship). Generalized cross validation (GCV) criterion was used to evaluate the models based on their goodness of fit. Chi-square test was used to compare two nested models. The preferred model is the one with the highest R^2 and smallest GCV. Quantile-Quantile plot of residuals, plot of residual vs. linear

predictors, histogram of residuals, and plot of observed values vs. linear predictors were also considered for model evaluation.

Statistical analysis of data was performed using R project software (ver. 3.1.3, Institute from Statistics and Mathematics, Vienna University of Economics and Business, Vienna, Austria). For GAM modeling, R package 'mgcv' was employed. Penalized regression splines were used as the smooth function and tensor product smooths were used for potential interactions between two predictor variables.

Results

Particulate Properties and *E. coli* - Particulate Attachment Results

Carbon content, surface area and size distribution for each of the particulates are listed in Table 1. Among three particles, Ca-Montmorillonite has the smaller size among three particles, while 2-line ferrihydrite and corn stover have similar size distribution. However, the surface areas of 2-line ferrihydrite and corn stover are different. The carbon content of corn stover is 38%, while the carbon content of Ca-Montmorillonite is 0.055% and carbon content of 2-line ferrihydrite is zero.

For each *E. coli* strain, the attachment fraction to each particulate was obtained using the settling method. *E. coli* - particulate attachment fractions range from 0.00 to 0.32. Analysis by ANOVA found the impact of particulate type on *E. coli* attachment fractions are significant (p-value=5.1×10^{-4}). Figure 2 shows the boxplots of attachment fractions analyzed by each particulate type. The mean attachment fraction is 0.062 for 2-line ferrihydrite, 0.053 for Ca-Montmorillonite, and 0.091 for corn stover. The attachment fractions to corn stover were significantly higher than the attachments to 2-line ferrihydrite (p-value=5.5×10^{-4}) and the attachments to Ca-Montmorillonite (p-value=0.0020). The attachment fractions to 2-line ferrihydrite and Ca-Montmorillonite were similar (p-value=0.82). Considering the results from particulate properties, under typical environmental conditions, *E. coli* attachment fraction likely increases as the surface area decreases and carbon content increases (36, 59).

In this study, 11% of the observed attachment fractions were zero. According to eq. 2, these are due to the uncertainty of plate counting. Plate counting of colonies is accepted by most researchers to estimate the concentration of viable bacteria in a sample. Countable range in 45-mm petri dishes was 20 to 80 colonies (56). To assess the uncertainty in our experimental procedures, 5 *E. coli* strains were cultured, harvested, diluted using the same procedures as in Fig. 1. Plating was repeated 6 times for each strain. According to the results, coefficient of variation (standard deviation divided by the mean) can reach 0.2 when 20 colonies was the average count, and the coefficient of variation decreased to 0.08 when the average is 50 colonies.

E. coli surface Characteristics Correlated with *E. coli* - Particulate Attachment

Although each *E. coli* strain was subjected to the same storage and growth conditions, differences in *E. coli* characteristics were observed in different environmental habitats. Briefly, stream sediment *E. coli* isolates had significantly greater hydrophobicity, greater EPS protein content and EPS sugar content, less negative net charge, and higher point of zero charge when compared to *E. coli* isolates collected from overlying stream water. For each particulate tested, the Kendall-tau correlation method found no significant linear relationship (p-value>0.05) between *E. coli* attachment fraction and any of the measured *E. coli* surface characteristics. Therefore, smooth functions are needed to include the range of analyzed cell characteristics as variables in predicting attachment fraction.

The histograms of cell characteristic results including zeta potential, EPS protein, EPS polysaccharide, ratio of EPS protein to polysaccharide, absolute value of net charge, and acidity indicated right-skewed distribution. The values of these characteristics were nature-logarithm (log) for better normality prior to use as predictor variables in GAM models. Tensor product smooths were used for potential interactions between two predictor variables. The model failed to predict *E. coli* attachment fractions to 2-line ferrihydrite. GAM models to fit *E. coli* attachment fractions to Ca-Montmorillonite and corn stover will be discussed in details, respectively.

GAM model to predict attachment to Ca-Montmorillonite

Values of log absolute values of *E. coli* surface charge (log |net charge|), log of EPS sugar content (log EPS sugar), and log of acidity (log acidity) were shown to be useful to model *E. coli* attachment fractions to Ca-montmorillonite. The interactions of log acidity with log net charge, and log EPS sugar with log acidity were also included. The model can be written as eq. 3:

$$\text{attachment fraction} = \alpha_0 + \alpha_1 \times \log|\text{net charge}| + f_1(\log \text{EPS sugar}) + f_2(\log \text{acidity}) + f_3(\log \text{acidity}, \log|\text{net charge}|) + f_4(\log \text{acidity}, \log \text{EPS sugar})$$

The value of R^2 for this model is 0.52 and model performance assessments are shown in Figure 3. The model failed to fit when the attachment fractions were zero but the Q-Q plot of residuals indicated a nearly normal distribution. Log |net charge| showed a significant positive linear impact on the attachment fractions to Ca-Montmorillonite (Table 2). Since all *E. coli* strains measured had negative surface net charge, we can conclude that *E. coli* strains with a more negative surface net charge are more likely attach to Ca-Montmorillonite. However, the impact from $f_1(\log \text{EPS sugar})$ was not significant (*p*-value=0.17) but $f_4(\log \text{acidity}, \log \text{EPS sugar})$ impacted the attachment fraction significantly (*p*-value=0.035). The contour plot of $f_4(\log \text{acidity}, \log \text{EPS sugar})$ (data not shown) indicated that the attachment would increase as log acidity increases or log EPS sugar decreases. Log acidity was included as $f_2(\log \text{acidity})$, $f_3(\log \text{acidity}, \log|\text{net charge}|)$ and $f_4(\log \text{acidity}, \log \text{EPS sugar})$, thus the impact from log acidity was complicated. Briefly, when the value increased from -11.5 to -9.5, attachment to Ca-Montmorillonite increased, but the attachment decreased as the value of log acidity increased from -9.5 to -8.5.

GAM model to predict attachment to corn stover

Values of log |net charge|, log EPS sugar, and hydrophobicity were useful to model *E. coli* attachment fractions to corn stover. The model can be written as eq. 3:

$$\text{attachment fraction} = \alpha_0 + \alpha_1 \times \log|\text{net charge}| + f_1(\log \text{EPS protein}) + f_2(\text{hydrophobicity})$$

The R^2 for this model is 0.23 and the model performance assessment is shown in Figure 4. Although the R^2 is lower than the R^2 for the Ca-Montmorillonite GAM model, the distribution of the residuals still suggests this model is acceptable. From Figure 4A, we can observe that the model failed to address when the attachment fractions were zero.

Log |net charge| was included in the model but no significant impact on the attachment fractions to corn stover was observed (Table 2). Moreover, the 90% confidence intervals of log |net charge| for Ca-Montmorillonite and corn stover did not overlap, which indicated that the impacts from cell net surface charge on these two particles were significantly different at α=0.10. In addition, according to the model, the impacts from log EPS sugar and hydrophobicity were neither linear nor significant. Therefore, except for our measured cell characteristics, there were other factors which could impact the attachment fractions to corn stover.

Discussion

To explore the impact of cell characteristics and particulate properties on *E. coli*, we conducted an attachment assay using one *E. coli* strain and pure particulates under lab conditions. The attachment fractions obtained from this study were lower than the findings from previous studies using environmental samples. Krometis et al. (2007) found between 20 and 35% of *E. coli* are associated with settleable during normal flow conditions. Another study conducted by Schillinger et al. (1985) determined that 10–20% of fecal coliform cells are adsorbed to suspended particles in untreated stormwater runoff. The difference indicated bacterial attachment in environmental waters might be different than our single strain and pure particulate assays. In this study we used $CaCO_3$ solution to mimic the environment with pH 8 and ionic strength 10mM, while solution in environmental water samples would have more completed ionic components. Moreover, there are always multiple types of microorganisms and particulates in environmental water samples, therefore the attachment mechanism could be complicated

The uncertainty in estimating the cell count in supernatant and reminder would cause errors in measuring attachment fractions, and the uncertainty is larger when the plate counts were smaller than 50 CFU. The uncertainty derived by cultivation of microorganisms can contribute to both laboratory bias (subsampling, dilution, pouring-plating) and measurement error (60, 61). In this study, 11% of the observed attachment fractions were zero and the models failed to model the zero attachment fractions, which caused model insufficiency. Therefore, 50 to 80 colonies per 45-mm petri dish is recommended to reduce the uncertainty of measuring attachment fractions.

This large diversity in cell characteristics, particulate properties and attachment fractions among different *E. coli* strains and particulates clearly demonstrates the inadequacy of using a static parameter or linear coefficient to assess the general attachment behavior of all *E. coli* strains. For example, in Soil & Water Assessment Tool (SWAT), it assumes linear regression between attached and planktonic bacteria and the linear coefficient depends on the percentage of clay in sediment (11). The results from this study could be referred to the future study with multiple microorganisms and particulates, and therefore help with improve bacterial transport modeling in streams.

Below is a summary of the major conclusions:

- Attachment fractions to corn stover were significantly larger than the attachments to 2-line ferrihydrite (p-value=5.5×10^{-4}) Ca-Montmorillonite (p-value=0.0020). Considering the results from particulate properties, *E. coli* attachment fractions would likely increase as the particulate surface area decreases or carbon content increases.
- Value of log |net charge| showed a significant positive linear impact on the attachment fractions to Ca-Montmorillonite (p-value=0.013) but no significant impact on the attachment to corn stover (p-value=0.36).

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Tables

Table 4.1. Particulate properties.

	Carbon content (%)	Surface area (m^2/g)	Size distribution (μm)		
			$D_x^*(10)$	$D_x(50)$	$D_x(90)$
2-line ferrihydrite	0	142	9	41	118
Ca-Montmorillonite	0.055	111	1	4	12
corn stover	38	3	18	53	106

* D_x under size distribution indicates the maximum diameter of particulates (in μm) in 10, 50, and 90% of the entire population (i.e., 10 percent of the ferrihydrite particles have diameters < 9 μm)

Table 4.2. Estimated regression coefficients of log |net charge| for deriving from GAM models to estimate attachment fractions.

Particulate	Estimate	SE	*p*-value	90% confident interval
Ca-Montmorillonite	0.025	0.010	0.013	(0.0091, 0.042)
corn stover	-0.006	0.007	0.362	(-0.020, 0.0073)

Figures

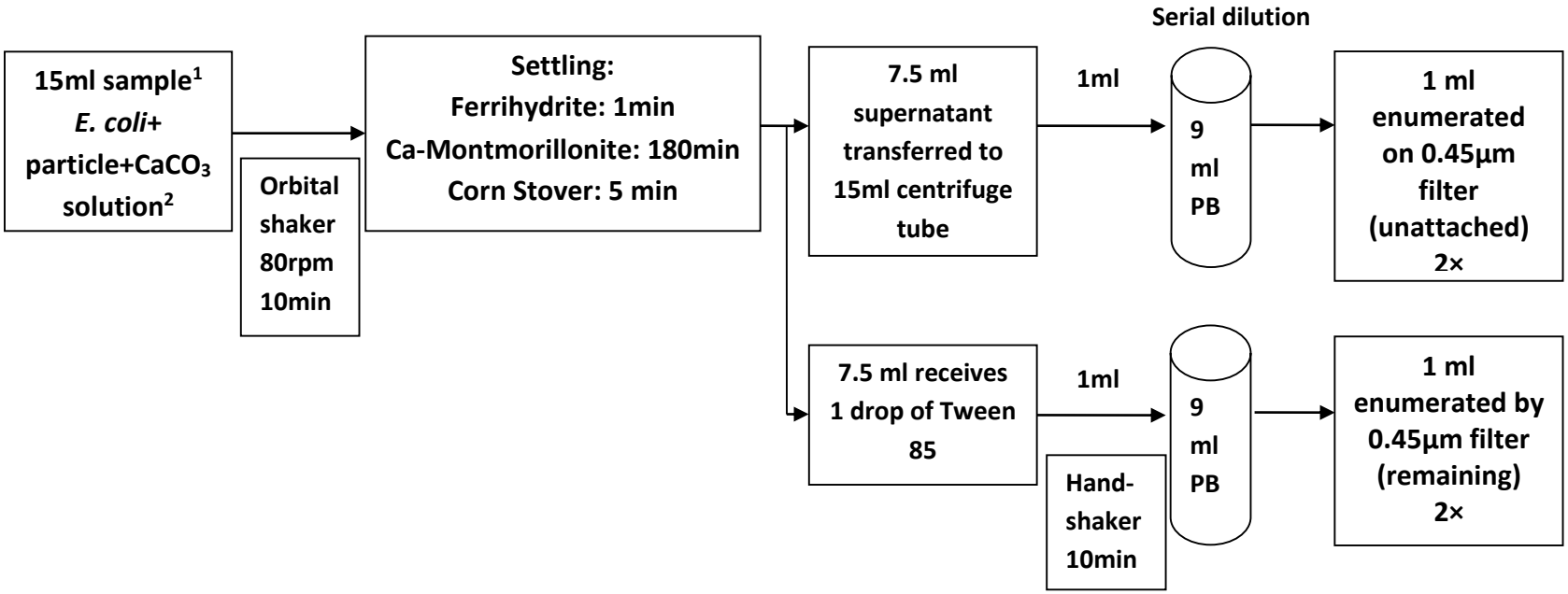

Fig. 4.1. Flow chart describing the attachment assay

1. *E. coli* concentration: 10^3 cfu/ml; Ca-Montmorillonite concentration: 108 mg/ml; Ferrihydrite concentration: 1.06 mg/ml; triplicate samples for each assay.

2. Solute: $CaCO_3$; solvent: autoclaved deionized water. This solution has pH 8, with 10mM as ionic strength

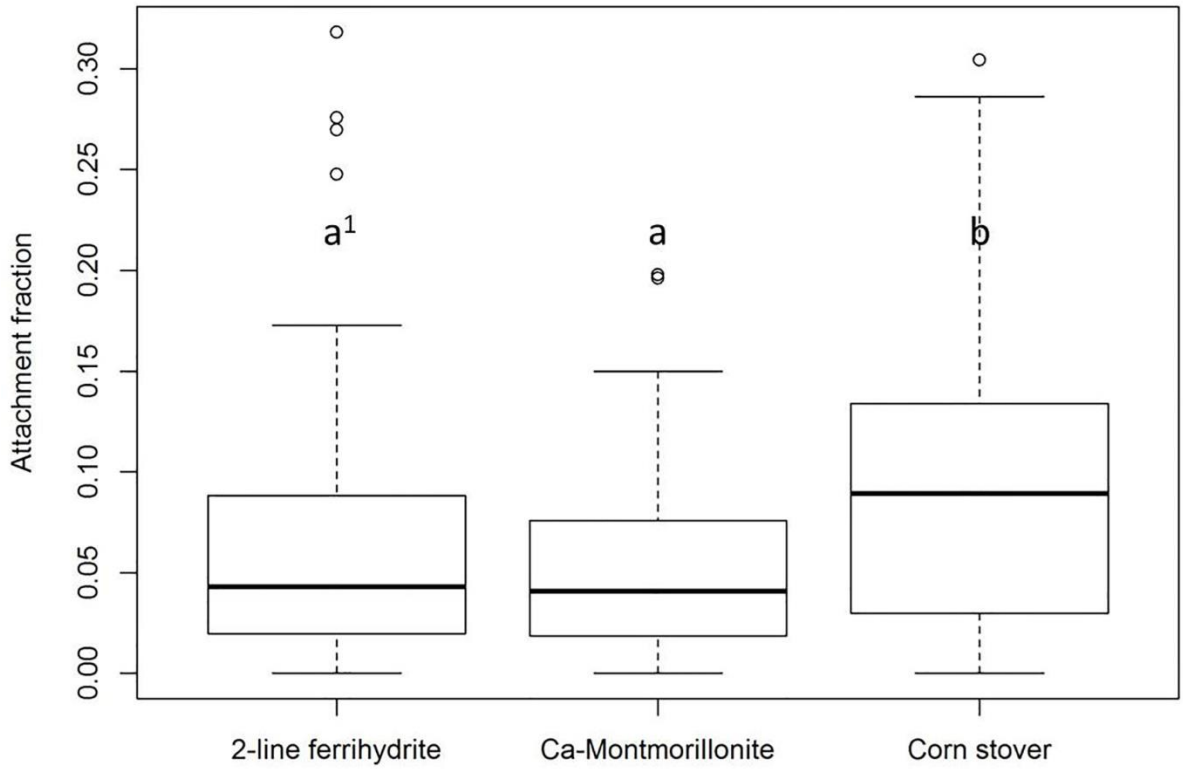

Fig. 4.2. Boxplot[2] of attachment fractions analyzed by each particulate type.

1. Within each subplot, values with the same letter are not different at the significant level. The difference is determined by a Wilcoxon test with significance level set at α=0.05.
2. Each plot show five numerical values: the smallest observation (Q1=Q2-1.5(Q4-Q1), the low end of the whisker), 25% quartile (Q2, low boundary of box), median (Q3, the band near the middle of box), 75% quartile (Q4, high boundary of box), and largest observation (Q5=Q4+1.5(Q4-Q1), the high end of the whisker). Outliers, if any, are indicated by dots.

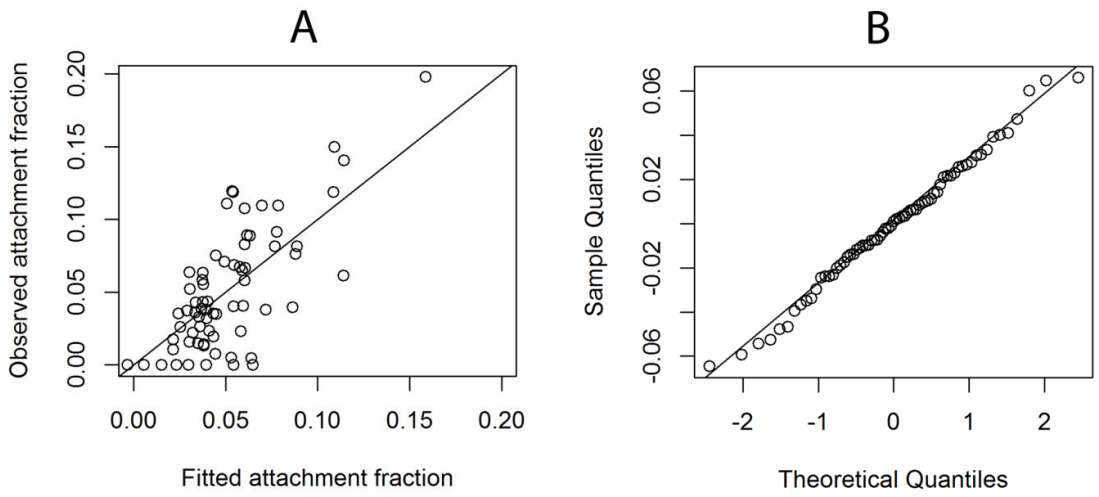

Fig. 4.3. Plots for checking the performance of model for predicting *E. coli* attachment to Ca-Montmorillonite: A. plot of observed attachment fractions. Vs fitted attachment fractions; B. Q-Q plot of residuals.

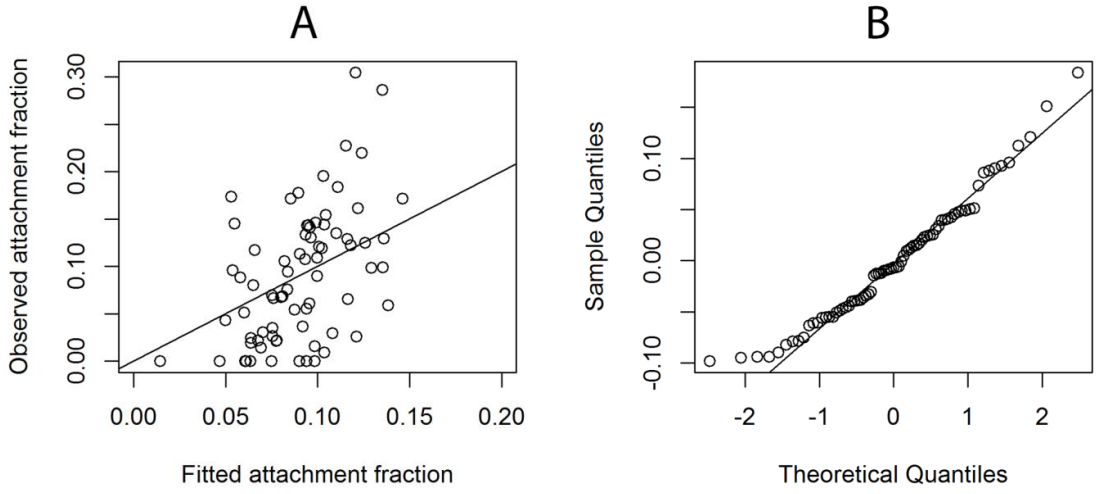

Fig. 4.4. Plots for checking the performance of model for predicting *E. coli* attachment to corn stover: A. plot of observed attachment fractions. Vs fitted attachment fractions; B. Q-Q plot of residuals.

CHAPTER 5. ATTACHMENT OF *E. COLI* TO MANURE COMPONENTS

A paper to be submitted to *Journal of Environmental Quality*

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Core Ideas

- Developed procedures to construct *E. coli* attachment to manure particles analysis.
- Explored attachment mechanisms of *E. coli* to two manure components by fitting attachment data to common adsorption isotherms.
- Our results suggest that a more heterogeneous mechanism for *E. coli* attachment to methylene chloride soluble manure particles than insoluble particles.

Abstract

Manure-borne bacteria can move as planktonic cells, or attached to soil or manure particles in the aquatic environment. In this study, we explored the mechanism of *E. coli* attachment to manure particles. Three genomically different beef manure *E. coli* strains (A, B and C) and a genetically modified version of *E. coli* O157:H7 ATCC 43888 were analyzed for their attachment to two types of beef manure particles differentiated by their solubility in methylene chloride: methylene chloride insoluble and methylene chloride soluble. Flow cytometry was employed to measure attachment percentages for 6 different *E. coli* concentrations and the attachment data were fitted to common adsorption isotherms. Our results showed that the Freundlich adsorption model provided a good fit of *E. coli* attachment to manure particles. From plotting the equilibrium adsorption isotherms, we can conclude that the attachment mechanisms were similar for two different manure components (methylene chloride insoluble and soluble) when the strain was A, B, or ATCC 43888. However, the isotherm patterns for two manure components were quite different for strain C. Considering the Freundlich parameters, for strain A, C and ATCC 43888, the value of *n* was smaller when the particle type was methylene

chloride soluble, which indicated more heterogeneous mechanisms for *E. coli* attachment to methylene chloride soluble manure particles. Our study showed that the heterogeneity of attachment sites of manure particles can impact *E. coli* attachment to manure.

Introduction

Nearly 15% of waters across the United States are classified as impaired because of elevated pathogen levels determined by presence of fecal indicator bacteria (FIB) such as *E. coli* (USEPA, 1986, USEPA, 2009). Agriculture is a significant contributor of bacteria in the environment. According to U.S. EPA, United States annually produces 1.37 billion tons of solid animal manure (USEPA, 2005), which may contain various pathogenic microorganisms. Two major sources of bacteria in streams are from land application of manure from confined animal systems and direct deposit by grazing animals (Soupir, Mostaghimi, et al., 2006).

In 2007, there were 22 million acres treated with manure on 300,000 farms across the United States (USDA, 2009); some land-applied manure can be carried to the stream by runoff or overland flow (Larsen, Miner, et al., 1994). In addition, direct deposit of cattle manure into streams is also technically classified as nonpoint source pollution according to U.S. EPA (USEPA, 1994). Although both manure application and direct deposits contribute to fecal contaminations in water, direct deposit has been found to cause greater relative increases in instream fecal bacteria concentrations (Line, 2003, McDaniel, Soupir, et al., 2013, Sheffield, Mostaghimi, et al., 1997). To prevent disease outbreaks associated with water-borne pathogens and to identify when there is a critical threat to public health, better models of the fate and transport of pathogenic organisms to drinking and recreational water supplies are needed.

During transport in the aquatic environment, manure-borne bacteria move as planktonic cells, or attached to soil or manure particles (Guber, Pachepsky, et al., 2007, Pachepsky, Sadeghi, et al., 2006, Tyrrel and Quinton, 2003). Guber et al. (2007) found that the presence of manure colloids reduced bacterial attachment to soils. This was attributed to three possible reasons: i) competition for the limited attachment sites on soil surfaces between bacteria and soluble organic matter, ii) modification of soil mineral

surfaces by soluble manure constituents, or iii) modification of bacterial surfaces by dissolved organic matter (Guber, Pachepsky, et al., 2007, Guber, Shelton, et al., 2005, Guber, Shelton, et al., 2005). However, batch studies have so far remain unsuccessful in identifying the dominant mechanisms responsible for microbial association with manure particles. A thorough understanding of pathogen adhesion to manure particles is of great importance for assessing the fate of pathogens in manure and aquatic environments.

The goal of this study was to elucidate how *E. coli* attach to particles in livestock manure. Assessed manure components were methylene chloride insoluble particles and methylene chloride insoluble particles from beef manure. The objectives were: 1) to develop procedures to assay *E. coli* attachment to manure particles; and 2) to explore and compare the attachment mechanisms of *E. coli* to these two components by fitting attachment data to common adsorption models. An improved understanding of microorganisms-manure attachment mechanism will help to improve modeling both local and watershed-scale models of bacterial transports.

Materials and Methods

A flow cytometry method was used to partition between *E. coli* attached to manure particles and freely suspended *E. coli* (Liang, Soupir, et al., 2014). To investigate the mechanisms of bacterial attachment, we measured the attachment percentages with various *E. coli* strains, particles, and *E. coli* concentration. Properties of the bacteria were considered by using different environmental strains from beef manure, as described below and a known modified pathogenic strain (strain A, B, C, and ATCC 43888); experiments were conducted on two types of manure particles (methylene chloride soluble and insoluble); while environmental conditions such as ionic strength, temperature and pH were held constant.

E. coli Sampling and Analysis

E. coli strains were isolated from beef manure samples collected from the Iowa State Beef Nutrition Research Farm (latitude 42.0591, longitude -93.6835) near Ames, Iowa. Fresh beef manure samples were collected twice in June and November 2013. Immediately after collection, samples were placed on ice and transferred to the lab. One

gram of manure was added to 9 mL autoclaved deionized water in a test tube. And then 1 mL was serially diluted in 9 mL autoclaved deionized water six times. One mL of the resulting suspension was filtered through a 0.45-µm cellulose filter paper (EMD Millipore; Pittsburg, PA). *E. coli* strains were incubated on the filter paper using modified mTEC agar plates (USEPA, 2002). One single colony was selected from each agar plate and the plate-sticking method was applied to ensure that the selected colony was formed by only one *E. coli* strain. Forty strains were isolated from the manure samples and the strains were inoculated in Luria-Bertani liquid media (BD Biosciences; San Jose, CA), grown to the stationary phase, and stored at -80˚C in 15% glycerol.

Repetitive Sequence-Based PCR (Rep-PCR) was employed to distinguish *E. coli* strains at subspecies and strain level. $(GTG)_5$ with the sequence of 5'-GTGGTGGTGGTGGTG-3' was used as the primer (Ma, Fu, et al., 2011, Mohapatra, Broersma, et al., 2008, Mohapatra and Mazumder, 2008, Rademaker and deBruijn, 1997). Briefly, the PCR mixture contained 12.5 µL PCR-master- mix (2X, Qiagen), 10 µL primer (50 pmol) and 2.5 µL water for a total volume of 25 µL. A small fraction of a fresh *E. coli* colony was transferred to the PCR mixture as the template by using a 1-µL inoculation loop. PCR was conducted in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) and the program was set for an initial denaturation at 95°C for 2 min, 32 cycles of denaturation (94°C for 3 sec and 92°C for 30 sec), annealing (40°C for 1 min) and extension (65°C for 8 min), then a final extension at 65 °C for 8 min. After PCR progress, 10 µl of resulting PCR products and 2 µL of 6X loading dye mixture (Life Technology, Grand Island, NY) were loaded onto 1.5% agarose gel. The 1Kb Plus DNA ladder (Life Technology, Grand Island, NY) was loaded into every tenth well and was used as an external control for normalization. Electrophoresis was applied at 4°C and 80 V for 14 hours, and the sample was stained for 20 minutes in TAE solution containing 0.5 µg mL^{-1} ethidium bromide. Gel pictures were captured by a Molecular Imager ChemiDoc (Bio-Rad, Hercules, CA).

The resulting gel image files were imported into Bionumerics (version 7.1, Applied Maths, Kortrijk, Belgium) for normalization, band identification, and cluster analysis. Similarity coefficients for each strain pair were generated for Bionumerics programming by Pearson's correlation method with a band matching tolerance of 0.5%,

and an optimization value of 0.5% (BioNumerics, 2013). The technique of unweighted pair groups with mathematical averages (UPGMA) was used for clustering and generating the dendrogram. Strains with similarity less than 80% were considered genomically different. Three genomically different strains were randomly selected and labeled as A, B, and C for attachment analysis. A pathogenic strain was also considered, ATCC^{TM} 43888, a genetically modified version of *E. coli* O157:H7, with Shiga-like toxin I and II producing genes removed.

Manure Particles

Waste-derived organic particles were obtained from beef manure in the Iowa State Beef Nutrition Research Farm, the same source as the *E. coli* isolates. Particles greater than 2 mm were discarded. The manure particles were then sorted into two size fractions (<53 μm and > 53 μm, termed coarse particles and fine particles, respectively). Samples of each size fraction were assayed for *E. coli* populations, and the <53 μm fraction (harboring the greater bacterial population) was used in screening attachment studies. It has been suggested that the particles in manure within this size range include bacterial floc, single cells, and organic residuals (Rodriguez Andara and Lomas Esteban, 2002). The fine particles were further divided into two compositional fractions by solubility or insolubility in methylene chloride using a separatory funnel (Kimble, Vineland, NJ). Methylene chloride insoluble (water-soluble phase) particles were considered more polar and less hydrophobic than methylene chloride soluble particles (Oasmaa, Kuoppala, et al., 2003, Simoneit, Elias, et al., 2004). These fractions provided reproducible reference points corresponding to chemical characteristics relevant to bacterial attachment to the particles. The methylene chloride insoluble fraction was autoclaved at 121°C for 1 hr for sterilization and then freeze-dried at -80°C (SP scientific, Warminster, PA) for 7 days to obtained dry methylene chloride insoluble manure particles. The methylene chloride soluble particles were obtained by removing of methylene chloride in a rotary evaporator (Buchi, Switzerland). Dry particle samples were then placed under 254-nm handheld UV wand (American Air & Water, Hilton Head Island, SC) to inhibit growth of microorganisms. Figure 1 shows a flow chart of manure particle preparations.

Flow Cytometry: Measurement of *E. coli* Attachment Percentage

Flow cytometry was employed to measure attachment percentages to improve understanding of bacterial attachment mechanisms. *E. coli* strains were grown in stock suspension of Luria-Bertani broth (BD Biosciences; San Jose, CA) for 18 hours at 37˚C to reach the stationary stage of the growth curve. *E. coli* suspension was centrifuged for 3 min at 1,878 ×*g* (Eppendorf, Hauppauge, NY) at 4˚C. The supernatant was discarded, and the cell pellet was washed three times using phosphate-buffered saline (PBS) (HACH, Loveland, CO; pH 7.4). The PBS was pretreated by filtering through a 0.45-µm filter paper to remove any potential particles and then autoclaving at 121˚C for 15 min. The final pellet was resuspended using PBS to a 0.5 McFarland standard (approximately 1.0×10^{8} CFU mL^{-1}) according to the Clinical and Laboratory Standards Institute (2006). The manure particle suspension was prepared with manure particles (methylene chloride soluble or insoluble) and PBS. Two DNA stains, SYTO 11 green fluorescent nucleic acid stain (5 mmol L^{-1} solution in DMSO; Life Technologies; Grand Island, NY) and propidium iodide (PI) (4.3 mmol L^{-1} solution in water; BD Biosciences; San Jose, CA) were used to distinguish live/dead cells while performing attachment analysis (Liang, Soupir, et al., 2014). SYTO 11 is a cell-impermeant dye that labels both live and dead cells, enabling discrimination of cells from background electronic noise and debris. Propidium iodide (PI) is impermeable to cells with intact membranes, but permeates dead cells.

Fixed volumes of *E. coli*, manure, PBS, and SYTO 11 were combined to a volume of 250 µl (BD Biosciences; San Jose, CA) for attachment analysis. Six different *E. coli* concentrations (from approximately 2×10^{6} cells mL^{-1} to 2×10^{7} cells mL^{-1}) were used to mimic different bacterial concentrations associated with manure and the concentrations were similar to the recommended range for optimal performance by the flow cytometer (Liang, Soupir, et al., 2014). Seven controls were required for each test: PBS+ two stains, PBS+ *E. coli*, PBS+ *E. coli*+ PI, PBS+ *E. coli*+ SYTO 11 (live and dead cells), 30% (w/w) ethanol+ *E. coli* +two stains (dead cells), PBS+ *E. coli*+ two stains, and PBS+ *E. coli*+ two stains+ beads (969 beads/µL, BD Biosciences; San Jose, CA). The *E. coli* concentration was determined by the control with beads and manure concentration was 2.4×10^{-3} g mL^{-1}. Samples were shaken by hand for 10 min to increase

bacteria and particle interactions. Stains were added immediately before analysis by the flow cytometer to prevent exposure to light. The analysis procedure was revised from the previous work of Liang et al. (2014). Briefly, the samples were tested at-488 nm wavelength on a FACSCanto flow cytometer (BD, Franklin Lakes, NJ) and analyzed by BD FACSCanto Clinical Software (BD, Franklin Lakes, NJ). For SYTO 11, the FL1 channel was used as the optical detector and the bandpass filter was 530/30 nm. The gate for identifying *E. coli* is based on forward scatter channel (FSC) signal and SYTO 11 fluorescence. The FL3 channel was used as the optical detector, and the bandpass filter was 610/20 nm for PI. The area for live *E. coli* is defined based on control samples of *E. coli* stained with SYTO 11 only, and the area for dead *E. coli* is defined based on control samples of ethanol-fixed *E. coli* stained with both SYTO 11 and PI. The distinction between free *E. coli* and those attached to manure particles was established by examining the side scatter channel (SSC) signal of cells. Free *E. coli* are defined as those cells with low SSC, and *E. coli* attached to manure particles are defined as those cells with high SSC. The number of events within each gate was provided by the flow cytometer output. The attachment percentage were calculated as

$$\frac{\#\text{ of live attached } E.coli}{\#\text{ of live attached } E.coli + \#\text{ of live free } E.coli} \times 100\% \qquad [1]$$

The mean attachment percentage was achieved by averaging results from duplicates.

Adsorption Models and Data Analysis

The concentration of free *E. coli* (C in cells mL^{-1}) and the concentration of attached *E. coli* (C_s in cells g^{-1} manure particles) were calculated using sample *E. coli* concentration and mean attachment percentage. The attachment data was modeled using common adsorption models: linear, Langmuir, and Freundlich. The nonparametric Wilcoxon signed-rank test was used to determine if attachment percentages varied between two particles or any two *E. coli* strains. Isotherm fitting and statistical analysis of data was performed using R project software (ver. 3.2.0, Institute from Statistics and Mathematics, Vienna University of Economics and Business, Vienna, Austria).

Results

E. coli Strain Selection and Attachment Percentages

By computer-assisted rep-PCR DNA fingerprint analysis, 14 (35% of 40 strains) were considered genomically different on the basis of the 80% similarity criterion. Fig. 2 shows the electrophoresis gel images of rep-PRC figure print banding patterns for these 14 strains and ATCC 43888. Among 14 genomically different environmental strains, No. 1, 5 and 25 were randomly selected for attachment analysis and labeled as A, B and C.

Attachment percentages were measured by flow cytometer. For example, Fig. 3 shows the flow cytometer output for strain B (concentration as 4.98×10^6 cells mL^{-1}) to methylene chloride soluble manure particles. Fig. 3-a was used to determine the area of *E. coli* based on the forward scatter channel (FSC) signal and SYTO 11 fluorescence. In Fig. 3-b, the area for live *E. coli* is based on control samples of *E. coli* stained with SYTO 11 only, and the area for dead *E. coli* is based on control samples of ethanol-fixed *E. coli* stained with both SYTO 11 and PI. Fig. 3-c presents only the live cell events and shows that the distinction between free *E. coli* and those attached to manure particles was established by examining the side scatter channel (SSC) signal of cells. The gate of free *E. coli* was defined by control samples of *E. coli* stained with SYTO 11.

The percentages of *E. coli* attached to manure particles ranged from 2% to 47%. The percentages to the methylene chloride insoluble component ranged from 2% to 30% while the percentages ranged from 5% to 47% for attachment to methylene chloride soluble component. For each *E. coli* strain and manure particle combination, the variations among different concentrations were minor. The average percentages of attached *E. coli* for both of components are listed for each strain in Table 1, as well as the standard errors. Comparing the two manure particles, the average attachment percentages to methylene chloride insoluble particles was 21% lower than the average attachment percentages to methylene chloride soluble particles over all *E. coli* strains. According to the Wilcoxon signed-rank test with significance level set as $\alpha=0.05$, for strain A and B, the percentages of *E. coli* cells attached to the methylene chloride soluble component were lower but not statistically significantly different from the percentages attached to the methylene chloride insoluble component, with *P*-value= 0.0578 and 0.0585, respectively.

However, the percentages were significant higher for strain C and for strain ATCC 43888 when attached to the methylene chloride soluble component than to the methylene chloride insoluble component (*P*-value= 0.0313 and 0.0313).

The same Wilcoxon signed-rank test and significance level were applied to compare attachment percentages between any two *E. coli* strains. For attachment to the methylene chloride insoluble component, strain ATCC had significantly lower attachment percentages than strain A (*P*-value= 0.0313), but no significant differences were indicated for strain A, B and C (*P*-value>0.05). The attachment percentages to the methylene chloride soluble component by strains A and ATCC were similar (*P*-value>0.05), but they were significantly lower than the results from strain B ((*P*-value=0.0355). Furthermore, attachment percentages for strain C were significantly higher than these for strain B (*P*-value=0.0340).

Effects of Particles on *E. coli* Attachment

The attachment data conformed to the Freundlich adsorption model using non-linear regression, which employs a power function:

$$C_s = K_f \cdot C^n \qquad [2]$$

where C is the *E. coli* concentration in solution (cells mL^{-1}), C_s is the number of *E. coli* cells attached per unit mass of particles (cells g^{-1}), K_f is the coefficient related to attachment capacity, and n is the linearity exponent. The equilibrium adsorption isotherm of *E. coli* to methylene chloride insoluble particles and methylene chloride soluble particles are shown in Fig. 4. The number of attached *E. coli* increased with the initial *E. coli* concentration. The values of K_f and n are listed in Table 2, as well as the coefficient of determination (R^2) for each isotherm fitting. Even though there were variations among the values of K_f, no K_f values were significantly different from zero (*P*-value>0.05). When we compared the n values of attachment to two different manure components for the same strain, the n value of methylene chloride soluble particles is always smaller than the n value of methylene chloride insoluble particles, except when strain was B. However, the differences of n values of two different manure components were not significant for all *E. coli* strains at significance level=0.10.

Discussion

From the adsorption patterns in Fig. 4, we can conclude that the attachment mechanisms were similar for two different manure components (methylene chloride insoluble and soluble) when the strain was A, B, and ATCC 43888. However, for strain C, the adsorption patterns for the two manure components were quite different. Considering the particle properties, methylene chloride soluble particles would be more hydrophobic and less polar (Oasmaa, Kuoppala, et al., 2003). We can hypothesize the mechanisms of strain C adsorbing to two manure components were different because of the hydrophobicity and surface charge interaction between strain C cells and the manure particles. Measurements of bacterial hydrophobicity and surface charge for strain C cells in the future would provide us supportive information for this hypothesis.

Additionally, considering the Freundlich parameter of strain C, the *n* value for adsorption to methylene chloride insoluble component is 1.15 with 95% confidence interval (0.851, 1.46), which indicating an approximately linear adsorption; while the *n* value for adsorption to methylene chloride soluble component is 0.681 with 95% confidence interval (0.322, 1.04), indicating the attachment mechanism of strain C to methylene chloride soluble manure is more heterogeneous than to methylene chloride insoluble manure but the difference was not significant at level $\alpha=0.5$. Similarly, for strain A and ATCC 43888, the value of *n* of methylene chloride soluble particles was smaller but not significantly different the value of *n* of insoluble particles. Moreover, the value of *n* for ATCC 43888 to insoluble particles was significantly greater than 1 at $\alpha=0.05$, indicating a cooperative adsorption (Dada, Olalekan, et al., 2012, Shahbeig, Bagheri, et al., 2013).

Previous studies have not been successful in identifying the dominant mechanisms responsible for microbial association with manure particles. In our study, we explored and compared the attachment mechanisms of *E. coli* to two beef manure components (methylene chloride insoluble and soluble) by fitting attachment data to the Freundlich isotherm. Below is a summary of the major findings and implications for future research:

- The attachment of *E. coli* to manure particles can be influenced by the hydrophobicity and surface charge interaction between cells and manure particles. Future work on measuring the hydrophobicity and surface charge of each *E. coli* strain was recommended.
- The attachment mechanism is more heterogeneous for *E. coli* to methylene chloride soluble particles than to insoluble particles but the difference was not significant. Measuring the interaction energy in future research will provide more supportive information.

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Tables

Table 5.1. Average percentage attached for each particle, and *E. coli* strain.

***E. coli* strain**	**methylene chloride insoluble manure particle**	**methylene chloride soluble manure particle**	***P*-value†**
A	14(±1) ‡ A§ a#	10(±2) A a	0.0578
B	16(±1) A a	12(±0) A b	0.0585
C	25(±3) A a	43(±2) B c	0.0313
ATCC 43888	6(±1) A b	9(±1) B a	0.0313
AVG	15(±2) A	19(±3) A	0.650

†. The *P*-value of attachment difference between the two particles.

‡. Standard error for each percentage is listed in parenthesis.

§. Within each row, values with the same upper case letters are not significantly different based on Wilcoxon-test with significance level set at α=0.05.

#. Within each column, values with the same lower upper case letters are not significantly different based on Wilcoxon-test with significance level set at α=0.05.

Table 5.2. Freundlich† parameters for *E. coli* adsorption to manure particles.

***E. coli* strain**	**methylene chloride insoluble manure particle**				**methylene chloride soluble manure particle**			
	K_f	n			K_f	n		
	Estimate	Estimate	Standard error	R^2		Estimate	Standard error	R^2
A	1.01×10^5	0.632	0.123	0.921	8.72×10^5	0.445	0.047	0.970
B	1.46×10^5	0.751	0.162	0.913	19.1	1.16	0.108	0.985
C	49.1	1.15	0.155	0.969	2.29×10^5	0.681	0.183	0.860
ATCC 43888	0.0486	1.48	0.104	0.993	0.794	1.33	0.182	0.971

†. The Freundlich equation is expressed as $Cs=K_f \cdot C^n$, where K_f is the coefficient related to adhesion capacity and n is the linearity exponent.

Figures

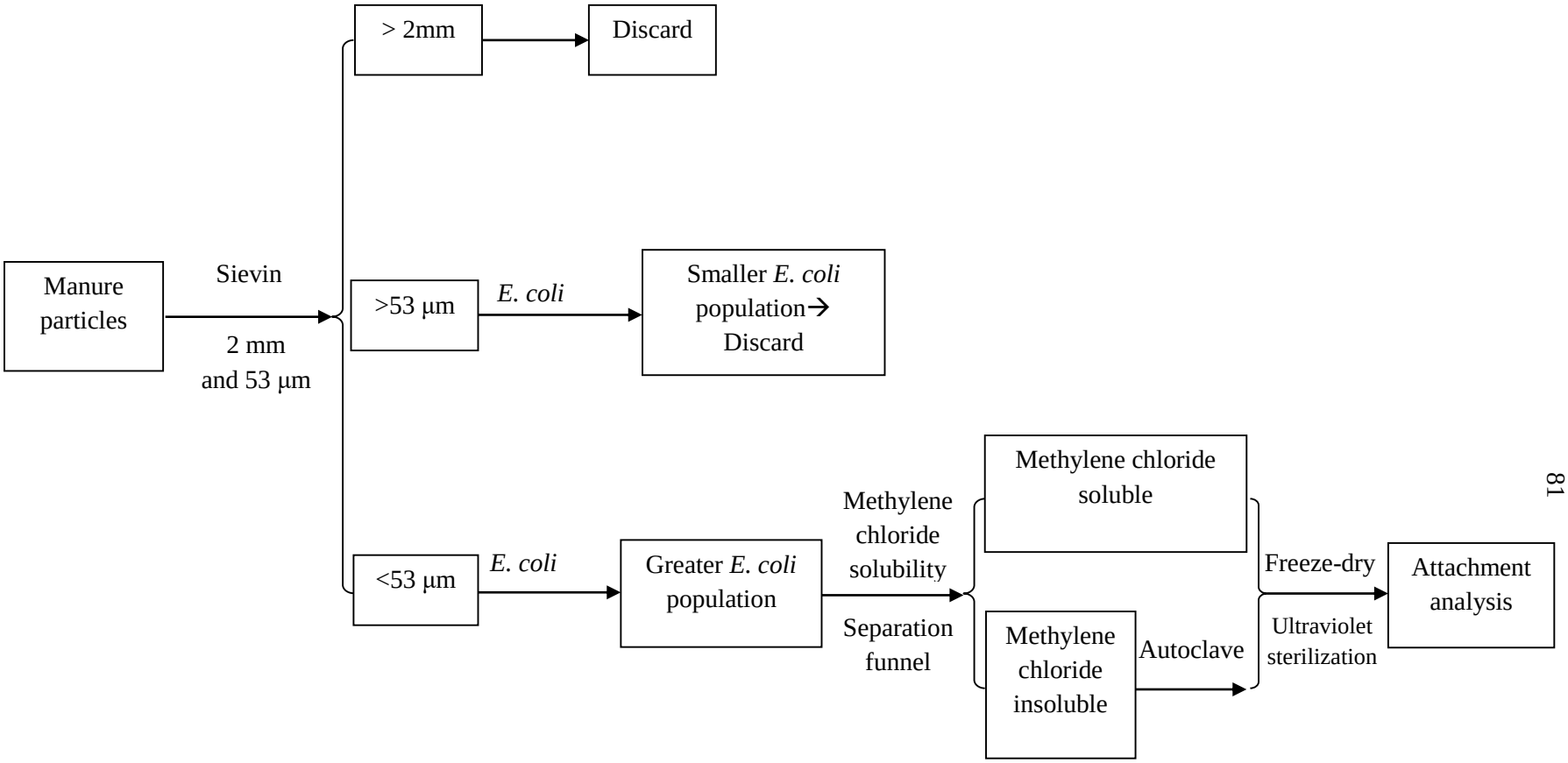

Fig. 5.1. Flow chart describing the preparation of manure particles.

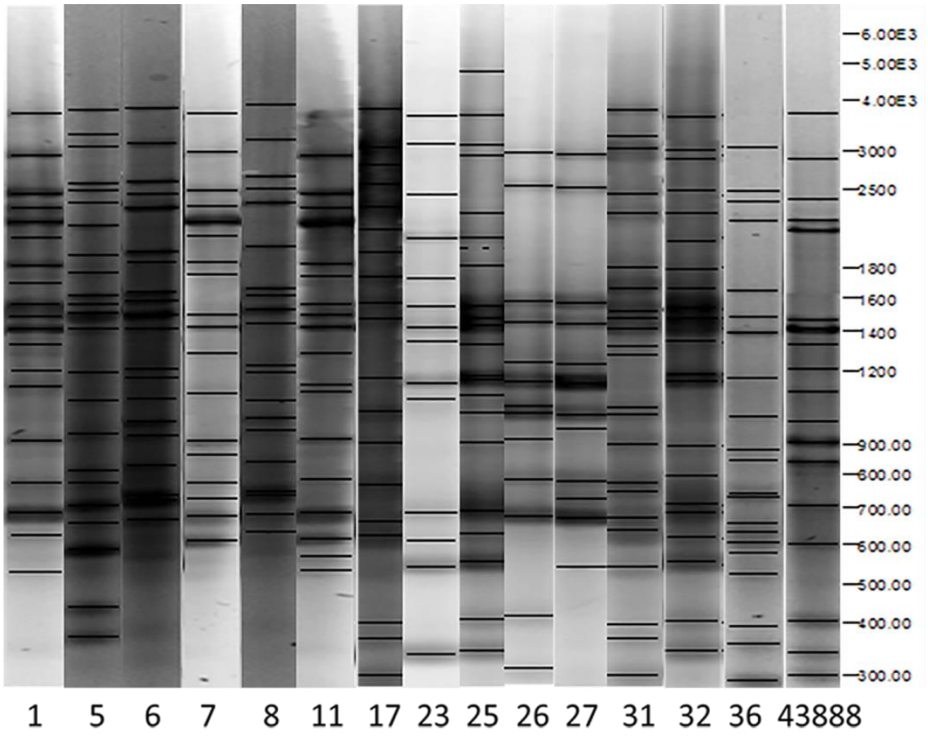

Fig. 5.2. The gel images show rep-PCR fingerprint banding patterns for 14 genomically different strains (out of environmental 40 strains) and ATCC 43888. Similarity of all strain pairs are smaller than 80%, based on UPGMA cluster analysis.

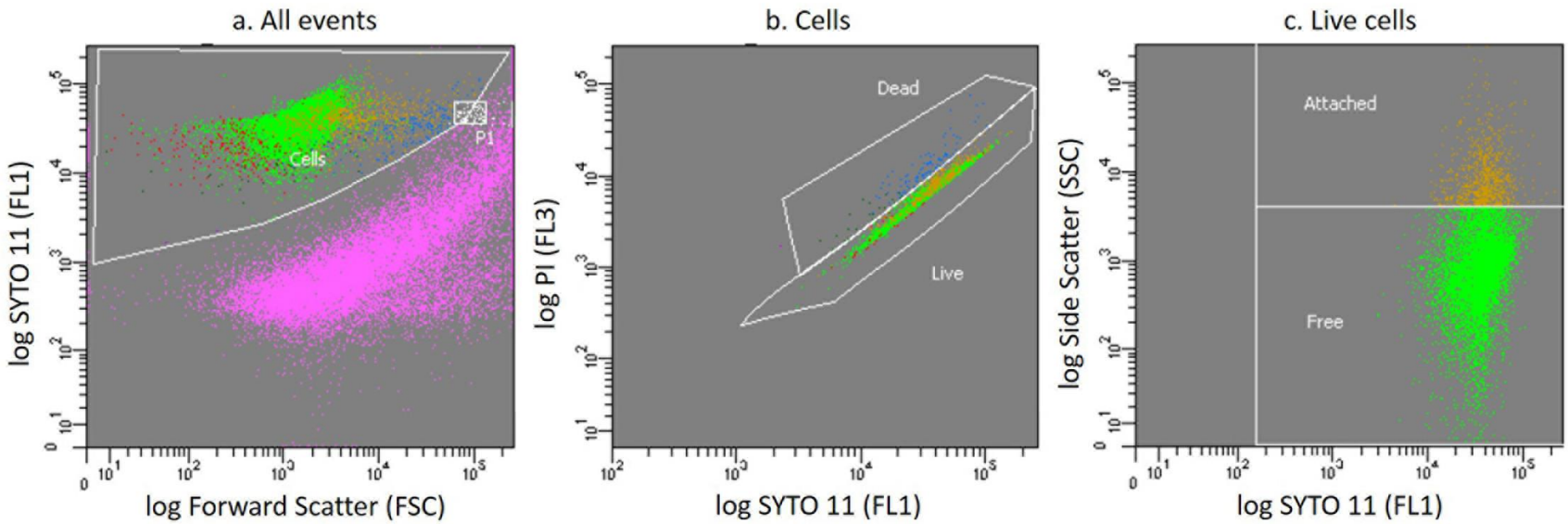

Fig. 5.3. Flow cytometer output for strain B (concentration as 4.98×10^{6} cells mL^{-1}) to methylene chloride soluble manure particles: (a) determination of the area of *E. coli* was based on forward scatter channel (FSC) signal and SYTO 11 fluorescence; (b) the area for live *E. coli* is defined based on control samples of *E. coli* stained with SYTO 11 only, and the area for dead *E. coli* is defined based on control samples of ethanol-fixed *E. coli* stained with both SYTO 11 and PI; (c) live cell events, showing that the distinction between free *E. coli* and those attached to manure particles was established by examining the side scatter channel (SSC) signal of cells. The gate of free *E. coli* was defined based on control samples of *E. coli* stained with SYTO 11.

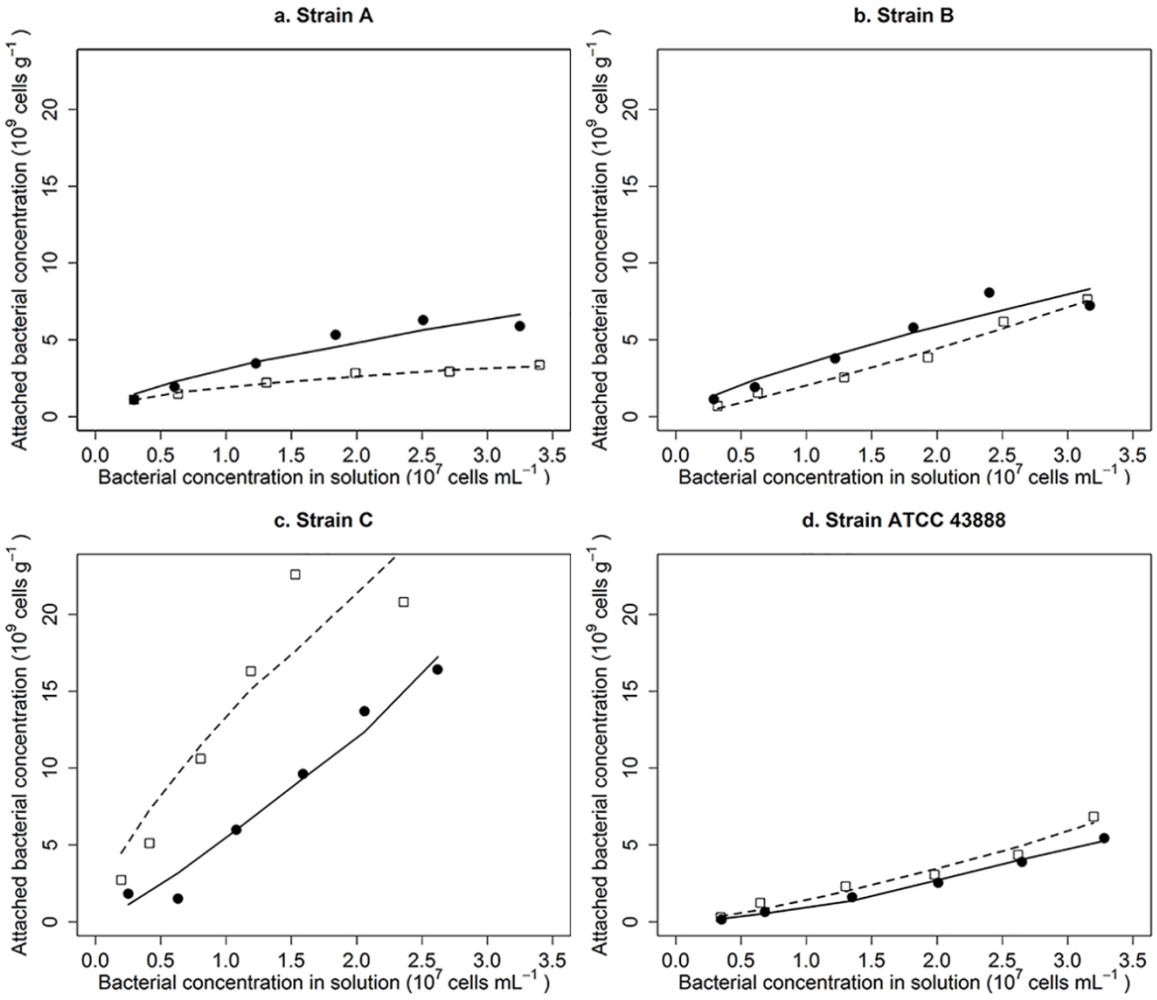

Fig. 5.4. Equilibrium adsorption isotherm of *E. coli* to manure particles. The filled black circles and solid lines represent observed and fitted values for methylene chloride insoluble manure particles; hollow squares and dashed lines represent observed and fitted values for methylene chloride soluble manure particles.

CHAPTER 6. GENERAL CONCLUSION

General conclusions

The goal of this study was to improve understanding of bacterial attachment in the aquatic environment. Attachment of stream *E. coli* to stream particulates, and attachment of manure *E. coli* to manure particulates were investigated. This study indicated *E. coli* attachment to particulates can be impacted by bacterial, particulate, and environmental factors.

The first objective of this study was to determine if differences in environmental *E. coli* cell surface properties are due to extrinsic (environmental) or intrinsic (genomic) properties, or an interaction of these two. This objective was addressed by analyzing the surface properties of *E. coli* collected from two environmental habitats (stream bottom sediment and overlaying water). Our results indicated that there were variations in hydrophobicity, EPS components, net charge and point of zero charge between stream sediment *E. coli* and water *E. coli*, and these variations were due to the interaction of extrinsic and intrinsic properties.

The second objective was to identify the impact of bacterial and particulate properties on *E. coli* attachment fractions. Attachment assays were constructed using a single stream *E. coli* strain and one model particulates (ferrihydrite, Ca-Montmorillonite, or corn stover) with environmentally relevant concentrations. Among three particles, attachment fractions to corn stover were significantly larger than the attachments to 2-line ferrihydrite and the attachments to Ca-Montmorillonite. Furthermore, Generalized Additive Model (GAM) successfully predicted the attachment fractions to Ca-Montmorillonite using cell characteristics as predictor variables. Net charge showed a significant positive linear impact on the attachment fractions to Ca-Montmorillonite but no significant impact on the attachment to corn stover.

The third objective was to assess the mechanisms of *E. coli* attachment to particulates in beef manure. The studied *E. coli* included three genomically different beef manure *E. coli* strains (A, B and C) and one *E. coli* O157:H7 (ATCC 43888), and two beef manure components (methylene chloride insoluble and soluble) with size smaller

than 53 µm were investigated. Flow cytometry was used to measure attachment fractions for 6 different *E. coli* concentrations. The attachment data was fitted to common adsorption models and the Freundlich isotherm provided a good fit. The isotherm results indicated a more heterogeneous mechanism for *E. coli* attachment to methylene chloride soluble manure particles. The study also suggested of the hydrophobicity and surface charge interaction between cells and manure particulates would possibly impact the attachment of *E. coli* to manure particulates.

Implications

Studies suggest that microbe-particulate interactions play an important role in predicting the movement of microorganisms in streams. In this study, we developed a systematic study on parameters influencing bacterial attachment to stream and manure particulates. It will improve our scientific understanding of *E. coli* transport in surface water systems and further help with developing techniques to reduce pathogen impairment in rivers and stream. Moreover, the lack of understanding about the mechanism driving microbe-particulate interactions has led to various assumptions in previous modeling of bacterial environmental transport. The results from this study will improve scientific basis in modeling bacterial fate and transport in aquatic environment.

The results from this study can also be referred to agricultural practices. Under typical environmental conditions, *E. coli* attachment fraction likely increases as the surface area decreases and carbon content increases. Therefore, impact of subsurface water quality from manure application is expected to be less critical for the fields with smaller soil particle size and higher soil carbon content, since more microorganisms could be retained within soil particles via attachment. Moreover, organic coatings on soil particles could also increase the negativity of surface charge. According to the results from this study, it can further significantly impact microorganism attachment to soil particles.

Recommendations for future research

There is clearly a need for more information on bacteria attachment to environmental particulates to assist water quality modeling efforts. This study indicated bacterial attachment could be impact by bacterial, particulate and environmental factors. However, there are still some limitations of this study. Before a clear mechanism can be established, more research must be conducted. Here are some suggestions for further study:

- Uncertainties in measuring attachment fractions using the settling method need to be considered; new techniques need to be developed for measuring bacterial attachment fraction under environmentally relevant concentrations.
- Single strain was analyzed for its properties and attachment in this study; further research with mixed bacteria populations is required and the results could be related to the results from this study.
- Other than environmental habitat, surface properties can be analyzed on bacteria collected in different temperature and nutrient level.
- For the research with manure particles, we used fixed mass of manure particles; varying the mass of particles would improve the understanding of attachment mechanism.
- Measuring surface charge and hydrophobicity of both *E. coli* and manure particles, and their interaction energy in future research will provide more supportive information for attachment mechanism.