Differences in survival among *Enterococcus faecalis* subspecies in two freshwater creek sediments

Yucheng Feng[1], Peter G. Hartel[2], Jared A. Fisher[2], Karen Rodgers[2], Bin Liu[1] and Kerry J. Ritter

Abstract

While bacterial source tracking (BST) has proven to be a useful and relatively inexepensive method for detecting human and bird fecal contamination in environmental waters, research suggests that some subspecies of fecal indicator bacteria are better adapted than others to survive different environmental conditions and stresses. We determined the survival of seven different *E. faecalis* subspecies, three from different wastewater treatment plants and one from a latrine (presumably all of human origin), two from birds, and the American Type Culture Collection (ATCC) type strain, in two freshwater creek sediments, Saugahatchee Creek in Alabama and Tanyard Creek in Georgia. Ribotyping was used to identify different subspecies. Survival was determined in modified sentinel chambers (Microfilterfuge™ tubes sealed at both ends with ≤0.45-µm membranes). Each Enterococcus ribotype was inoculated into sentinel chambers at approximately $10^{5\text{-}6}$ bacteria per g dry weight of sediment, and the Most-Probable Number (MPN) was estimated at Days 0 and 11. The MPNs of the seven *E. faecalis* subspecies declined 62.4 to 95.2% in Alabama sediment; MPNs of the same subspecies declined 95.4 to 99.6% in Georgia sediment. There was no positive correlation among the subspecies that survived better in Alabama sediment and those that survived better in Georgia sediment. Statistical analyses suggest that not only did differences in survival exist among subspecies of *E. faecalis*, but also that differences existed between locations. However, in terms of BST, the rapid decreases in MPNs suggested that any subspecies adaptation to environmental conditions was minor.

Introduction

Bacterial source tracking (BST) identifies sources of fecal contamination in water. Such identification can be done indirectly with chemical methods (e.g., fluorometry), or directly with genotypic (e.g., ribotyping) or phenotypic (e.g., antibiotic resistance) methods. Because there are many potential users that cannot afford expensive BST methods, we have concentrated on inexpensive methods.

One inexpensive BST method for identifying human or bird fecal contamination is determining the percentage of *E. faecalis* isolates among all the fecal enterococci in an environmental water sample. Generally, *E. faecalis* is common in the feces of humans and birds and rare in the feces of other animals (Wheeler *et al*. 2002, Kuntz *et al*. 2004). Thus, waters with a high percentage of this bacterium (≥30%) are likely contaminated with human or bird feces.

However, Gordon *et al*. (2002) showed that some subspecies of *Escherichia coli*, another fecal indicator bacterium widely used in BST research, are better adapted to survive environmental conditions than other subspecies. Because we found high numbers of *E. faecalis* exist in sediment (>10^3 MPN) per g dry weight; McDonald *et al*. 2006), we suspected that some subspecies of *E. faecalis* were also better environmentally adapted in sediment than other subspecies. This survival would have the potential to confound BST results because if some subspecies survive better than other subspecies, then the longer-living subspecies could indicate long-past fecal contamination.

Therefore, we used ribotyping to identify seven different subspecies of *E. faecalis*, four presumably from humans, two from birds, and the American

[1] *Auburn University, Department of Agronomy and Soils, Auburn, AL*
[2] *University of Georgia, Department of Crop and Soil Sciences, Athens, GA*

Type Culture Collection (ATCC) type strain, and determined their survival in two freshwater sediments, one from Alabama and the other from Georgia.

Methods

The sediments from two freshwater creeks were chosen. The chemical and physical characteristics of both creeks were measured with a Hydrolab Quanta (Austin, TX). The first creek was the 113-km long Saugahatchee Creek in Alabama, which originates in Chambers and Lee counties, and flows westward through parts of Macon and Tallapoosa counties as part of the Lower Tallapoosa Sub-basin. The land use of the 570-km^2 watershed is 67% forest, 12% agriculture, 7% urban, and 14% other. Portions of the creek are on the State of Alabama's 303(d) list as being impaired for nutrients. During the study period (Fall 2003), the creek was pH 7.5, the dissolved oxygen ranged from 7 to 8 mg L^{-1}, and the water temperatures ranged from 15 to 25˚C.

The second creek was Tanyard Creek in Georgia, which flows entirely in Clarke County. The land use of the 2-km^2 watershed is 74% urban, 21% forested, and 5% other. The creek is on the State of Georgia's 303(d) list as being impaired for fecal coliforms. During the study period (Fall 2003), the creek was pH 6.7 to 7.1, the dissolved oxygen ranged from 7 to 9 mg L^{-1}, and the water temperatures ranged from 20 to 23˚C.

Sediment was collected during baseflow conditions with a 60-ml syringe (which had its tip cut off for easier sampling), and the sediment was transferred to 1-L bottle. Only the uppermost layer (<0.5 cm) of sediment was removed. The sediment was allowed to settle for at least 1 hour before excess water was removed and discarded. The sediment was reshaken, and 20-g amounts were placed in 160-ml milk dilution bottles. These 20-g portions of sediment were autoclaved for 30 minutes. After cooling, 100 ml of sterile distilled water was added aseptically, the contents shaken, and the overlying water removed after 1 hour of resettling. In this manner, any potentially undesirable soluble products formed during autoclaving (e.g., nitrite) were minimized. A 200-µg portion of nonsterile sediment was added to each 20-g sample of sterile sediment. In this manner, the sterile sediment was made nonsterile again, but any potentially high background numbers of fecal enterococci in the sediment were reduced 100-fold.

A preliminary experiment was conducted with the *E. faecalis* type species to determine the final sampling time before the MPN declined to below the limit of detection (<10^3 per g dry weight of sediment). The type species was obtained from the ATCC (Manassas, VA), and E. faecalis ATCC #19433 was streaked onto a plate containing Tryptic Soy Agar (Becton Dickinson, Sparks, MD) and incubated at 35°C for 24 hours. A small amount of inoculum was removed from the streak plate and was added to 100 ml of phosphate buffer (0.65 g KH_2PO_4, 0.35 g K_2HPO_4; pH 7.0) to give a lightly turbid suspension (approximately 10^7 MPN per ml). A 200-µl portion of this suspension was inoculated into the 20.2-g portion of nonsterile sediment. In this manner, the starting density of the type species was 10$^{5-6}$ MPN per g dry weight of sediment. In addition, a control was created in exactly the same way except the sediment was inoculated with *E. coli* ATCC #11775. The sediment was mixed thoroughly with an ethanol-flame sterilized spatula, and a 0.7-ml portion of sediment was transferred to each modified sentinel chamber with a pipetter.

Sentinel chambers were modified after a design by Jenkins *et al*. (1999). The chambers were obtained by removing the insert to a Microfilterfuge™ tube (Rainin Instrument, Oakland, CA; Figure 1). A 1-cm hole was cut aseptically in the cap with a heated cork borer. After adding inoculated, nonsterile sediment to the chamber, the chamber was sealed by inserting a 2- by 2-cm piece of sterile, 0.22-µm Versapor membrane (an acrylic copolymer on a nonwoven nylon support that withstands autoclaving; Gelman Sciences, Ann Arbor, MI) under the cap, and adding three zinc washers to the outside of the chamber to ensure that the chamber did not float. The original sentinel chamber was designed with a 60-µm nylon mesh and no washers. The base of the chamber was notched with a scalpel for easy identification. Chambers were grouped with a fishing leader running through the opening created by tying the washer to the cap, and were kept in phosphate buffer before transferring them to the creek. At each site, the fishing leader was connected to a secured line that kept the chambers in place. To avoid any effects of UV light, the chambers were placed in a heavily shaded portion of each creek.

The MPN of *E. faecalis* ATCC #19433 was estimated on Days 0, 3, 7, 10, and 14 with the Enterolert system (IDEXX Laboratories, Westbrook, ME). There were three replicates for each sampling day, except Day 0, which was done in duplicate. At each sampling date, sediment samples were serially dilut-

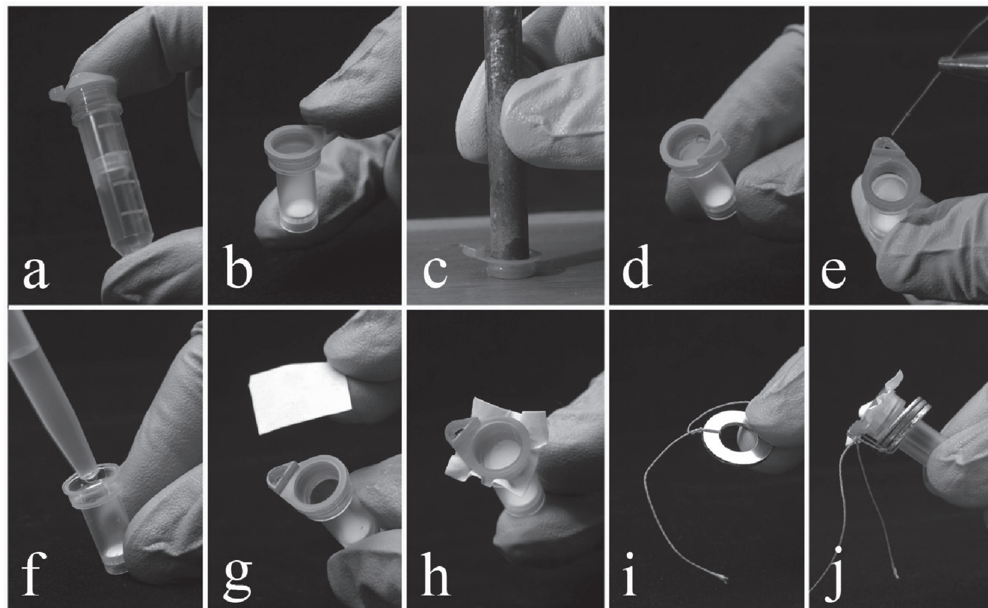

Fig. 1. Construction of a modified sentinel chamber. The insert (b) to Microfilterfuge™ tube (a) was removed, and the center of the insert cap was cut out with a cork borer (c, d). After inoculation (f), the chamber was sealed with a 2- by 2-cm piece of sterile, 0.22-µm Versapor membrane (g, h). A hole melted in the cap with nichrome wire (e) allowed the chamber to be weighed down with washers (i) and secured with braided fishing line (j). Each sentinel chamber contained approximately 0.7 ml of sediment.

ed to 10^{-3} and 10^{-5} in bottles containing sterile distilled water. A package of powdered Enterolert reagent was added to each bottle.

After the reagent was dissolved, the contents of each bottle were added to a Quanti-tray, a sterile disposable panel containing 97 wells. Each Quanti-tray was mechanically sealed, which distributed the sample uniformly into the wells. Each Quanti-tray was incubated for 24 hours at 41±0.5°C. Fluorescing (positive) wells were counted under a 365-nm UV light. In order to express MPNs on a g dry weight of sediment basis, the sediment weight was determined gravimetrically by adding three separate 20-g portions of sediment to preweighed aluminum dishes and drying the dishes overnight at 95°C.

The preliminary experiment was repeated with different subspecies of E. faecalis, including the type strain, except that the sentinel chambers were only sampled on Days 0 and 11. Multiple isolates of E. faecalis from robin (*Turdus migratorius*) and laughing gull (*Larus atricilla*) were obtained as described by Kuntz *et al*. (2004); isolates of *E. faecalis* from a latrine and different wastewater treatment plants were obtained in the same manner (Hartel unpublished). Based on fecal loading rates, the isolates from the wastewater plants were likely to be of human origin.

Isolates were processed on a RiboPrinter (DuPont Qualicon, Wilmington, DE) using the restriction enzyme *Pst*I as described by the manufacturer, and were analyzed by BioNumerics (Version 3.0, Applied Maths, Kortrijk, Belgium). The ribotype patterns were converted to binary data (where 0 represented the absence and 1 represented the presence of a band class) and were exported into SAS (Version 9.0, SAS Institute, Cary, NC) and S-PLUS (Version 6.2, Insightful Corp., Seattle, WA). The distribution of ribotype patterns was computed from an *E. faecalis* host origin database as a whole. Distinct ribotype patterns were defined according to the Dice coefficient and a similarity index (percentage agreement between two patterns) of 100%. This index was chosen because intra- and inter-gel variations with the RiboPrinter and the *E. faecalis* ATCC #19433 strain were 100% (an exact match). From these banding patterns, distinctly different ribotypes were chosen to represent different subspecies. Statistical analyses of differences among locations and species were based on t-tests, assuming unequal variances. A Bonferroni correction was used to adjust statistical significance when reporting across multiple comparisons.

Results

In Alabama and Georgia sediments, *E. faecalis* ATCC #19433 declined from ~10^{5-6} MPN g^{-1} dry weight sediment to below levels of detection (<10^3 MPN g^{-1} dry weight of sediment) in 14 days (data not shown). The control was consistently below the limit of detection. Based on these data, 11 days was selected as the final sampling date for the *E. faecalis* subspecies.

As a set, all *E. faecalis* subspecies decreased in numbers after 11 days, more so in Georgia sediment than in Alabama sediment ($p = 0.024$; Table 1). Subspecies decreases, calculated in terms of percentage of initial densities, ranged from 62.4% to 95.2% for Alabama sediment and from 95.4 to 99.6% for Georgia sediment. There was no positive correlation between the percentage survival of the seven subspecies in Alabama sediment compared to the percentage survival of the seven subspecies in Georgia sediment. For example, the ATCC type strain, which was the second-best survivor in Alabama sediment (78.9% decline), was the worst survivor in Georgia sediment (99.6% decline). Likewise, *E. faecalis* subspecies GA-3, the best survivor in Georgia sediment (95.4% decline), was one of the worst survivors in Alabama sediment (92.2% decline).

When the *E. faecalis* subspecies were compared at one location, their survival differed in the two sediments. After Bonferroni adjustment for multiple comparisons, only numbers of *E. faecalis* DE-457 decreased significantly in Alabama sediment. In contrast, all *E. faecalis* subspecies showed statistically significant decreases in Georgia sediment except *E. faecalis* GA-3. However, care should be taken when interpreting the lack of significance because there were few replicates for each species (i.e., low power) and Bonferroni adjustment is conservative. In all cases, the p-value for each *E. faecalis* subspecies was <0.05. With regards to the control, the MPN was consistently below the limit of detection.

Discussion

Given that some *E. coli* subspecies are better adapted to environmental conditions than other *E. coli* subspecies (Gordon *et al*. 2002), we suspected that some subspecies of *E. faecalis* are better environmentally adapted to survive in sediment than other subspecies. Such differential survival has the potential to confound BST results because if some subspecies survive better than other subspecies, then the longer-living subspecies could indicate long-past fecal contamination. However, overall, the results suggested that survival differences in moist sediment among the subspecies were minor, and differential survival was unlikely to be a confounding factor. Therefore, the only confounding factor for fecal enterococci continues to be their enhanced survival in desiccated sediments (Hartel *et al*. 2005).

Even so, there were differences in survival

Table 1. Survival of selected *Enterococcus faecalis* subspecies in freshwater creek sediments from Alabama and Georgia. The fecal isolates obtained from the wastewater treatment plants and the latrine were presumably of human origin considering their fecal loading rates. The low limit of detection was <10^3 Most-Probable-Number (MPN) per g dry weight of sediment. ATCC, American Type Culture Collection (Manassas, VA); NA, not applicable.

		Alabama (Saugahatchee Creek Sediment)				Georgia (Tanyard Creek Sediment)			
Source	Isolate Number	log_{10} MPN g^{-1} dry weight of sediment		Decline %	Rank	log_{10} MPN g^{-1} dry weight of sediment		Decline %	Rank
		Day 0	Day 11			Day 0	Day 11		
Wastewater	DE-384	5.16 ±0.10	4.74 ±0.34	62.4	1	6.03 ±0.04	3.81 ±0.46	99.4	6
ATCC	Type	4.28 ±0.09	3.60 ±0.23	78.9	2	5.64 ±0.19	3.27 ±0.50	99.6	7
Wastewater	DE-205	5.36 ±0.07	4.51 ±0.27	85.8	3	6.02 ±0.02	3.84 ±0.40	99.3	3
Laughing Gull	GA-14	5.21 ±0.06	4.16 ±0.33	91.1	4	6.30 ±0.04	4.13 ±0.31	99.3	3
Robin	GA-3	5.43 ±0.02	4.32 ±0.27	92.2	5	6.02 ±0.13	4.68 ±0.18	95.4	1
Latrine	DE-2	4.97 ±0.16	3.83 ±0.34	92.8	6	5.94 ±0.11	4.33 ±0.41	97.6	2
Wastewater	DE-457	5.20 ±0.02	3.88 ±0.08	95.2	7	6.25 ±0.03	4.08 ±0.64	99.3	3
Escherichia coli	Control	<3.00[c]	<3.00	NA	NA	<3.00	<3.00	NA	NA

among subspecies of *E. faecalis* at each location and differences between the two locations. Differences in location may be because of biotic and abiotic factors. The most likely limiting abiotic factors were nutrients and temperature, because reasonable explanations exist to eliminate the four other major abiotic factors, oxygen, pH, ultraviolet light, and moisture. Oxygen was an unlikely factor because fecal enterococci are microaerophilic and can tolerate both oxygen-rich and oxygen-poor conditions; pH was an unlikely factor because both creeks were neutral or near-neutral and this range is optimal for survival of fecal enterococci; ultraviolet light was an unlikely factor because the chambers were placed in the shade; and moisture was an unlikely factor because it was not limiting. Although nutrients were not measured, temperature may be a factor because the temperature optima for the fecal enterococci are around 37°C.

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