MONITORING, IDENTIFICATION, AND GENETIC CHARACTERIZATION OF TETRACYCLINE-RESISTANT ENTEROCOCCUS FROM MILLER CREEK, ROBERTSON COUNTY, TENNESSEE

MARY H. McREYNOLDS

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A Thesis
Presented for the
Master of Science
Degree
Austin Peay State University

Mary H. McReynolds

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DEDICATION

This thesis is dedicated to my parents, William F. and Alice McReynolds, who have always encouraged and helped me to obtain a good education.

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Sincerely,

Mary McReynolds

Mary McReynolds

ABSTRACT

Tetracycline-resistant *Enterococcus* (TRE) have been detected from a watershed in Robertson County, TN, which had previously been shown to contain densities of *Enterococcus* that exceeded EPA guidelines for recreational contact. Low concentrations of tetracycline derivatives are common livestock feed additives used to enhance weight gain and thus could select for tetracycline-resistant bacteria within the livestock. Thirty-four TRE were isolated on selective mEnterococcus agar supplemented with tetracycline (16µg/mL) and then identified to species using standard biochemical tests. Ten species of *Enterococcus* were identified and all are typical cattle flora. Southern analysis using a 294 bp segment of the *tet*(M) gene from the conjugative transposon Tn*916* to probe *Sau*3AI-digested genomic DNAs extracted from each of the 34 TRE identified seven unique profiles. The same probe was also used in Southern analyses of 20 undigested DNAs from the 34 TRE separated by CHEF pulsed field gel electrophoresis to determine the location of the tetracycline-resistance element. The tetracycline-resistance element was found on chromosome-sized genetic elements in all isolates and 4 of

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CHAPTER I

INTRODUCTION

Agricultural Use of Antibiotics

Half of the antibiotics produced in the world are used on farm animals for nutritional or therapeutic purposes. Animal feed supplemented with low doses of antibiotics ($\leq$200g/ton) promotes animal growth and improves digestion efficiency (Pelczar et al., 1993). Animals receiving antibiotics in feed gain four to five percent more body weight (Witte, 1998). These antibiotics suppress the normal microbiota in the small intestine so that nutrient absorption is improved; however, low concentrations can also select for antibiotic-resistant bacteria within the livestock (Tortora et al., 1995).

The acquisition of antibiotic resistance has important medical implications. Antibiotic-resistant, non-pathogenic bacteria that survive production processes (e.g. those in raw cured sausage or raw milk cheese) can move through the food chain to other hosts where the opportunity to pass their genes to pathogenic bacteria exists (Perreten et al., 1997).

The World Health Organization (WHO) considers antibiotic-resistant bacteria a global concern. It recommends international cooperation in monitoring the development of antibiotic-resistances in bacteria, including bacteria isolated from food animals (WHO, 1997). In 1997, seventy members of human and animal health fields met in Berlin; they unanimously agreed that because of increasing health risks to humans, the excessive use of antibiotics should be reduced, especially when used to promote weight gain in food animals (WHO, 1997).

Tetracyclines are among the most common antibiotics added to animal feeds (Tortora et al., 1995). Chlortetracycline, a derivative of tetracycline, is an antibiotic found in animal feeds used by ranchers in Robertson County, Tennessee. In preliminary studies, a small percentage of tetracycline-resistant bacteria were identified among the bacteria in fecal pollution from farm run-off (McReynolds and Dailey, 1998; 1999). Those tetracycline-resistant bacteria belong to the genus *Enterococcus*.

Enterococcus

Enterococcus is a natural flora of the intestinal tract of nearly all animals, from cockroaches to humans

(Huycke et al., 1998). They are readily recovered from the environment, including surface waters contaminated with animal excrement or raw sewage (Huycke et al., 1998). *Enterococcus* is one of the bioindicators used by the Environmental Protection Agency (EPA) to measure water quality. The EPA advised that waterways with densities of *Enterococcus* in excess of 33 colony-forming units (CFU) per 100 mL of surface water are not safe for recreational contact (U.S.E.P.A., 1986).

Although *Enterococcus* is a useful indicator of environmental quality, they are also a prominent cause of nosocomial infections in the United States (English and Chesney, 1996). Infections caused by these Gram-positive cocci include urinary tract infections, endocarditis, neonatal sepsis, meningitis, nosocomial infections related to indwelling vascular catheters (English and Chesney, 1996), and burn wound infections (Heggers et al., 1998). Because enterococcal infections are generally restricted to immunocompromised individuals, the genus is considered much less virulent than many other pathogenic bacteria.

It is difficult to treat enterococcal infections because they are intrinsically resistant to many antibiotics such as cephalosporins, macrolides,

lincosamides, penicillins, aminoglycosides, and quinolones (Zareba and Hryniewicz, 1995; Leclereq, 1997). Some of these antibiotics such as aminoglycosides, however, are made more effective when used synergistically with another type of antibiotic such as a beta-lactam that disrupts the cell wall and promotes the uptake of the aminoglycoside (Moellering, 1992).

Successful treatment is made even more difficult by the ability of these bacteria to acquire antibiotic-resistance genes. Murray (1998) suggests that the numerous ways by which *Enterococcus* transferred DNA among cells reflected how *Enterococcus* developed such broad-spectrum antibiotic-resistance. One of the more prominent *Enterococcus*, *Enterococcus faecalis*, exchanges genetic material through pheromone-responsive conjugation (Murray, 1998). Pheromones are chemical signals that promote migration of responsive cells towards one another. A second type of transfer occurs among Gram-positive organisms. A broad host-range plasmid mediates the conjugation and may be responsible for the transfer of resistance genes among Staphylococcus, Streptococcus, and *Enterococcus* (Murray, 1998). The third type of exchange involves conjugative transposons, which mediate the most

nonspecific transfer between unrelated cells. Conjugative transposition might explain how resistance genes have spread from Gram-positive bacteria to Gram-negative species (Murray, 1998).

Conjugative transposons are linear mobile elements that possess the genetic machinery necessary for transfer to other bacteria through a circular intermediate (Rice, 1998). Conjugative transposons have been extensively studied and are very different from classical transposons, which are confined within the genome of an individual cell (Scott, 1992). When conjugative transposons insert into a DNA sequence, they do not duplicate the target sequence and the transposon-recipient bacteria develop the ability to act as a conjugational donor (Scott, 1992).

Perhaps another reason *Enterococcus* is so capable of accumulating and spreading multiple antibiotic resistances is because they are such a hardy taxa (Murray, 1998). They can survive a broad range of growth conditions: they tolerate temperatures ranging from 10°C to 45°C; they are facultative anaerobes; and they survive hypotonic, hypertonic, acidic and alkaline environments (Huycke et al., 1998). Sodium azide and concentrated bile salts, which cannot be tolerated by most microorganisms, are used

in selective medias for isolating *Enterococcus* (Huycke et al., 1998). The ability of *Enterococcus* to adapt and survive under such varied conditions might be linked in part to the promiscuous ability of these bacteria to exchange genetic material as indicated above.

Tetracycline-resistance Gene

Franke and Clewell (1981) found that the gene encoding tetracycline-resistance in *Enterococcus faecalis*, *tet*(M), is located on the conjugative transposon, Tn*916*. Tn*916* is the smallest of the conjugative transposons at 16.4 kilobases (Scott, 1992) and the most prominent representative that has been found in streptococci (Bertram et al., 1991). The specific mechanisms of the Tet(M) protein for enabling cells to resist tetracycline remain unclear; however, the gene product has been purified and it is structurally and functionally similar to elongation factor G for translation (Burdett, 1991).

The target for tetracycline is the 30S ribosomal subunit inside the cell where it inhibits protein synthesis. Tetracycline prevents the attachment of aminoacyl-tRNA to the accepter site on the mRNA ribosome complex.

Elongation factor G is important in the binding of charged t-RNA molecules to the ribosome and translocation of the ribosome along the mRNA transcript. Since tetracycline binds reversibly to the t-RNA acceptor site on the ribosome it is plausible Tet(M) promotes release of the tetracycline from the ribosome (Hamel et al., 1971).

Study Site

Tennessee's Department of Environmental Conservation (TDEC) designated the Sulphur Fork Creek/Red River (SFC/RR) watershed as one of Tennessee's priority watersheds (TDEC, 1998). Austin Peay State University's (APSU) Center for Field Biology has been monitoring the SFC/RR watershed for the past four years. Center researchers have consistently found high levels of fecal bacteria, primary algal productivity reduction, and sediment deposition (Dailey et al., 1998; Kinsey, 1998). A preliminary study conducted at multiple sites along SFC and RR in Robertson County, TN and Logan County, KY revealed that a small percentage ($\leq$1%) of the enterococcal population exhibited resistance to tetracycline (McReynolds and Dailey, 1998; 1999).

Miller Creek in Robertson Co., TN (Figure 1-1) is a tributary of SFC, which has been listed on the 303(d) list for impaired/limited water quality (TDEC, 1998). It is

approximately 12.8 km long, 5.5 m wide, and has a surface area of 7.04 hectares (Kinsey, 1998). Miller Creek drains nearly 6070 hectares of privately-owned livestock pasture and cropland in southwestern Robertson County (Kinsey, 1998).

Two sites were selected along Miller Creek for sampling (Figure 1-2). The lower Miller site (LM) (Figure 1-2A) was located ten kilometers northeast of I-24, approximately 0.4 km downstream from the Carr Road bridge in a 3rd order section of the stream. The upper Miller site (UM) (Figure 1-2B) was located along Henry Gower Road in a 2nd order section of the stream, 0.8 km south of the intersection with Sandy Springs Road (Lebkeucher and Houtman, 1999). The LM-site bisected a cattle pasture. Although there was a tree-lined barbed-wire fence on both banks, the fence was in such a state of disrepair that cattle had direct access to the banks and streambed. At the UM-site, a narrow vegetative buffer zone separated the west bank from cropland. Pasture for grazing cattle bordered the cropland acreage. The shoulder of Henry Gower Road is on the east bank and across the road is a house and yard.

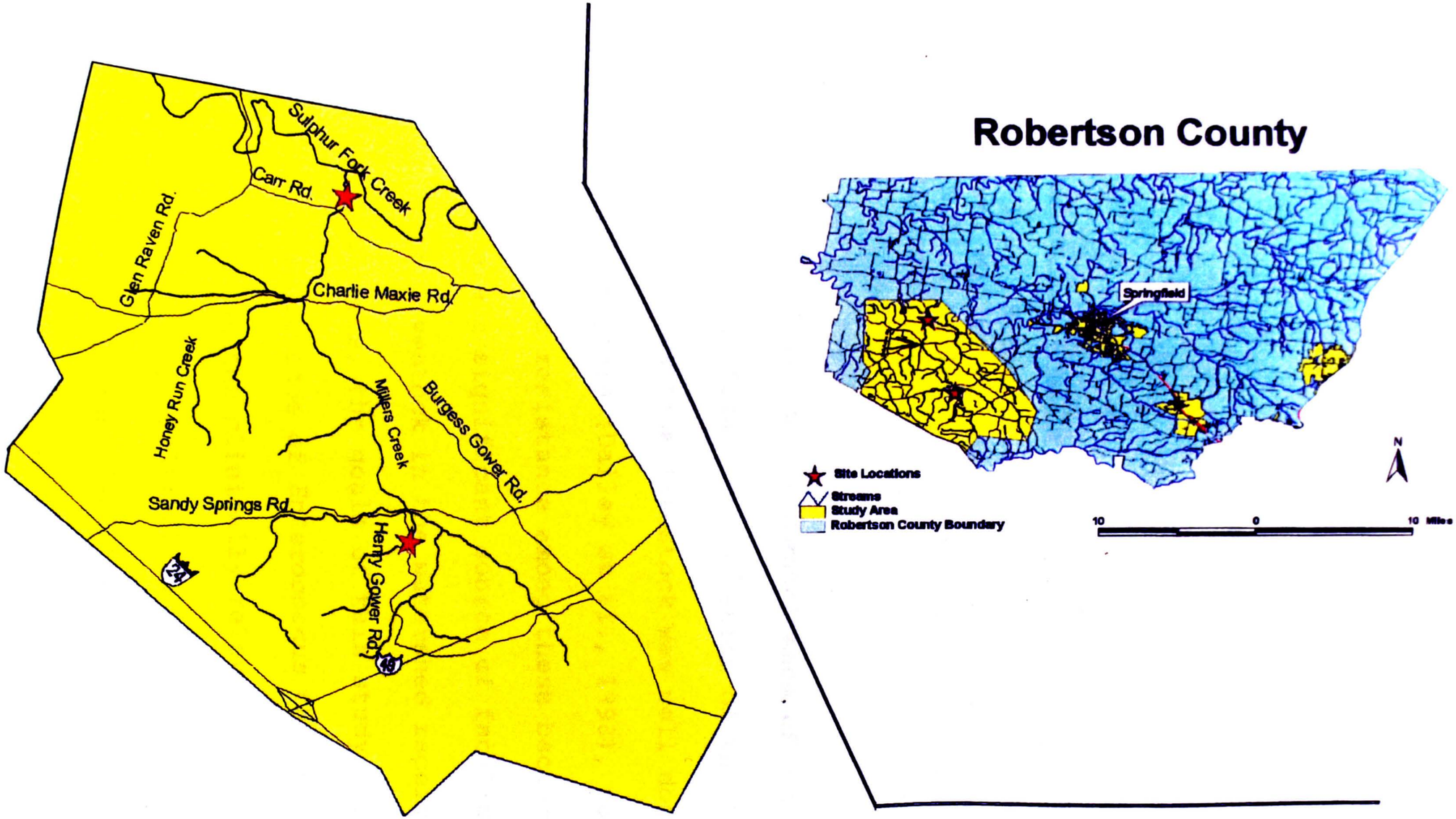

Figure 1-1. Map of water collection sites on Miller Creek. Miller Creek is located in the southwestern region of Robertson County, Tennessee. Robertson County is located in the middle of the state and forms part of the northern boundary of Tennessee with Kentucky. The lower Miller site was located ten kilometers northeast of I-24, approximately 0.4 km downstream from the Carr Road bridge in a 3rd order section of the stream. The upper Miller site was located along Henry Gower Road in a 2nd order section of the stream, 0.8 km south of the intersection with Sandy Springs Road (Lebkuecher and Houtman, 1999).

Figure 1-2. Sites of water collection. Photograph A is the lower Miller collection site and photograph B is the upper Miller collection site. The direction of water flow is indicated by the red arrow.

Purpose and Significance of Study

Even though fecal contamination of the SFC watershed with *Enterococcus* from livestock was well documented over the past four years (Dailey et al., 1998), no studies dealt with antibiotic resistance among these bacteria. Since livestock are a significant source of fecal pollution and some of the livestock in the watershed receive antibiotic-containing feeds, the goals of this study were: 1) to monitor the presence of *Enterococcus* that were resistant to tetracycline at the clinically-relevant concentration of 16 µg/mL (N.C.C.L.S., 1993); 2) to identify selected tetracycline-resistant isolates to species using standard biochemical tests (Krieg and Holt, 1984); 3) to detect the presence of the tetracycline-resistance gene, *tet*(M),

within these bacteria; 4) to determine the location of the tetracycline resistance gene(s); and 5) to qualify and quantify the frequency of mobility for the genetic elements that harbored the tetracycline resistance gene(s).

This project has very important environmental significance because for the first time it documents the presence of tetracycline-resistant *Enterococcus* in Miller Creek and its potential for spread throughout the watershed and into communities of northwestern Tennessee and southwestern Kentucky.

CHAPTER II

MATERIALS AND METHODS

Isolation of Tetracycline-resistant *Enterococcus*

Water sampling

Water samples were collected from two sites along Miller Creek once each month from July through October of 1999. The water was collected in two sterile, one-liter plastic bottles from steadily flowing water within the thalweg of the creek. Water temperature was recorded at each site on the day of sampling.

Isolation of Enterococcus

For isolation and enumeration of *Enterococcus*, three 10-mL and three 1-mL samples were vacuum filtered through sterile membrane filters (0.45 µm-pore size) within 6 hours of water collection, plated on mEnterococcus agar (EA), and incubated at 42°C for 48 hours in accordance with EPA water monitoring guidelines (Dailey et al., 1998; U.S.E.P.A., 1986). Three 300-mL samples were also filtered as above and plated on EA supplemented with 16 µg/mL tetracycline (EA-Tet) and incubated as above. After the 48-hour incubation, all reddish-pink colonies were enumerated and

recorded for each plate. To confirm that the colonies represented *Enterococcus*, random isolates were Gram stained, tested for catalase reaction, and assayed for their ability to hydrolyze esculin in the presence of bile. In addition the colonies on the EA-Tet were retested for tetracycline-resistance by transferring random colonies to Todd-Hewitt agar containing 16 µg/mL tetracycline (TH-Tet) and examined for growth following a 48-hour incubation at 37°C.

Relevant nonparametric statistical tests that did not require finite number information such as the Rank Sum were used to test for significant differences or correlations between sites, species, and various other parameters.

Speciation of Enterococcus

Five random colonies were selected from the EA and EA-Tet plates from each site for complete identification. Species were identified using standard biochemical tests as described in Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984).

Detection of *tet*(M) Gene

Bacterial strains and DNA extraction

A bacterial clone, *E. coli* CG120, was supplied by Dr. Donald Clewell, University of Michigan, Schools of Medicine

and Dentistry in Ann Arbor, Michigan. This bacterium contains the plasmid, pAM120, which harbors a conjugative transposon, Tn*916*, that contains the *tet*(M) gene. The culture was maintained on LB agar supplemented with 4 µg/mL tetracycline and 25 µg/mL ampicillin (Gawron-Burke and Clewell, 1984).

Plasmid DNA was extracted using a plasmid miniprep kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Five milliliters of TH-Tet broth was inoculated with a TRE isolate and incubated at 37°C for 48 hours. The cells were harvested from 2 mL of the culture by centrifugation and were lysed. The DNA was purified in a special silica matrix that has high affinity for DNA, washed with ethanol, and eluted with deionized water.

Genomic DNA was extracted from each of the 34 isolates of tetracycline-resistant *Enterococcus* and one isolate of tetracycline-sensitive *Enterococcus* using the Wizard Genomic DNA kit (Promega, Madison, WI) as directed by the manufacturer. DNA was extracted from 1.5 mL of a 10mL-overnight cell culture, grown at 37°C in BHI broth with or without tetracycline, depending on tetracycline sensitivity. The cells were collected from the broth

medium by centrifugation and then lysed. The cells and nuclei were lysed and RNAs were degraded. The genomic DNA was precipitated with sodium acetate and ethanol, pelleted, and resuspended in 1X TE.

Restriction enzyme analysis and agarose gel electrophoresis

A 10 µl volume containing approximately 8 µg of genomic DNA was digested with the restriction endonuclease, *Sau*3AI, at 37°C for 1 hour. Digested and undigested DNAs were each separated by size on an 1% agarose gel containing ethidium bromide (1 µg/mL) in 1X TAE buffer for three hours at 80V. Gels were visualized and photographed on an UV transilluminator (Bloom et al., 1996).

Southern transfer

To facilitate the mobilization of the DNA out of the gel and onto the nylon membrane, the DNA underwent an acid depurination pretreatment step. The gel was soaked in 0.25M HCl for 30 minutes at room temperature. Next the gel was placed in denaturation solution (1.5M NaCl, 0.5M NaOH) for 1 hour to denature the DNA and then neutralized in neutralization solution (1M Tris-HCl, pH 8.0 and 1.5M NaCl) for 1 hour. Then a standard Southern transfer of the DNA to a nylon membrane was performed (Bloom et al., 1996).

The nylon membrane was then allowed to air dry overnight, instead of baking for 30 minutes at 70°C.

Probe preparation

The probe was prepared from the plasmid pAM120 by using the PCR DIG Probe Synthesis Kit according to the manufacturer's instructions (Boehringer-Mannheim Corporation, Indianapolis, IN). This procedure incorporates a nucleotide analog labeled with the steroid derivative, digoxigenin, into the amplified DNA sequence.

The PCR primers used to amplify the probe sequence were developed from the *tet*(M) gene sequence found in Tn*916* using the bioinformatics program Primer3 (Rozen and Skaletsky, 1998). See ORF 11 of GenBank accession number U09422, which is the entire nucleotide sequence of Tn*916* isolated from *E. faecalis* DS16, for the tet(M) gene. This particular entry was used because it was submitted by Dr. Clewell and is the exact DNA sequence of the Tn916 in the plasmid clone, pAM120. The forward primer, 5'-TTGATGCCCTT-TTGGAAATC-3', begins at nucleotide 13207 of the GenBank entry and the reverse primer, 5'-ACTGCATTCCACTTCCCAAC-3', ends at nucleotide 13500, to produce a 294 base pair product.

An Amplitron II thermocyler (Barnstead| Thermolyne, Dubuque, IA) was programmed with the following PCR protocol: an initial 2 minute jumpstart at 95°C, followed by 10 cycles of 10 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, and 2 minutes of elongation at 72°C, then 20 cycles of 10 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, 2 minutes of elongation at 72°C with 5 seconds added to each elongation cycle, and ending with a 7 minute post-dwell at 72°C.

Hybridization

Probe hybridization and chromagenic detection was performed using the Boeringer-Mannheim's Genius Detection kit with slight modification of the procedure described by Bloom et al. (1996). The nylon membrane was soaked in prehybridization solution for two hours at 50°C with agitation. For hybridization, the nylon membrane was removed to a hybridization bag (Gibco BRL). Ten microliters of the DIG-labeled PCR product, generated above, was denatured for 5 minutes at 95°C and promptly added to 3 mL of fresh prehybridization solution. This solution was quickly poured into the hybridization bag with

the membrane and heat-sealed. The hybridization reaction was incubated overnight at 50°C with agitation.

Chromogenic detection

Chromogenic detection was performed exactly as described by Bloom et al. (1996). The membrane was washed three times to eliminate non-specific binding of the probe. Then the membrane was treated with a blocking reagent that reduces background coloration during the development procedure. Next the anti-DIG antibody conjugated with alkaline phosphotase (supplied with the Genius Detection kit) was applied. It complexed with the DIG-labeled nucleotide analogs that were incorporated into the probe. The addition of the chromogenic substrates nitroblue tetrazolium salt (NBT) and bromo-chloro-indolyl-phosphate (BCIP) revealed the location of the DIG-labeled probe.

Gene Mobility *In Vitro*

Bacterial strains

Enterococcus faecium ATCC 19434^{T} (American Type Culture Collection, Manassas, VA) is the recipient bacterium reported in the literature for filter mating procedures to measure the frequency of mobility of tetracycline-resistance genes (Quadnau et al., 1998). *Enterococcus faecium* 19434 is resistant to rifampicin (50 µg/mL) and

fusidic acid (20 µg/mL). Fusidic acid was unavailable for initial culture of the bacterium and consequently, the bacterium lost its resistance to fusidic acid. The resulting fusidic acid-sensitive strain of *E. faecium* 19434 was used as the recipient strain in the gene mobility experiments. The bacterium was grown at 37°C for 48 hours on TH agar supplemented with 50 µg/mL rifampicin (TH-Rif. All thirty-four TRE isolated from Miller Creek were tested as donors to transfer their tetracycline-resistance marker.

Filter mating

The filter mating procedure described by Quadnau et al. (1998) was used to determine mobility of the genetic element conferring resistance to tetracycline in the *Enterococcus* isolated from Miller Creek. Presumptive donor tetracycline-resistant *Enterococcus* and recipient *E. faecium* ATCC 19434^T, were cultured independently for 48 hours in 50 mL of TH-Tet broth and TH-Rif broth, respectively. Following growth, all samples were washed twice in 0.9% NaCl. The cell densities for the two broth cultures were then determined using a spectrophotometer at a wavelength of 600 nm. Cell concentrations were calculated using the formula: $A_{600} \times 10^8$ CFU x DF x C = number of cells/mL, such that A_{600} represents the absorbance

at 600 nm wavelength, CFU is colony forming units, DF is dilution factor for the spectrophotometer reading, and C is the dilution factor of the cell suspension.

The cell populations were then combined at a donor: recipient ratio of 1:10, mixed, and vacuum filtered onto a 47mm-nitrocellulose membrane filter containing 0.2 µm-sized pores (Fisher Scientific, Pittsburg, PA) and placed on a brain heart infusion (BHI) agar plate.

Transconjugants

Following incubation at 37°C for 18 hours, the cells were harvested by scraping the filter membrane and resuspending the cells in 1 mL of 0.9% NaCl. Transconjugants were selected by plating 100 µL of 10-fold serial dilutions of the resuspended cells on duplicate plates of BHI agar containing 50 µg/mL rifampicin and 16 µg/mL tetracycline. After a 24-hour incubation period at 37°C, conjugation frequency was calculated by dividing the total number of transconjugants by the starting cell density of donor parent cells.

Location of the *tet*(M) gene

Pulse field gel electrophoresis (PFGE) was used to investigate the location of the tet(M) gene among the genomic DNAs. PFGE allows for the separation of larger

sized DNA molecules (200,000 to 2.5 million bases) than can be achieved using standard agarose gel electrophoresis. In PFGE, the electric field changes periodically, which facilitates separation of the larger sized DNAs through the agarose gel matrix. PFGE typically requires longer electrophoresis times (12-24 hours) and thus operates with much lower voltage to reduce breakage of large DNAs.

Two liters of commercial 0.5X TBE (Bio-Rad Laboratories, Hercules, CA) were added to a level electrophoresis chamber. The system was equilibrated to 14°C using a cooling module (Bio-Rad Laboratories, Inc.) and the electrophoresis was programmed to run at a constant voltage of 6V/cm with pulse times ramping linearly from 5 to 35 seconds for 15 hours. Following electrophoresis, the gel was stained with ethidium bromide (1 µg/mL) and the DNA was visualized and photographed on an UV-transilluminator. The electrophoresed samples were then subjected to Southern transfer, probe hybridization, and chromagenic detection as described above, except that the time allotted for the Southern transfer was extended to 48 hours due to the large size of the DNA fragments.

CHAPTER III

RESULTS

Isolation and Identification of *Enterococcus*

Enterococcal Enumeration

Monitoring of water from the upper and lower sections of Miller Creek revealed concentrations of *Enterococcus* that exceeded EPA recommended allowances of 33 colony forming units/100 mL for recreational waters (Fig 3-1). Small percentages of the *Enterococcus* from both sites were found to be resistant to tetracycline (16 µg/mL) throughout the sampling period. At the upper Miller site, the population of tetracycline-resistant *Enterococcus* (TRE) ranged from 0.09% to 1.83% of the overall enterococcal population (Fig 3-1A) while at the lower Miller site, the TRE densities comprised 0.04% to 0.37% of the total (Fig 3-1B). Water temperatures were recorded during each collection and no correlation was observed between enterococcal cell densities and water temperature.

Species Identification

Eighty samples of *Enterococcus* were planned to be isolated from Miller Creek by randomly choosing five

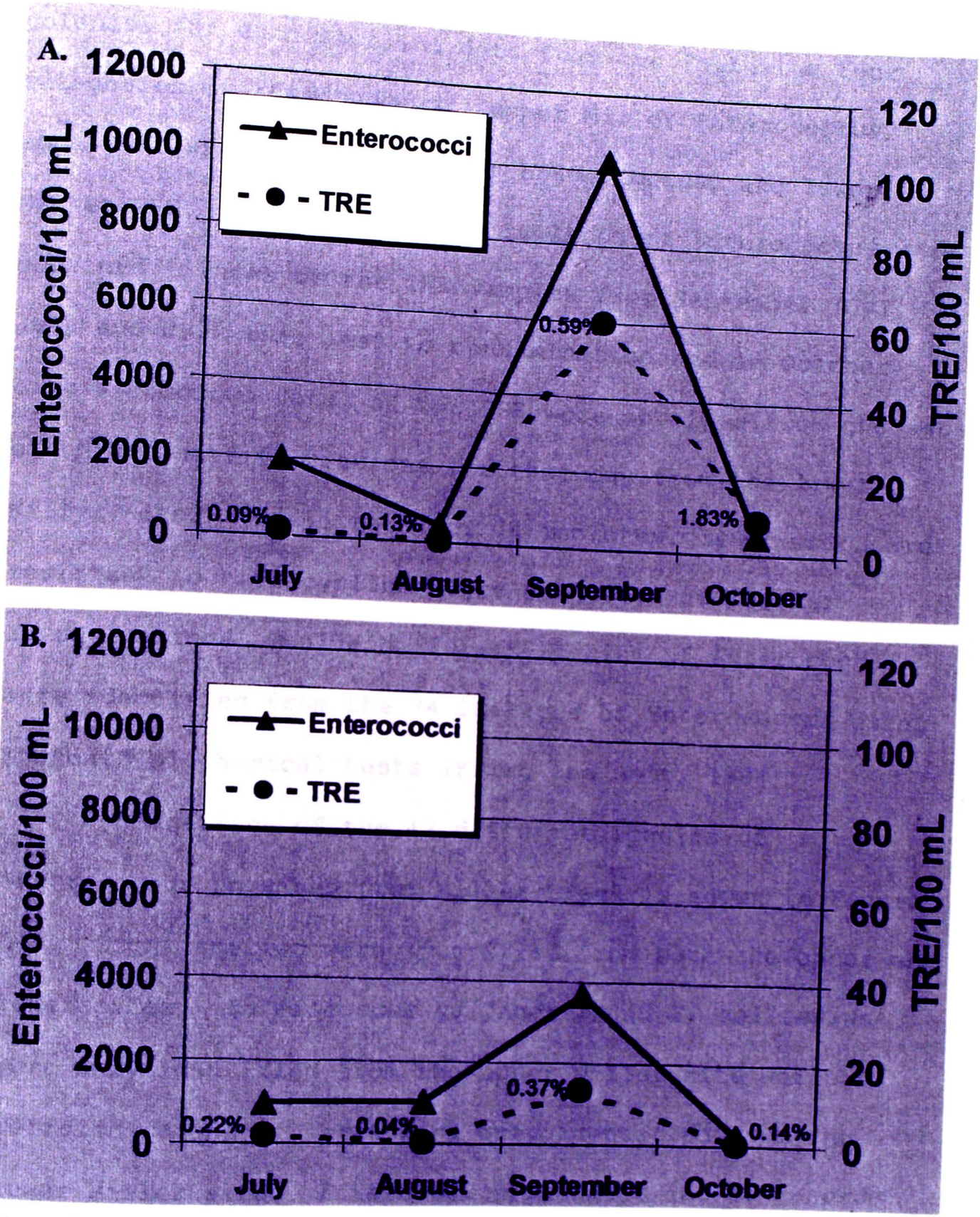

Figure 3-1. Comparison of the densities of *Enterococcus* in Miller Creek at the upper Miller site (A) and the lower Miller site (B) during the summer and fall of 1999. *Enterococcus* spp. were enumerated by plating serial dilutions of the water on mEnterococcus agar. Each point represents an average of 3 replicate samples. The percent of the population that was determined tetracycline-resistant is listed to the side of each of the TRE data points. Note the scale differences for *Enterococcus* and TRE to facilitate both sets of data on a single graph.

colonies for each sampling date from the following four categories of *Enterococcus*: upper Miller *Enterococcus*, upper Miller TRE, lower Miller *Enterococcus*, and lower Miller TRE. However, only 74 isolates of *Enterococcus* were obtained. Three of the TRE samples from September (Jy1, Jy7, and Jy9) were lost to contamination and in October, only a combined total of two TRE were obtained from the set of three water filtrations for that day from the lower Miller site. Thus a total of 74 isolates, 34 of which are resistant to tetracycline, were subjected to further investigation. Twelve different species of *Enterococcus* were identified from the 74 isolates of *Enterococcus* using standard biochemical tests (Krieg and Holt, 1984).

A comparison of the 12 different species of *Enterococcus* obtained from Miller Creek is shown in Figure 3-2. Eight species were identified from both the upper and lower sites. *Enterococcus gallinarum* and *E. solitarius* were only identified from the upper Miller site while *E. seriolicida* and *E. hirae* were only identified from the lower Miller site. *Enterococcus faecalis* and *E. cecorum* were the more frequently isolated *Enterococcus* from the upper Miller site (Fig 3-2A) whereas *E. faecalis* was isolated more frequently when antibiotic resistance was

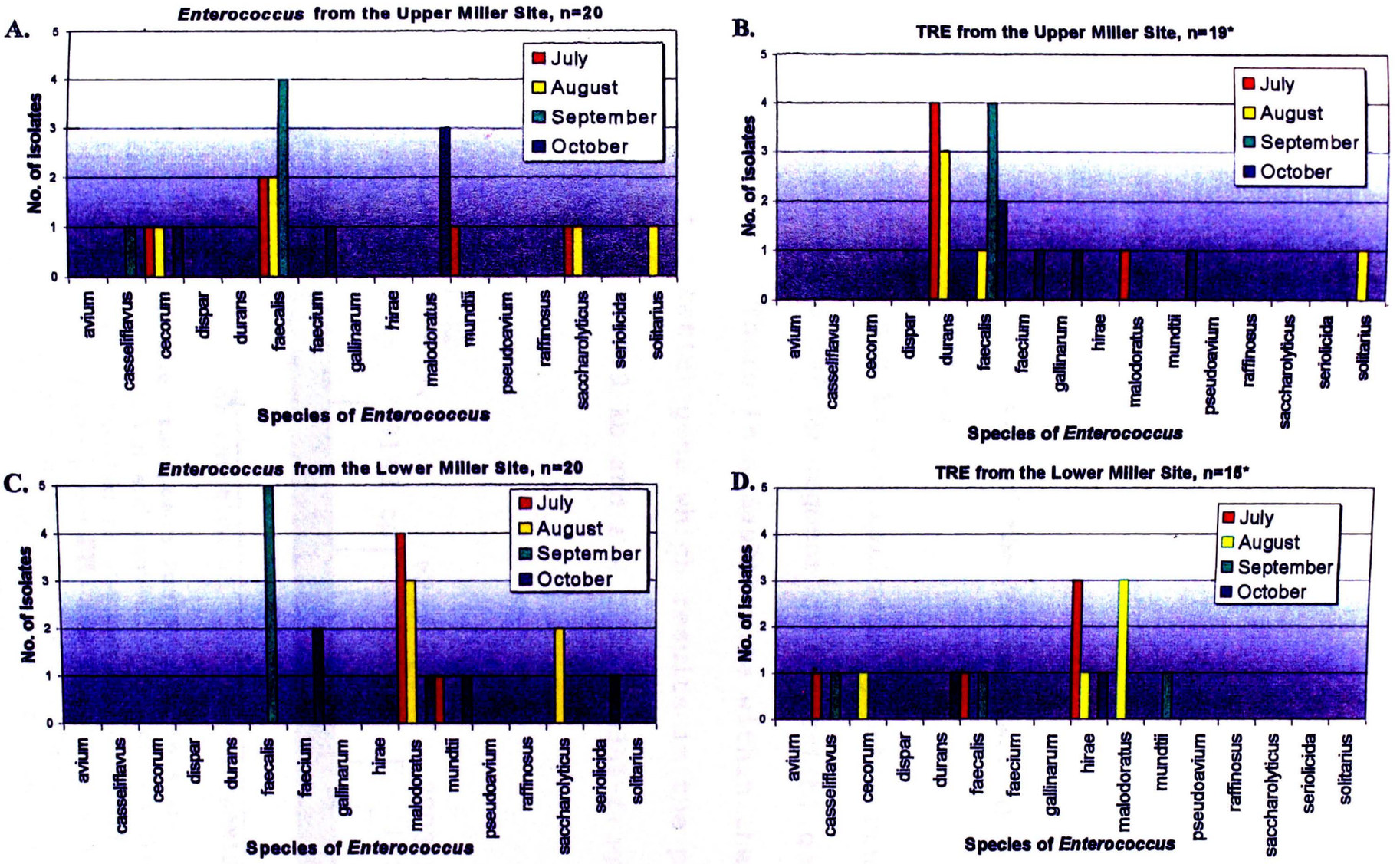

Figure 3-2(A-D). Comparison of the species of *Enterococcus* isolated from Miller Creek. The species of *Enterococcus* obtained from the upper Miller site are shown in (A) and the TRE from the same site are in (B). The species of *Enterococcus* obtained from the lower Miller site are shown in (C) and the TRE from the same site are in (D). Five isolates of *Enterococcus* and TRE were obtained from each site during each of the four months July, August, September, and October. Vertical bars represent the number of isolates identified to each species and are color-coded according to the month when they were isolated.

*One September TRE isolate from the upper Miller site was lost to contamination, two September TRE isolates from the lower Miller were lost to contamination, and only two TRE isolates were obtained from the water sampled from the lower Miller site in October.

selected (Fig 3-2B). Frequency of isolation is determined by the number of different months in which the species were identified rather than the number of isolates obtained. At the lower Miller site, *E. malodoratus* was encountered more frequently (Figure 3-2C), however, *E. hirae* was most commonly identified among the tetracycline-resistant *Enterococcus* (Figure 3-2D).

Detection of the Tet(M) Gene(s)

Probe characterization

The probe used in the following genetic studies corresponds to a 294 bp segment of the *tet*(M) gene of Tn*916* (Figure 3-3). There is a *Sau*3AI site within the probe region of the *tet*(M) gene which results in the probe hybridizing to a 2.9 kb and a 4.1 kb *Sau*3AI-fragment of

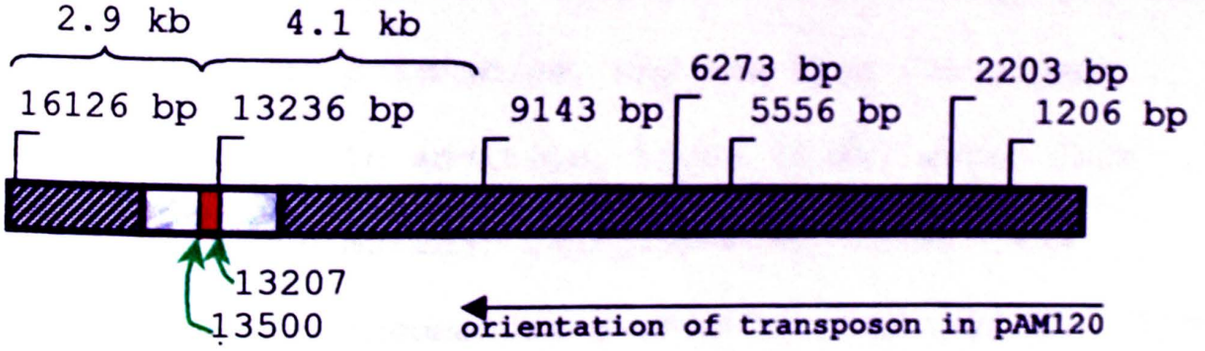

Figure 3-3. Conjugative transposon Tn*916*. The 16.4 kb conjugative transposon, Tn*916*, was cloned and sequenced by Dr. Don Clewell from the University of Michigan. Labeled on the map are Tn*916* ▨, *tet*(M) ▭, the region recognized by the probe ▬, and the recognition sites for the *Sau*3A1 restriction endonuclease ⌈. The nucleotides are numbered according to GenBank accession number U09422. The green arrows indicate the first and last base of the probe sequence.

Tn*916*. The *Sau*3AI site divides the 294 bp region disproportionately such that 264 nt (90%) of the probe sequence recognizes the 2.9 kb fragment and 10% recognizes the 4.1 kb fragment.

A search was then performed to identify related sequences among tetracycline resistance genes published in the searchable database hosted by the National Center for Biotechnology Information using our *tet*(M) probe sequence and the molecular biology internet service, BLASTn 2.1.1 (Altschul et al., 1997). Twelve sequences were identified that demonstrated greater than 85% homology with our probe sequence. All twelve were *tet*(M) genes sequenced from transposons and plasmids from very diverse bacteria: four from *Enterococcus faecalis*, two from *Gardnerella vaginalis*, two from *Neisseria gonorroeae*, one from *Neisseria meningitidis*, one from *Staphylococcus aureus*, one from *Streptococcus pneumoniae*, and one from *Ureaplasma urealyticum*. In addition, three *tet*(O) genes from *Streptococcus mutans*, *Campylobacter jejuni*, and *Streptococcus pneumoniae* shared 58% homology with the probe and a *tet* gene of unspecified class from *Listeria monocytogenes* was 46% homologous.

Probe detection

Southern blot analysis revealed that all TRE isolates had gene sequences complementary to the 294 bp probe sequence (Figure 3-4). The 34 TRE were classified into 7 unique DNA profiles according to the size variability of the *Sau*3AI fragment(s)hybridized by the probe (Table 3-1). The majority of the isolates, 18 (52.9%), formed Group I and had DNA fragments comigrating with the 2.9 kb and 4.1 kb bands of Tn*916* (Figure 3-4, lane 2). Groups II and IV each contained 6 isolates while each of the remaining groups were defined by single isolates (Table 3-1).

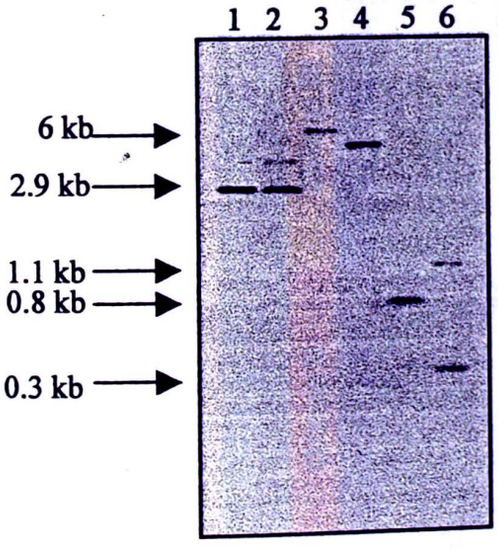

Figure 3-4. Bands representing the seven DNA profiles (I-VII) of the *tet*(M) gene demonstrated in the 34 TRE. Lane 1=pAM120 (positive control) with 4.1 kb and 2.9 kb bands, lane 2=Group 1 (represented by Sp2) with 4.1 kb and 2.9 kb bands, lane 3=Group IV (represented by Sp4) with 6 kb band, lane 4=Group II (represented by Sp5) with 5 kb band, lane 5=Group VII (represented by Sp6) with 0.8 kb band, and lane 6=Group VI (represented by Ag5) with 1.1 kb and 0.3 kb bands. Two profiles not pictured are Group III whose members exhibit bands approximately 5 kb and 2.9 kb and Group V with a 0.3 kb band.

Table 3-1. Classification of 34 TRE into 7 groups according to band size-locations of the *tet*(M) gene

Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
2.9kb +/- 4.1kb	5kb	2.9kb + 5kb	6kb	0.3kb	0.3kb + 1.1kb	0.8kb
Jy4 *E. malodoratus*		Jy3 *E. durans*		Jy7 *E. casseliflavus*		Sp6 *E. casseliflavus*
Jy6 *E. hirae*						
Jy8 *E. faecalis*						
Jy9 *E. hirae*						
Jy10 *E. hirae*						
Ag1 *E. solitarius*						
Ag2 *E. faecalis*						
Ag3 *E. durans*						
Ag4 *E. durans*						
Sp2 *E. faecalis*						
Sp8 *E. faecalis*						
Sp10 *E. mundtii*						
Ot1 *E. faecalis*						
Ot2 *E. faecium*						
Ot3 *E. mundtii*						
Ot4 *E. gallinarum*						
Ot5 *E. faecalis*						
Ot6 *E. durans*						

Further Analysis of the *Tet*(M) Gene

Frequencies of transconjugation

Since many of the tetracycline-resistance genes were suggestive of *tet*(M), we assayed each of the TRE from all 7 groups for the ability to transfer tetracycline resistance. A standard filter mating assay was used and demonstrated that all TRE isolates were capable of transferring tetracycline resistances to a tetracycline-sensitive *E. faecium*. The frequencies of mobility ranged from 10^{-8} to 10^{-1} per donor (Table 3-2).

Location of tet(M)

Twenty randomly selected TRE isolates were subjected to pulsed-field gel electrophoresis (PFGE) to investigate the location of the *tet*(M) genetic element. The gels were subjected to Southern blotting using the same 294 bp probe described above (Fig 3-5). All of the TRE demonstrated hybridization consistent with a chromosomal location for the tetracycline-resistance gene (Fig 3-5, lanes 4-9). In addition, isolates *Jy5*, *Ag8*, *Ag9* and *Ag10* showed bands suggestive of extrachromosomal locations (Fig 3-5, lane 8).

Table 3-2: Frequencies of mobility of the tetracycline-resistance gene(s) for the Miller Creek TRE isolates

Isolate	Species	Mobility[1]
Jy1	durans	1.078 E-2
Jy2[2]	durans	1.767 E-1
Jy3	durans	3.593 E-6
Jy4	malodoratus	5.390 E-6
Jy5 [3]	durans	2.607 E-2
Jy6	hirae	4.030 E-7
Jy7	casseliflavus	6.890 E-8
Jy8	faecalis	1.040 E-5
Jy9	hirae	2.850 E-6
Jy10	hirae	4.030 E-2
Ag1	solitarius	2.392 E-6
Ag2	faecalis	2.140 E-7
Ag3	durans	4.240 E-5
Ag4	durans	8.160 E-7
Ag5	durans	3.140 E-8
Ag6	cecorum	1.127 E-5
Ag7	hirae	4.630 E-6
Ag8	malodoratus	3.440 E-2
Ag9	malodoratus	1.880 E-2
Ag10	malodoratus	5.330 E-3
Sp2	faecalis	6.201 E-7
Sp3	faecalis	1.400 E-6
Sp4	faecalis	9.004 E-7
Sp5	faecalis	1.602 E-6
Sp6	casseliflavus	9.302 E-4
Sp8	faecalis	6.980 E-6
Sp10	mundtii	1.700 E-8
Ot1	faecalis	2.000 E-6
Ot2	faecium	1.184 E-6
Ot3	mundtii	2.600 E-5
Ot4	gallinarum	1.100 E-6
Ot5	faecalis	1.700 E-6
Ot6	durans	1.700 E-8
Ot7	hirae	1.400 E-4

[1]Mobility is defined as the frequency that the tetracycline-resistance gene was transferred from the donor TRE isolates to *E. faecium* ATCC 19434 recipients using the filter mating procedure. This frequency was calculated by dividing the number of transconjugants by the cell count of the limiting parent, which was always the donor, since the mating ratio was 10(recipient):1(donor) throughout the experiments.

[2]Undigested DNAs extracted from the TRE samples shown in red were analyzed by PFGE. The tetracycline-resistance element was detected on chromosome-sized genetic elements.

[3]Red ovals indicate TREs analyzed by PFGE and Southern blotting that showed smaller genetic elements suggestive of plasmid DNA.

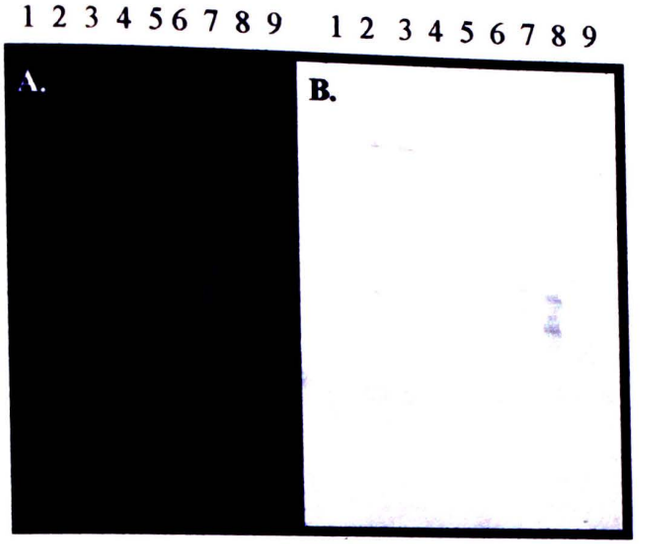

Figure 3-5. PFGE (A) and Southern transfer of TRE's (B). Approximately 5 µg undigested genomic DNA was loaded in each lane: Lane 1= Tet-sensitive enterococcal (TSE) DNA (negative control), Lane 2=TSE + pAM120, Lane 3= pAM120 (positive control), Lane 4=Jy4, Lane 5=Jy6, Lane 6=Ag4, Lane 7=Ot7, Lane 8=Jy5, and Lane 9=Jy9.

CHAPTER IV

DISCUSSION

Introduction

Miller Creek, which is located in rural Robertson County, Tennessee, has been documented over the past five years to contain levels of *Enterococcus* that exceed EPA guidelines for recreational contact (Dailey et al. 1998; Dailey, unpublished data). Since Miller Creek flows alongside and through several cattle farms, we sought to characterize the impact of these cattle on the bacteria in the creek. We present the following findings concerning *Enterococcus* at Miller Creek: (1) confirmed the previous data documenting high levels of *Enterococcus*; (2) demonstration that 0.04% to 1.83% of the *Enterococcus* was tetracycline-resistant *Enterococcus* (TRE); (3) the TRE were distributed over 10 species; (4) all TRE carried a genetic element showing sequence homology with the *tet*(M) class of tetracycline-resistance genes; and (5) all tetracycline-resistance elements were capable of mobilization to other isolates of *Enterococcus*.

The EPA recognizes *Enterococcus*, which were formerly classified as fecal streptococci, as a bioindicator of water quality. An enterococcal density of 33 CFU/100 mL of fresh water has been proposed as a guideline for when gastroenteritis and other water-borne diseases associated with fecal contamination is a significant risk from

Table 4-1. Some common microorganisms associated with water supplies contaminated by fecal material that are pathogenic to humans

Organisms	Diseases	Symptoms	Reservoirs
Balantidium coli	Balantidiasis	Diarrhea, abdominal cramps, fever, and nausea	Swine and monkeys
Campylobacter jejuni	Campylobacteriosis	Fever, abdominal pain, and diarrhea	Cattle, sheep, dogs, cats, rodents, and fowl
Cryptosporidium parvum	Cryptosporidiosis	Diarrhea, stomach cramps, upset stomach, and slight fever	Wide variety of animals including mammals, reptiles, and fish
Entamoeba histolytica	Amebiasis	Loose stools, stomach pain, and stomach cramping	Humans
Escherichia coli	Gastroenteritis	Stomach cramps, nausea, vomiting, and watery diarrhea	Wide variety of animals including piglets, calves, and humans
Giardia lamblia	Giardiasis	Diarrhea, abdominal cramps and nausea	Animals such as beaver or muskrats
Hepatitis A virus	Hepatitis	Fever, chills, anorexia, abdominal discomfort, jaundice, and dark urine vomiting, and leg cramps	Human contact with feces/sewage, contaminated shellfish and other filter-feeders
Salmonella spp.	Enteritis	Nausea, vomiting, diarrhea, fever, abdominal cramps, myalgias, and headache	Virtually all animals: poultry, reptiles, livestock, rodents, domestic animals, birds and humans
Vibrio cholerae	Asiatic cholera	Severe diarrhea, vomiting, and leg cramps	Humans and some crustaceans
Yersinia enterocolitica	Enterocolitis	Diarrhea, fever, abdominal pain	Source is rarely identified but has been isolated from water, milk, and wild and domestic animals

Source: Murray et al., 1994

recreational contact (U.S.E.P.A., 1986). Several waterborne pathogens can cause gastroenteritis (Table 4-1). We did not test for any of these pathogens in Miller Creek. Interestingly, others have shown that many of these human pathogens such as *Campylobacter* (Gaunt and Piddock, 1996), *Escherichia coli* (Levy, 1976; Tschäpe, 1994), *Salmonella*, *Yersinia*, and *Enterococcus* have acquired antibiotic-resistance as a result of the use of antibiotics as growth promoters in livestock feed (Witte, 1998). Since the densities of *Enterococcus* recorded from Miller Creek do correlate well with the presence of pathogenic microorganisms (Cabelli, 1980; Dufour, 1984; Kay et al., 1994; and Miescier and Cabelli, 1982) and nearly half of the antibiotics produced in the USA are used for farm animals (Tortora et al., 1995), we tested for the presence of tetracycline-resistant *Enterococcus* (TRE) in Miller Creek.

Water Monitoring and TRE

Miller Creek is a tributary to Sulphur Fork Creek. Results from a preliminary study in 1998 conducted at sites along Sulfur Fork Creek and Red River waters detected the presence of low levels of TRE (McReynolds and Dailey, 1998;1999). As in previous studies of the watershed, the

concentrations of *Enterococcus* at Miller Creek were always found exceeding EPA allowances for recreational waters. Additionally, the results of all four sampling dates for this study, between July and October of 1999, revealed the presence of *Enterococcus* resistant to tetracycline at the clinically-relevant concentration of 16 µg/mL.

Although the TRE demonstrated considerable quantitative variability throughout the study period, they were present in all samples. The fluctuation observed in enterococcal densities could be the result of changing environmental conditions such as variation in rainfall, farm runoff, or water temperature. Nevertheless, the increases and decreases of the TRE population mimicked the same trends as the general population of *Enterococcus* closely, and the percentages of the *Enterococcus* that were TRE remained consistently low. They ranged from 0.04% to 1.83% and averaged 0.66% at the upper Miller site and 0.19% at the lower Miller site. This difference was not considered significant by the Rank Sum test.

Although the TRE always represented a small percentage of the overall enterococcal contamination of the water, it is inaccurate to rank the quantity of TRE as "negligible." During the month of September the concentration of TRE by

itself was enough to exceed EPA guidelines for enterococcal contamination at the upper Miller site. Perhaps this was a result of a lack of any rainfall greater than one half inch in any 24-hour time period since July 24 as recorded nearby by the USGS rain gauging station located at Port Royal, Tennessee on the Red River (Lowery, 2000). Nearly a half inch of rain fell between the September and October water collection times (Lowery, 2000) explaining why the enterococcal densities were reduced in October.

Identification of TRE to Species

In *Bergey's Manual of Determinative Bacteriology* are descriptions of sixteen species of *Enterococcus* (Krieg and Holt, 1984). Twelve species were identified among the isolates of *Enterococcus* inhabiting Miller Creek but only ten were represented in the sample of 34 TRE isolated for the purpose of identification and further characterization. Although the sample size was not large, Figure 4-1 shows an overview of the more common *Enterococcus* that were identified. *Enterococcus faecalis*, *E. malodoratus* and *E. cecorum* were the species of general *Enterococcus* encountered most often and *E. faecalis*, *E. durans*, and *E. hirae* were the three most common TRE.

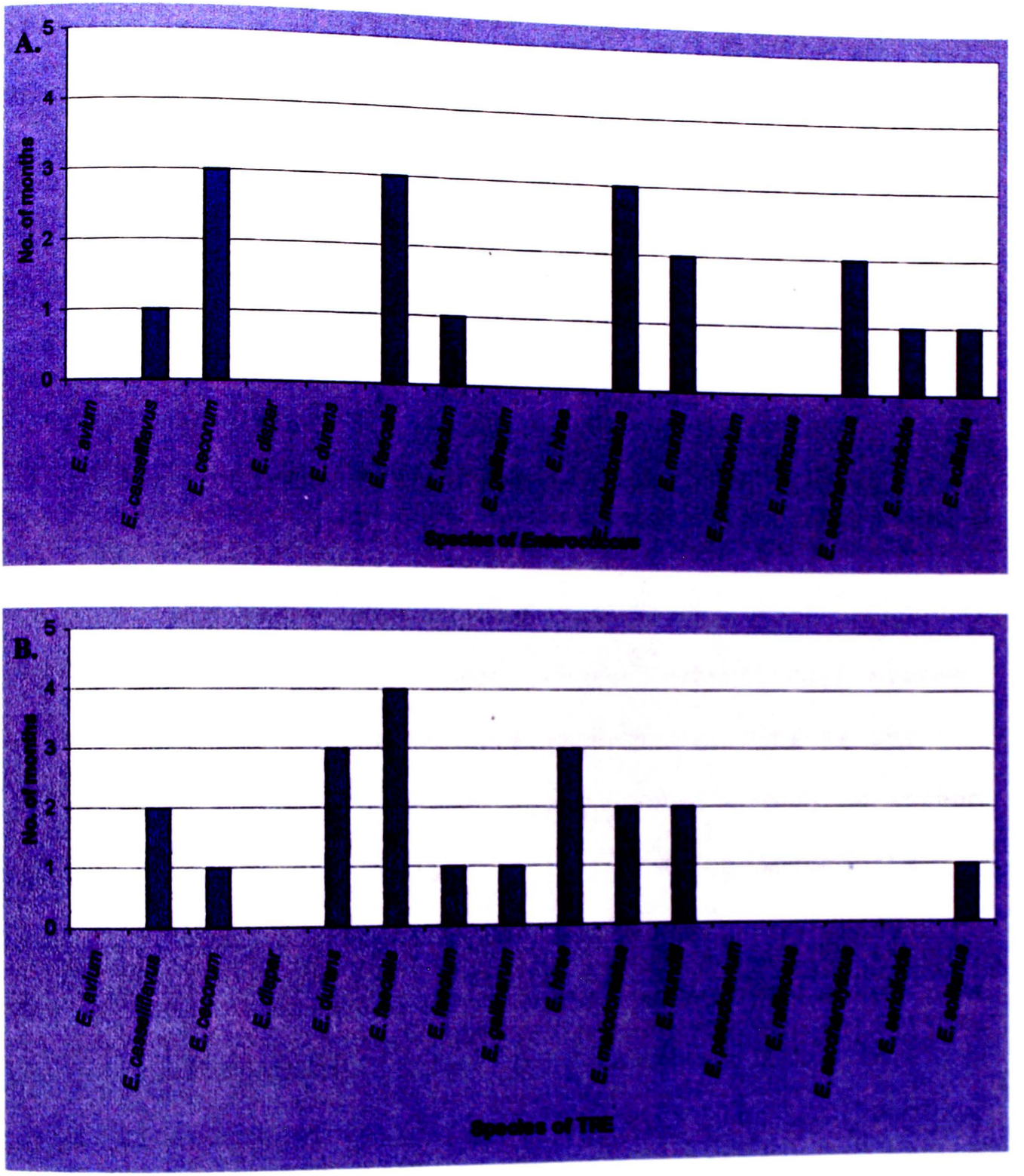

Figure 4-1. Frequencies of isolation for the 16 species of *Enterococcus* from the upper and lower sites of Miller Creek between July and October 1999. Because only a maximum of 5 colonies of enterococci and TRE were identified to species each month, we have measured frequency by the number of months each species was isolated. The enterococcal population (n=40) is represented in (A) and the tetracycline-resistant population (n=34) is shown in (B).

Many attempts have been made to distinguish sources of *Enterococcus* but few have shown great reliability or specificity (Wiggins et al., 1999). The difficulty is due to the fact that fecal bacteria have unstable phenotypes, low sensitivity at the intraspecies level, and limited specificity (Stull et al., 1988). Table 4-2 is provided as a guide indicating where some of the species of *Enterococcus* have been isolated. Although the more prevalent species of *Enterococcus* identified in this study have all been found in cattle or other food animals, most are also common to humans (Devriese et al., 1995).

Nevertheless cattle were surely a significant source of the fecal pollution in Robertson County because the cattle were frequently given unrestricted access to stream beds and were often sighted in the creek or around the banks of various tributaries of RR and SFC, including Miller Creek. Furthermore it seems highly likely that the cattle were responsible for the presence of TRE because chlortetracycline, an analog of tetracycline, is commonly found in livestock feed.

Table 4-2. Reported sources of enterococcal isolates

Species	Description
E. avium	Obtained from avian, canine, and human sources (Koneman et al. 1992).
E. casseliflavus	Rare in animals and humans (Niemi et al. 1993). Recovered from plants, soil, feces of chickens (Koneman et al. 1992)
E. cecorum	Isolated from chickens (Devriese et al. 1983).
E. dispar	Isolated from human feces (Koneman et al. 1992).
E. durans	Prevalent in milk and dairy products (Kielwein 1978).
E. faecalis	Dominant species shed in human feces; also found in cattle, pig, poultry, dogs, sheep, and goats (Devriese et al. 1995; Koneman et al. 1992).
E. faecium	Present in human waste and prevalent in milk and dairy products (Devriese et al. 1995). It is also found in other animals (Koneman et al. 1992)
E. gallinarum	Isolated from chicken feces (Koneman et al. 1992).
E. hirae	Seems to be rarely isolated from humans (Devriese et al. 1995). Obtained from chickens, cattle, pigs, canines, horses, sheep, goats, and rabbits (Koneman et al. 1992).
E. malodoratus	Isolated from Gouda cheese and unpasteurized milk products (Koneman et al. 1992).
E. mundtii	Isolated from plants and soils (Collins et al. 1986). Also recovered from gastro-intestinal tracts of cattle, pigs and horses (Koneman et al. 1992).
E. pseudoavium	Isolated from a case of bovine mastitis (Koneman et al. 1992).
E. raffinosus	Isolated from human infections (Koneman et al. 1992).
E. saccharolyticus	Isolated mainly from straw bedding in cattle stables (Koneman et al. 1992).
E. seriolicida	Has not been isolated from humans but causes disease in yellow tail fish (Koneman et al. 1992).
E. solitarius	Isolated from ear exudate (Koneman et al. 1992).

Genetic Characterization of TRE

Conjugative transposons are often associated with the dissemination of antibiotic resistance in Gram positive bacteria (Clewell et al. 1995). Most conjugative transposons carry *tet*(M) or a closely-related tetracycline-resistance gene, especially in *Streptococcus* and *Enterococcus* (Rudy and Scott, 1994; Salyers, 1995). It was of interest to determine if the isolates of TRE from Miller

Creek possessed the *tet*(M) gene on a mobile genetic element.

We chose three characteristics that are somewhat diagnostic of the *tet*(M) gene for the genetic characterization of the TRE isolates:

- probed with a fragment of the DNA sequence for the *tet*(M) gene found in conjugative transposon Tn*916* that is deposited in GenBank, accession number U09422 (Flannagan et al, 1994).
- tested for mobility of the genetic element(s) conferring resistance to tetracycline in our TRE to a tetracycline-sensitive host and compared frequencies of mobility with those reported from other mating experiments, and
- determined the genomic location (i.e. chromosomal or plasmid) of the sequences that were recognized by the probe for the *tet*(M)-like genes.

The data from these investigations suggested the tetracycline resistance gene in all of the TRE isolated from Miller Creek was *tet*(M). The Southern blotting experiments identified seven distinct DNA profiles, which we designated Group I-VII among the 34 TRE isolates. In addition, all 34 TRE isolates were capable of transferring the tetracycline-resistance element. The transfer frequencies ranged from 10^{-8} to 10^{-1} per donor, which was quite similar to the broad range of 10^{-9} to 10^{-5} per donor, reported for the conjugative transposon Tn916 (Gawron-Burke and Clewell, 1982). Finally all of the TRE analyzed by CHEF PFGE suggested the *tet*(M) gene was located on the

chromosome as frequently reported for *Enterococcus* (Clewell, 1993). Interestingly, four isolates also showed recognition of smaller genetic elements suggestive of plasmid DNA.

Group I contained the largest number of isolates (18/34) and provided the results consistent with the sequence of the *tet*(M) gene from the pAM120 clone with Tn*916*. Although it seems unfortunate that a Sau3AI recognition site occurred in the sequence of DNA recognized by the probe, it did provide a certain degree of specificity in recognizing those isolates harboring the Tn916-like genetic elements. A BLASTn search (Altschul et al, 1997) of all the gene sequences in GenBank found that this restriction site is not conserved in all of the *tet*(M) genes. There were 16 entries of *tet*(M) that closely matched the 294 bp region recognized by our probe. Twelve of the entries featured the *Sau*3AI restriction site: "gatc," located at nucleotides 29-32 of the probe's sequence. All four of the variants from the BLASTn search shared a substitutional point mutation of a "c" in place of the "t," such that they read "gacc." If some of the TRE from Miller Creek had this alteration, then it could explain some of the diversity found in the DNA profiles.

Interestingly, in each of the Group II TRE, the *tet*(M) gene was located on a fragment estimated to be 5kb. Isolates that featured this pattern (with the exception of one) exhibited a higher frequency of mobility than reported for Tn*916*, averaging 10^{-2} per donor. Surprisingly, all of the isolates that possessed extrachromosomal DNA as determined by the CHEF PFGE experiment belonged to this group. Perhaps all of the members of Group II have plasmid copies, which could contribute to their higher frequency of mobility.

It is possible that some bacterial strain duplication exists in Group II. Isolates Jy1 and Jy5 could be the same bacterial strain and Ag8, Ag9, and Ag10 could be another. Each of these sets were of the same species, same section of Miller Creek, same month, same banding pattern, and share very similar mobility frequencies from the mating experiments. This is an important point to consider because if they are not different strains, then the size of Group II is over-represented.

Differences in fragment sizes recognized by the *tet*(M) probe characterize the other five groups as well. Their mobility frequencies from the mating experiments generally fall within the broad range characteristic of conjugative

transposon Tn*916*; therefore, the isolates could have mutated *Sau*3AI sites or the variability in fragment sizes and mobility frequencies of conjugative transfer could be attributed to a transposon other than Tn*916*. Composite transposons, which are a merger of several conjugative transposons, have been reported that contain the *tet*(M) gene (Horaud et al., 1991). It would be interesting to compare the DNA sequences from each of the groups and analyze just how different these *tet*(M) genes actually are.

Conclusions

In summary, the results of this study show that: 1) Miller Creek was contaminated with TRE along with high levels of *Enterococcus* which as a bioindicator species correlates very closely with the presence of human pathogens in recreational waters (Cabelli, 1980; Dufour, 1984; Kay et al., 1994, and Miescier and Cabelli, 1982), 2) the tetracycline-resistance genes were mobile and demonstrated genetic similarity with the *tet*(M) gene, and 3) the cattle are impacting the water quality of Miller Creek. In conclusion, it seems highly likely that resistance to tetracycline is being transferred to more pathogenic bacteria in Miller Creek and therefore the need for exclusion of cattle from the creek is most urgent.

More research on problems associated with the sub-therapeutic use of antibiotics in livestock feeds, development of easy and more accurate methods for detecting pathogens in bodies of water, and the need for exclusion of cattle from streams is needed. Cattle can be effectively diverted from streams when provided with an alternate water source, such as a spring-fed trough or a solar pump system (Hirschi and Funk, 1997). Theoretically the less time the cattle spend in and near the creeks, the less *Enterococcus* will enter the creeks through fecal waste. Such practices could reduce the concentrations of *Enterococcus* and other bacteria in the water, minimizing the chances for each tetracycline-resistant bacterium from establishing contact with another bacterium, and thereby reduce the spread of antibiotic-resistance. However, regardless of the source of the TRE, multiple species of *Enterococcus* were detected in Miller Creek and all have the capability of transferring their tetracycline resistance gene(s) to other *Enterococcus* and potentially more pathogenic bacteria.

REFERENCES

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. **25**: 3389-3402.

Bertram, J., M. Stratz, and P. Durre. 1991. Natural transfer of conjugative transposon Tn916 between Gram-positive and Gram-negative bacteria. J. Bacteriol. **174**:443-448.

Bloom, M.V., G.A. Freyer, and D.A. Micklos (eds.). 1996. Southern hybridization of λ DNA, pp.191-215. *In Laboratory DNA Science*. The Benjamin/Cummings Publishing Company, Inc., New York.

Burdette, V. 1991. Purification and characterization of *tet*(M), a protein that renders ribosomes resistant to tetracycline. J. Biol. Chem. **266**:2872-2877.

Cabelli, V. J. 1980. Health effects criteria for marine recreational waters. *U.S. Environmental Protection Agency publication no. EPA 600/1-80-031.* U.S. Environmental Protection Agency, Washington, D.C.

Clewell, D.B. and S.E. Flannagan. 1993. The conjugative transposons of Gram-positive bacteria. *In* D.B. Clewell (ed), Bacterial Conjugation. Plenum Press, New York, p.387.

Clewell, D.B., D.D. Jaworski, S.E. Flannagan, L.A. Zitzow, and Y.A. Su. 1995. The conjugative transposon Tn*916* of *Enterococcus faecalis*: structural analysis and some key factors involved in movement. *In* J. J. Ferretti, M. S. Gilmore, T. R. Klaenhammer, F. Brown, (ed), *Genetics of Streptococci, Enterococci and Lactococci. Dev. Biol. Stand.* **85**:11-17.

Collins, M.D., J.A.E. Farrow, and D. Jones. 1986. *Enterococcus mundtii* sp. nov. Int. J. Syst. Bacteriol. **36**:8-12.

Dailey, D.C., L.F. Barber, H.J. Semrau, C.L. Taylor, and M.T. Finley. 1998. Levels of fecal indicator bacterial associated with the Sulphur Fork Creek Watershed, Robertson Co., Tennessee. Proceedings of the Eighth Tennessee Water Resources Symposium, Nashville. P-5.

Dailey, D.C. and M.T. Finley. 2001. Fecal bacteria in the Red River and Sulphur Fork Creek watersheds: a 5 year study. Manuscript in preparation.

Devriese, L.A., G.N. Dutta, J.A.E. Farrow, A. Van De Kerckhove, and B.A. Phillips. 1983. *Streptococcus cecorum*, a new species isolated from chickens. *Int. J. Sys. Bacteriol.* **33**:772-776.

Devriese, L.A., B. Pot, L. Van Damme, K. Kersters, and F. Haesebrouck. 1995. Identification of *Enterococcus* species isolated from foods of animal origin. Int. J. Food Microbiol. **26**:187-197.

Dufour, A.P. 1984. Health effects criteria for fresh recreational waters. *U.S. Environmental Protection Agency publication no. EPA 600/1-84-004.* U.S. Environmental Protection Agency, Washington, D.C.

English, B.K., and P.J. Chesney. 1996. Infections due to drug-resistant pneumococci, enterococci, and *Mycobacterium tuberculosis*. Pediatr. Ann. **25**:502-510.

Flannagan, S.E., L.A. Zitzow, Y.A. Su, and D.B. Clewell. 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid **32**:350-354.

Franke, A.E. and D.B. Clewell. 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. *J. Bacteriol.* **145**:494-502.

Gaunt, P.N. and L.J.V. Piddock. 1996. Ciprofloxacin-resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. J. Antimicrob. Chemother. **37**:747-757.

Gawron-Burke, C. and D.B. Clewell. 1982. A transposon in *Streptococcus faecalis* with fertility properties. *Nature* (London) **300**:281-284.

Hamel, E., M. Koka, and T. Nakamoto. 1972. Requirement of an *Escherichia coli* 50S ribosomal protein component for effective interaction of the ribosome with T and G factors and with guanosine triphosphate. *J. Biol. Chem.* 247:805-814.

Heggers, J.P., L. McCoy, B. Reisner, M. Smith, P. Edgar, and R.J. Ramirez. 1998. Alternate antimicrobial therapy for vancomycin-resistant enterococci burn wound infections, March 18-21, 1998, pp. 399-403. *In* 30th Annual Meeting of the American Burn Association. American Burn Association Shriners Burn Institute, Galveston, Texas.

Hirschi, M. and T. Funk. 1997. Alternative water for cattle: a tempting option. *In* M. Hirschi, R. Frazee, G. Czapar, and D. Peterson, (eds.), *60 Ways Farmers*

Can Protect Surface Water. University of Illinois Extension, University of Illinois at Urbana-Champaign, p160.

Horaud, T., G. De Cespédès, D. Clermont, F. David, and F. Delbos. 1991. Variability of chromosomal genetic elements in streptococci, p. 16-20. *In* G.M. Dunny, P.P. Cleary, and L.L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.

Huycke, M.M., D.F. Sahm, and M.S. Gilmore. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emer. Infect. Dis. **4**:239-249.

Kay, D., J.M. Fleisher, R.L. Salmon, F. Jones, M.D. Wyer, A.F. Godfree, Z. Zelenauch-Jacquotte, and R. Shore. 1994. Predicting likelihood of gastroenteritis from sea bathing: results from randomized exposure. *Lancet* **344**:905-909.

Kielwein, G. 1978. Vorkommen und Bedeutung von Enterokokken in Milch und Milchprodukten. Arch. Lebensmittelhyg. **29**:127-128.

Kinsey, J.J. 1998. Fish and macroinvertebrate communities in Miller Creek, Robertson County Tennessee [thesis]. Clarksville (TN): Austin Peay State University. 33 p. Available from: Felix G. Woodward Library, Austin Peay State University.

Krieg, N.R. and J.G. Holt (eds.). 1984. Enterococci, p.538-539. *In* Bergey's Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore/London.

Lebkuecher, J.G. and R.A. Houtman. 1999. Characteristics of photoautotrophic periphyton and unialgal cultures grown *in situ* within the Sulphur Fork Creek watershed. *In* Proceedings of the Eighth Symposium on the Natural History of Lower Tennessee and Cumberland River Valleys. The Center for Field Biology, Austin Peay State University, Clarksville, TN, p.47-55.

Leclereq, R. 1997. Antibiotic resistance in streptococci and enterococci, p. 419-427. *In* Streptococci and the Host, edited by Horaud et al. Plenum Press, New York.

Levy, S.B., G.B. FitzGerald, and A.B. Macone. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N. Engl. J. Med. **295**:583-588.

Lowery, J.F. 2000. Precipitation data from station number 03436100 Red River at Port Royal, TN for October 1998-

September 1999, and October 1999. United States Geological Survey, Tennessee.

McReynolds, M.H., and D.C. Dailey. 1998. Tetracycline-resistant *Enterococcus* sp. in the Sulphur Fork Creek Watershed, Robertson Co., Tennessee. Annual Meeting of the Tennessee Academy of Science. Tennessee Technological University, Cookville,TN.

McReynolds, M.H., and D.C. Dailey. 1999. Tetracycline-resistance in enterococci in Sulphur Fork Creek watershed, Robertson County, TN. Proceedings of the Eighth Symposium on the Natural History of Lower Tennessee and Cumberland River Valleys. The Center for Field Biology, Austin Peay State University, Clarksville, TN, p.61.

Miescier, J.J., and V.J. Cabelli. 1982. Enterococci and other microbial indicators in municipal wastewater effluents. J. Water Pollut. Control Fed. **54**:1599-1606.

Moellering, R.C. 1992. Emergence of Enterococcus as a significant pathogen. Clin. Infect. Dis. **14**:1173-1178.

Murray, B.E. 1998. Diversity among multidrug-resistant enterococci. Emer. Infect. Dis. **4**:37-47.

Murray, P.R., G.S. Kobayashi, M.A. Pfaller, and K.S. Rosenthal, (eds). 1994. Medical Microbiology, 2nd ed. Mosby-Year Book, Inc., St. Louis, MO.

National Committee for Clinical Laboratory Standards. 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved standard M7-A3. National Committee for Clinical Laboratory Standards, Villanova, PA.

Niemi, R.M., S.I. Niemelä, D.H. Bamford, J. Hantula, T. Hyvärinen, T. Forsten, and A. Raateland. 1993. Presumptive fecal streptococci in environmental samples characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **59**:2190-2196.

Pelczar, M.J., E.C.S. Chan, and N.R. Krieg (eds.). 1993. Antibiotics in animal feeds, p. 582. *In* Microbiology Concepts and Applications. McGraw Hill, Inc., New York.

Perreten, V., F. Schwarz, L. Cresta, M. Boeglin, G. Dasen, and M. Teuber. 1997. Antibiotic resistance spread in food. Nature **389**:801-802.

Quadnau, M., S. Ahrne, A.C. Petersson, and G. Molin. 1998. Antibiotic-resistant strains of enterococcus isolated from Swedish and Danish retailed chicken and pork. J. Appl. Microbiol. **84**:1163-1170.

Rice, L.B. 1998. Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob. Agents Chemother. **42**:1871-1877.

Rozen, S. and H.J. Skaletsky. 1998. Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Rudy, C.K. and J.R. Scott. 1994. Length of the coupling sequence of Tn*916*. J. Bacteriol. **176**:3386-3388.

Salyers, A.A. 1995. Mechanisms of gene transfer: bacterial diversity extends to sex, p. 81. *In* A.A. Salyers, Antibiotic resistance transfer in the mammalian intestinal tract: implications for human health, food safety and biotechnology. University of Illinois at Urbana-Champaign, Urbana, IL.

Scott, J.R. 1992. Sex and the single circle: conjugative transposition. J. Bacteriol. **174**:6005-6010.

Stull, T.L., J.J. LiPuma, and T.D. Edlind. 1988. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. **157**:280-286.

Tennessee Department of Environment and Conservaton. 1998. Final 1998 303(d) list, p.7. Division of Water Pollution Control, Nashville, TN.

Tortora, G.J., B.R. Funke, and C.L. Case (eds.). 1995. Antibiotics in animal feed linked to human disease, p.505. *In* Microbiology, an Introduction, 5th ed. The Benjamin/ Cummings Publishing Company, Inc., Reading, MA.

Tschäpe, H. 1994. The spread of plamsids as a function of bacterial adaptability. FEMS Microbiol. Ecol. **15**:23-32.

U.S. Environmental Protection Agency. 1986. Ambient water quality criteria for bacteria-1986. U.S. Environmental Protection Agency publication no. EPA 440-5.84-002. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C.

Wiggins, B.A., R.W. Andrews, R.A. Conway, C.L. Corr, E.J. Dobratz, D.P. Dougherty, J.R. Eppard, S.R. Knupp, M.C. Limjoco, J.M. Mettenburg, J.M. Rinehardt, J. Sonsino, R.L. Torrijos, and M.E. Zimmerman. 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. Appl. Environ. Microbiol. **65**:3483-3486.

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. Science **279**:996-997.

World Health Organization. 1997. The medical impact of the use of antimicrobials in food animals. http://www-pll.who.int/emc-documents/antimicrobial_resistance/whoemczoo974c.html. Berlin, Germany, October 13-17, 1997.

Zareba, T. and W. Hryniewicz. 1995. Patterns of antibiotic resistance among enterococcal strains isolated from clinical specimens and food in Poland. Eur. J. Clin. Microbiol. & Infect. Dis. **14**:69-71.

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