Microbial Population Analysis in Leachate From Simulated Solid Waste Bioreactors and Evaluation of Genetic Relationships and Prevalence of Vancomycin Resistance Among Environmental Enterococci

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Microbial Population Analysis in Leachate From Simulated Solid Waste Bioreactors

and

Evaluation of Genetic Relationships and Prevalence of Vancomycin Resistance Among Environmental Enterococci

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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MICROBIAL POPULATION ANALYSIS IN LEACHATE FROM SIMULATED SOLID WASTE BIOREACTORS AND EVALUATION OF GENETIC RELATIONSHIPS AND PREVALENCE OF VANCOMYCIN RESISTANCE AMONG ENVIRONMENTAL ENTEROCOCCI

BINA S. NAYAK

ABSTRACT
Degradation of the several million tons of solid waste produced in the U.S. annually is microbiologically mediated, yet little is known about the structure of prokaryotic communities actively involved in the waste degradation process. In the first study, leachates generated during degradation of municipal solid waste (MSW) in the presence (co-disposal) or absence of biosolids were analyzed using laboratory-scale bioreactors over an eight-month period. Archaeal and bacterial community structures were investigated by denaturing gradient gel electrophoresis (DGGE) targeting 16S rRNA genes. Regardless of waste composition, microbial communities in bioreactor leachates exhibited high diversity and temporal trends. Methanogen sequences from a co-disposal bioreactor were predominantly affiliated with the orders Methanosarcinales and Methanomicrobiales. Effect of moisture content on indicator organism (IO) survival
during waste degradation was studied using culture-based methods. Fecal coliform and *Enterococcus* concentrations in leachate decreased below detection limits within fifty days of bioreactor operation during the hydrated phase. IOs could be recovered from the bioreactor leachate even after a prolonged dry period. This study advances the basic understanding of changes in the microbial community during solid waste decomposition.

The purpose of the second study was to compare the ability of BOX-PCR to determine genetic relatedness with that of the “gold standard” method, 16S rRNA gene sequencing. BOX-PCR typing could clearly differentiate the strains within different *Enterococcus* species but closely related genera were not as distinguishable. In contrast, 16S rRNA gene sequencing clearly differentiates between closely related genera but cannot distinguish between different strains of *Enterococcus* species. This study adds to our knowledge of genetic relationships of enterococci portrayed by two separate molecular methods.

The incidence of vancomycin resistant enterococci (VRE) in environmental matrices, residential and hospital wastewater was also investigated. Low-level VRE (*vanC* genotype) were isolated from environmental matrices and residential wastewater. VRE isolates from hospital wastewater were identified as *E. faecium* and demonstrated resistance to ampicillin, ciprofloxacin and vancomycin (*vanA* genotype), but sensitivity to chloramphenicol and rifampin. Although no high-level VRE were isolated from surface waters, the high proportion of low-level VRE in environmental matrices is a cause for concern from the public health perspective.
MICROBIAL POPULATION ANALYSIS IN LEACHATE FROM SIMULATED SOLID WASTE BIOREACTORS

BACKGROUND

Waste disposal in landfills

The most common practice for disposal of waste generated in countries around the world is landfilling. With increasing global populations, the amount of waste generated each year is growing phenomenally. The world population passed the 6 billion mark at the turn of the century. In the US alone, 254 tons of municipal solid waste (MSW) was generated in 2007 (U.S. EPA 2008). The majority of the waste deposited in landfills is composed of MSW, which includes food waste, yard waste, paper, plastics, metals, glass, rubber, wood, leather, textiles, etc. This waste is comprised of approximately 40-50% cellulose, 12% hemicellulose, 10-15% lignin, and 4% protein (Barlaz et al. 1989). In addition to MSW, other types of wastes that are landfilled include biosolids from wastewater treatment facilities, residues from waste-to-energy (WTE) and other combustion processes, electronic wastes, construction and demolition wastes.

A consequence of growing populations in urban areas is the increased production of wastewater, coupled with a decrease in land availability for land application of associated biosolids. Treated and dewatered biosolids are used as fertilizers and soil amendments. The unutilized biosolids are disposed of in landfills (Reinhart 2003). Biosolids are rich in...
moisture and nutrients and contain diverse microbial populations that can provide supplemental inocula for waste degradation reactions (Rivard et al. 1990; Poggi-Varaldo 1992). Waste degradation in landfills is a slow, ongoing process occurring over decades and characterized by physicochemical reactions and microbial interactions.

**Leachate characteristics**

The liquid that percolates through the waste matrix is termed leachate, which solubilizes and mobilizes minerals associated with the waste (Kjeldsen et al. 2002), and also promotes microbially-mediated degradation of waste (El-Fadel 1999). The composition of leachate is related to the moisture content within landfills and biogeochemical reactions that occur within the waste matrix. In general, the amount of moisture available to support microbial activity depends on local climatic conditions, e.g. rainfall and temperature, the specific materials deposited in the landfill, and the frequency of leachate removal and/or recirculation (El-Fadel 1999). Among the dominant components of municipal waste are cellulose, hemicellulose, lignin and protein (Barlaz et al. 1989).

Leachates have been analyzed to determine the amount of dissolved organic matter (recorded as chemical oxygen demand or COD), volatile fatty acids (VFAs), inorganic components such as calcium (Ca$^{2+}$), magnesium (Mg$^{2+}$), potassium (K$^+$), ammonium (NH$_4^+$), iron (Fe$^{2+}$), sulfate (SO$_4^{2-}$) and hydrogen carbonate (HCO$_3^-$), heavy metals such as copper, lead nickel and zinc and trace amounts of arsenate, mercury, lithium, etc (Kjeldsen et al. 2002). Due to the differences in waste composition, age and landfilling practices the composition of leachate varies between landfills. The pH of leachate can vary from 4.5 to 9 depending on the stage of waste decomposition.
Landfill leachate can carry toxic metabolic products, heavy metals, pharmaceuticals and human pathogens (Mose and Reinthaler 1985; Gerba et al. 1995). Leachate contamination of groundwater due to improper waste management practices poses a threat to public health (Christensen et al. 1994; Roling et al. 2001). Leachate pooling at the bottom of the landfill is collected in a network of pipes called the leachate collection system and pumped to a storage tank. The collected leachate is then transported in tankers to a neighboring wastewater treatment plant where it is treated and disposed. Leachate recirculation is practiced in many landfills because it provides an additional inoculum of established microbial flora that can boost the rate of the degradation process (Chan 2002). It also reduces the amount of leachate requiring treatment and increases gas production (Reinhart 1996; Warith 1999). In contrast, excessive leachate recirculation can result in over-saturation of waste and have a toxic effect on sensitive organisms such as methanogens (Reinhart 1996). The frequency and amount of leachate recirculation also plays an important role in the development of certain microbial groups at different time points during waste degradation (Shen et al. 2001). Leachates collected from landfills carry a representative sample of the numerous microorganisms involved in degradation of that waste (Gurijala 1993; Pohland and Kim 2000). Studying the microbial community profile of this leachate over a period of time could provide interesting insights into the physical, chemical and biological processes occurring within the landfill.

**Microbial processes in landfills**

Landfills provide excellent environments for the development of diverse microbial populations due to the wide variety of substrates available to support the physiological requirements of microorganisms. The complexity and composition of microbial
communities in any particular landfill depends on several parameters such as the types of wastes deposited, moisture availability, landfill age and operating practices, toxicity of waste components and their breakdown products, and leachate management practices (Barlaz et al. 1989; Kjeldsen et al. 2002). These parameters have a direct or indirect effect on the pH, dissolved organic and inorganic carbon content, oxidation-reduction potential and temperature of the leachate.

Waste deposited in landfills is at different stages of degradation depending on the age and depth of the waste. In general, the process of waste degradation is presumed to progress in four stages from the time of deposition till maturation: aerobic, anaerobic, accelerated methane production and decelerated methane production (Barlaz et al. 1989). The first stage is dominated by aerobic heterotrophic bacteria that decompose cellulose and consume oxygen and nitrate present in the waste (Pourcher et al. 2001). This results in the production of carbon dioxide and possibly an increase in temperature. After oxygen is a consumed, anaerobic cellulolytic bacteria, i.e., Clostridium spp. and Eubacterium spp., hydrolyze cellulose and hemicellulose into monosaccharides that are further fermented to produce alcohols and carboxylic acids (Westlake 1995; Van Dyke and McCarthy 2002; Burrell et al. 2004). Acetogenic bacteria convert these organic acids and alcohols to acetate, carbon dioxide and hydrogen (Mackie and Bryant 1981). Acetogens can also act as hydrogen oxidizers by reducing carbon dioxide to produce acetate and other low molecular weight organic compounds.

Hydrogen has an important role in waste decomposition (Mormile 1996). Hydrogen-oxidizing methanogenic Archaea oxidize hydrogen and reduce carbon dioxide to methane(Griffin et al. 1997). Sulfate reducing bacteria (SRB), on the other hand, use
hydrogen as the electron donor and sulfate as the terminal electron acceptor (Daly et al. 2000). These two groups of microorganisms compete for available hydrogen in the landfill waste, which sometimes leads to the inhibition of methanogenesis by the SRBs (Mormile 1996; Raskin 1996). Carbon mineralization by methanogenesis is preferred for landfill functioning because sulfate reducers produce hydrogen sulfide, causing the phenomenon of “souring” (Gurijala 1993). Sourcing of waste reduces the pH and inhibits the activity of methanogens.

Methane production and recovery is environmentally and economically desirable due to its usefulness as a biogas for the production of energy in the form of heat and electricity. Unfortunately, methane recovery from landfills is affected by waste composition, microbial degradation dynamics and more importantly cost effectiveness. Methane is a potent greenhouse gas and the alternative to recovering methane, controlling methane emissions, is equally expensive. Methanotrophic bacteria present in the upper oxic region of the landfill use methane as their carbon and energy source and convert it to carbon dioxide (Whalen 1990; Wise et al. 1999). Therefore, in landfills where methane recovery is not an option, methanotrophs play an important role in controlling the emission of methane from landfill sites.

**Methanogenesis**

According to the US Environmental Protection Agency (EPA), in 2007, MSW landfills were responsible for approximately 23% of methane emissions in the U.S. making them the second largest anthropogenic source of methane (U.S. EPA 2009). Methanogens are classified under the domain *Archaea*, which includes three kingdoms: *Euryarchaeota*, *Crenarchaeota* and *Korarchaeota* (Winker and Woese 1991; Barns et al. 1996).
Methanogens belong to the kingdom *Euryarchaeota* and are divided into five orders: *Methanosarcinales, Methanomicrobiales, Methanobacteria, Methanococcales* and *Methanopyrales* (Bapteste et al. 2005). Methanogens are a strictly anaerobic, mostly autotrophic group with very diverse 16S rRNA sequences inhabiting varied ecological habitats ranging from the human digestive system to submarine volcanic vents (Ferry 1993). They utilize a limited range of substrates including hydrogen, acetate, formate, methanol and other methylated substrates.

Several genome-sequencing projects (*Methanobrevibacter smithii, Methanococcus maripaludis, Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*) have added to our knowledge of the complex metabolic pathway of methanogenesis (Bult et al. 1996; Smith et al. 1997; Hendrickson et al. 2004; Samuel et al. 2007). The most widely studied enzyme of methanogenesis pathways is methyl coenzyme M reductase (MCR), which catalyzes the final step of Methanogenesis whereby the methyl group of methyl-S-coenzyme M is reduced to methane. The MCR enzyme is comprised of α, β and γ subunits encoded by the *mcrA, mcrB* and *mcrG* genes, respectively (Bokranz and Klein 1987). The *mcrA* gene is highly conserved and is the gene that is most frequently used to detect methanogenic activity in varied environments (Lueders et al. 2001; Luton et al. 2002; Earl et al. 2003; Dhillon et al. 2005).

Methanogens can be detected and analyzed using molecular techniques such as fluorescence in-situ hybridization (FISH) and construction of clone libraries by targeting 16S rDNA (Mori et al. 2003), or biomarkers such as methanogen-specific DNA sequences (Hales et al. 1996; Earl et al. 2003; Dhillon et al. 2005). Some studies have developed methanogen clone libraries using the primers specific for the ubiquitous 16S
rRNA gene (Huang et al. 2003; Mori et al. 2003) while others have targeted the mcr gene encoding methyl coenzyme-M reductase (MCR) (Hales et al. 1996; Nercessian et al. 1999) or both (Springer et al. 1995; Lueders et al. 2001). Functional genes are used as biomarkers because their higher rates of evolutionary change enhance the ability to resolve sequences at the species level compared to the 16S rRNA gene (Braker et al. 2000; Junca and Pieper 2004). Currently, limited information is available regarding the composition of methanogen populations in landfills. The heterogeneous nature of waste makes it impossible to determine the type of substrates used by methanogens and the amount of methane generated during different stages of waste decomposition. This information could be useful in developing and improving methane management practices to efficiently recover the methane produced in landfills.

**Microbial characterization of leachate**

In several studies, culture-based methods such as heterotrophic plate counts have been applied to evaluate microbial numbers in leachates (Barlaz et al. 1989; Boothe et al. 2001). However, the utility of culture-based methods to enumerate and characterize environmental microorganisms is limited due to the highly specific and stringent growth requirements or many microorganisms, coupled with the need of many organisms to function as a consortium (Ward et al. 1992; Amann et al. 1995). Results from culture-based methods yield a biased subset of the total population and are not effective for assessing the microbial community structure (Hugenholtz and Pace 1996). Molecular methods such as fluorescence in-situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and sequencing of cloned DNA (clone libraries) have proven to be useful in identifying microbial species and community diversity in landfill waste
(Roling et al. 2001; Huang et al. 2002; Burrell et al. 2004; Huang et al. 2005). Complex community profiles can be statistically analyzed to determine the degree of diversity and levels of similarity among microbial populations in any given microbial ecosystem (Fromin et al. 2002).

Microbial ecosystem studies focus on microbial interactions with their environments and changes occurring in the community structures in response to shifts in environmental parameters. The extent of microbial diversity can be effectively captured by molecular biological techniques like genetic fingerprinting, temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla 1998). Bands from DGGE and TGGE gels can be excised and used for subsequent sequencing reactions. Complex community profiles obtained by using these techniques can be analyzed using statistical programs to determine the degree of diversity and levels of similarity between microbial populations in any given microbial ecosystem (Demba Diallo 2004). Huang et al used cloning and restriction fragment length polymorphism (RFLP) analysis to outline the archaeal community structure in landfill leachate (Huang et al. 2002). The cloning and sequencing approach was also used to study the bacterial diversity in landfill leachate by Huang et al in another experiment (Huang et al. 2005). Roling et al performed cloning and DGGE profiling of landfill leachate polluted aquifers to determine the correlation between the microbial community structure and hydrochemistry in the aquifers (Roling et al. 2001). To the best of our knowledge, no previous study has followed changes in archaeal and bacterial DGGE profiles over an extended period as attempted in this study.
Landfill management practices

Typically, leachate that is formed in landfills flows into leachate collection systems that are installed beneath a drainage layer, and is subsequently either recirculated through the landfill, treated on-site, or treated at a wastewater treatment facility. In some cases, clogging of leachate collection systems can occur and prevent drainage of the landfill, resulting in waste submergence (Fleming et al. 1999). Waste submergence causes accumulation of toxic compounds, inhibiting microbial activity and slowing waste degradation. Leachate may also leak through compromised pipes and contaminate the groundwater (Fleming et al. 1999). A host of factors have been implicated in the clogging of landfill leachate collection systems including waste characteristics (Cardoso et al. 2006), physical-chemical parameters (Ledakowicz and Kaczorek 2004; VanGulck 2004), and drainage system design (Rowe 2000b), however, limited information has been reported on the temporal variations of the microbial community structure associated with the clogging phenomenon. The deposition of inorganic chemicals such as calcium carbonate and hydroxyapatite, as well as the presence of biofilms presumably result in the phenomenon of clogging of leachate collection systems, thereby reducing the life of the landfill (Rowe 2000a; b; 2002). The “life” of a landfill is directly related to its capacity (area available for waste disposal) and the amount of waste deposited over a period of time. Upon “closure”, the landfill must be overlaid with a flexible membrane liner and earthen material and monitored for the next thirty years.

Bioreactor studies

The heterogeneous nature of the waste deposited in landfills makes it particularly challenging to design sampling strategies and to conduct analysis of leachate
composition. Therefore, several studies have employed the use of laboratory-scale bioreactors or lysimeters to simulate the conditions in landfills (Griffin et al. 1997; Pohland and Kim 2000; Burrell et al. 2004; Fleming 2004; Ledakowicz and Kaczorek 2004; Calli et al. 2005). Bioreactors make it possible to control or modify the various factors that influence waste degradation such as temperature, moisture and accumulation of toxic compounds. Furthermore, bioreactors facilitate the investigation of the effect of manipulating one or more of these variables on the overall process of waste decomposition.

Bioreactors have been employed to study the physical, chemical and microbiological changes occurring during the degradation of waste deposited in landfills. Effects of leachate recirculation, seasonal variation, amount of gas generation, development of microbial populations, attenuation of pollutants, aerobic versus anaerobic waste degradation and clogging of leachate collection systems have been the focus of several studies. Stessel and Murphy (1992) studied the effect of moisture and air on the rate of degradation with the aim of optimizing the quantities of these two variables to achieve reduction of waste within minimal amount of time (Stessel 1992). A comparison of laboratory versus in situ field bioreactors conducted in China revealed the feasibility of the use of bioreactors to mimic refuse decomposition in landfills (Youcai et al. 2002). Methanotroph community diversity and methane oxidation rates associated with different plant covers were studied in bioreactors by using methanotroph diagnostic microarrays (Stralis-Pavese et al. 2004). Burrell et al used methanogenic bioreactors to detect and identify cellulolytic Clostridium populations involved in anaerobic waste degradation (Burrell et al. 2004). In spite of the usefulness of bioreactors in reducing and controlling
variables associated with refuse decomposition, it is necessary to corroborate data obtained from bioreactor studies with data from actual landfill operation.

**Research goals**

Hypothesis 1: Microbial community structure will vary in bioreactors as a function of waste composition.

Methodology 1: Laboratory scale bioreactors were constructed and packed with only MSW or MSW combined with biosolids and ash from waste-to-energy processes. Leachate generated in the bioreactors was sampled once every week for a period of eight months and analyzed for total cell concentrations and microbial community structure.

Hypothesis 2: Microbial populations in bioreactors will exhibit temporal shifts, reflecting changes in the community structure with the progression of waste degradation.

Methodology 2: DGGE was used to evaluate the community development in terms of dominant members of the *Archaea* and *Bacteria*, providing a broad snapshot of changes in the microbial community over an eight-month period.

Hypothesis 3: Microbial concentrations in bioreactors supplemented with biosolids will be higher than in bioreactors packed with MSW only.

Methodology 3: Total microbial concentrations in the leachate sampled from bioreactors with different waste composition were assessed by fluorescence microscopy.

Hypothesis 4: Methanogens belonging to many different genera will be identified in the initial sample due to the complexity of the waste. As the waste “matures”, the sequence diversity of methanogens will decrease.
Methodology 4: Methanogen populations were analyzed from an early (day 50) sample and a late (day 218) sample of leachate by cloning and sequencing of the mcrA gene coding for the alpha subunit of the methyl coenzyme-M reductase (MCR) enzyme.

Hypothesis 5: Fecal coliforms and enterococci will be present at high concentrations during the initial stages of waste degradation. Other microbial community members such as cellulose degraders, sulfate reducing bacteria and methanogens will outcompete the indicator organisms as waste degradation progresses.

Methodology 5: Survival of indicator organisms such as fecal coliforms and enterococci during the process of waste degradation was studied by membrane filtration of leachate samples onto selective media. One bioreactor was subjected to a year long “dry period” and indicator organism concentrations in the leachate were evaluated after rehydration of the bioreactor.

Novel aspects of this study include the temporal profiling of *Archaea* and *Bacteria* populations using DGGE, identifying and comparing methanogen populations during the initial and later stages of waste decomposition and investigating the survival capability of indicator organisms through the waste degradation process. Portions of this work were published as a paper titled “Microbial population dynamics in solid waste bioreactors in the presence or absence of biosolids” in the Journal of Applied Microbiology (107(4): 1330-1339).
References


MICROBIAL POPULATION DYNAMICS IN LABORATORY-SCALE SOLID WASTE BIOREACTORS IN THE PRESENCE OR ABSENCE OF BIOSOLIDS

Abstract

Aims: Decomposition of solid waste is microbially mediated, yet little is known about the associated structure and temporal changes in prokaryotic communities. Bioreactors were used to simulate landfill conditions and archaeal and bacterial community development in leachate was examined over eight months.

Methods and Results: Municipal solid waste (MSW) was deposited in laboratory bioreactors with or without biosolids and combustion residues (ash). The near-neutral pH fell about half a log by day 25, but recovered to ~7.0 by day 50. Cell concentrations in bioreactors containing only MSW were significantly higher than those from co-disposal bioreactors. Archaeal and bacterial community structure was analyzed by denaturing gradient gel electrophoresis (DGGE) targeting 16S rRNA genes, showing temporal population shifts for both domains. mcrA sequences retrieved from a co-disposal bioreactor were predominantly affiliated with the orders Methanosarcinales and Methanomicrobiales.

Conclusion: Regardless of waste composition, microbial communities in bioreactor leachates exhibited high diversity and distinct temporal trends. The solid waste filled bioreactors allowed simulation of solid waste decomposition in landfills while also reducing the variables.
Significance and Impact: This study advances the basic understanding of changes in microbial community structure during solid waste decomposition, which may ultimately improve the efficiency of solid waste management.

Introduction

Disposal of municipal solid waste (MSW) in landfills supports the development of diverse microbial populations (Kjeldsen et al. 2002). The composition of microbial communities is influenced by many factors such as the types of wastes deposited, moisture availability, oxidation-reduction states, and temperature (Barlaz et al. 1989a; Kjeldsen et al. 2002). Co-disposal of waste includes MSW and other types of wastes, including biosolids from wastewater treatment facilities, ash residues from waste-to-energy and other combustion processes, electronic wastes, construction and demolition wastes.

Understanding microbial population development in landfills over a period of time is challenging due to the complexity of waste materials deposited and the spatial heterogeneity of landfills. Previous studies have focused on particular aspects of microbial populations in waste degradation processes. Group-specific primers were employed to detect cellulolytic clostridia (Van Dyke and McCarthy 2002) and fungi (Lockhart et al. 2006) in landfill leachate. Quantitative real time PCR was used to study the development of type I methanotrophic communities during composting of organic matter (Halet et al. 2006) and to determine the abundance of cellulolytic *Fibrobacter* species in landfills (McDonald et al. 2008). Sequencing of cloned DNA (clone libraries) was used to study archaeal populations in the leachate of a full-scale recirculating landfill and bacterial populations in the leachate of a closed landfill (Huang et al. 2002; Huang et
Several studies have also investigated methanogenic *Archaea* populations in landfills (Huang *et al.* 2003; Uz *et al.* 2003), yet none of them attempted a temporal comparison.

Methanogenesis is a process that generates useful methane gas during waste degradation in landfills (Barlaz *et al.* 1989a; Senior *et al.* 1990). However, methane recovery rates are affected by waste composition, microbial degradation dynamics and economic feasibility. Methane is also a potent greenhouse gas and landfills account for 34% of all methane emissions (U.S. EPA 1999). Methanogens can be detected and analyzed using molecular techniques such as fluorescence in-situ hybridization (FISH) (Calli *et al.* 2005) and construction of 16S rDNA clone libraries (Huang *et al.* 2003; Mori *et al.* 2003) or biomarkers (Hales *et al.* 1996; Nercessian *et al.* 1999; Earl *et al.* 2003; Dhillon *et al.* 2005). Functional genes are used as biomarkers because their higher evolutionary rates can enhance the resolution of sequences at the species level compared to the 16S rRNA gene (Braker *et al.* 2000; Junca and Pieper 2004).

Conditions characteristic of solid waste degradation in a landfill were mimicked in bioreactors filled with solid waste and maintained in the laboratory. The chemical data associated with the study have been published (Cardoso *et al.* 2006). The microbial data that were collected simultaneously with the chemical data are presented in this work. We hypothesized that the microbial community development would vary in bioreactors with different waste composition. Denaturing gradient gel electrophoresis (DGGE) was used to evaluate the community development in terms of dominant members of the *Archaea* and *Bacteria*, providing a broad snapshot of changes in the microbial community over an eight-month period. Methanogen sequences from leachate samples were obtained using
primers targeting the mcrA gene coding for the alpha subunit of the methyl coenzyme-M reductase (MCR) enzyme and compared over time.

**Materials and Methods**

*Bioreactor design*

Bioreactors were designed to simulate landfill disposal practices in the U.S. They were constructed from 1.4 m tall, 30.5 cm diameter PVC pipes. The waste mixtures were hydrated to field capacity and leachate was recirculated daily to simulate rainfall of 8 cm d⁻¹. The leachate collection system was designed to simulate field conditions and consisted of a perforated 32 mm diameter PVC. The leachate collection pipes were surrounded by gravel (50.8 mm) with geotextiles above and below the gravel layers. The drainage system separating the waste from the leachate collection pipe consisted of five inches of granular material (25.4 mm gravel or Cholee sand). The bioreactors were operated in duplicate. A diagram of the bioreactor design is presented in our previously published paper (Cardoso et al. 2006).

Four bioreactors were filled with either MSW alone or MSW co-disposed with biosolids and combustion residues from waste-to-energy facilities. The waste materials were obtained from the North County Resource Recovery Facility in Palm Beach County, FL. Duplicate bioreactors were packed with either 100% MSW or 60% MSW co-disposed with 30% combustion residues (6% fly ash + 24% bottom ash) and 10% biosolids (comprised of 50% material from drinking water treatment + 50% material from wastewater treatment).
Sample processing

Leachate samples were collected and three milliliters of each sample was filtered through 0.45 µm filters and the filters were stored at -20°C. Community DNA was extracted from the filters using the Ultraclean Soil DNA Kit (MoBio Laboratories, Inc.) per manufacturer’s instructions. The extracted DNA was stored at -20°C until further processing (1 week maximum).

Total microbial concentrations

Duplicate one ml samples of leachate from each bioreactor were individually centrifuged. The cells were washed in sterile phosphate buffered saline (PBS), stained with DAPI (4,6-diamidine-2-phenylindole) (1mg ml\(^{-1}\) DAPI) and filtered through a 0.2 µm polycarbonate filter (Millipore). The stained cells were observed under a fluorescent microscope using a UV2B filter. Cells from 5 different fields of view were counted and the average was used to calculate the cell concentration ml\(^{-1}\) of sample. Measurements in duplicate samples varied from one another by less than 10%.

Polymerase chain reaction (PCR) for community analysis

Direct amplification of the 16S rRNA genes of Archaea using the primer set 344f and 517r was not consistently successful; therefore a nested approach was used. The first round of PCR was performed using the primer set 21f and 958r (Delong 1992; Pearson et al. 2004). Acetamide was added to a final concentration of 2% (vv\(^{-1}\)) to increase the specificity of the reaction. PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. This procedure
was followed by a second round of PCR using the Archaea-specific forward primer 344f and a universal reverse primer 517r (Bano et al. 2004; Pearson et al. 2004). A 40 bp GC-clamp was attached to the 5’-end of the forward primer. Acetamide was excluded from the reactions. Templates were amplified using a “touchdown” PCR to increase primer specificity (Ferrari and Hollibaugh 1999). The archaeal primer sets used in this study amplify both euryarchaeotes and crenarchaeotes and do not amplify non-archaeal templates (Delong 1992; Raskin et al. 1994). Methanosarcina acetivorans strain C2A (DSM 2834) was used as the positive control.

Bacterial 16S rRNA genes were amplified using the primer set 1070f and 1392r (Ferris et al. 1996). A 40 bp GC-clamp was attached to the 5’-end of the reverse primer. PCR conditions were the same as those used for the first round of archaeal amplification. Escherichia coli ATCC 9637 was used as the positive control.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out using the Bio-Rad DCode Universal Mutation Detection System. A 1mm thick 7% (w v⁻¹) polyacrylamide gel containing a 40%-65% linear denaturing gradient of formamide and urea (100% denaturant = 7 M urea and 40% (v v⁻¹) formamide) was prepared for archaeal community analysis whereas a 45%-60% gradient was used for bacterial community analysis.

DGGE standards were created by loading GC-clamped PCR products (approximately 150 to 300 ng total DNA) of the small subunit rRNA gene from Aiptasia pallida (brown sea anemone) (18S rRNA), Gallus domesticus (chicken) (18S rRNA), Methanosarcina acetivorans strain C2A (DSM 2834) (16S rRNA), Clostridium perfringens (Sigma D5139) (16S rRNA), Escherichia coli ATCC 9637 (16S rRNA) and Streptomyces fradiae
ATCC 10745 (16S rRNA) mixed with 10 µl of loading dye. Two standard lanes were loaded per gel. Approximately 650 to 800 ng (total) of PCR product amplified from leachate samples was loaded in individual lanes of the gel. Gels were electrophoresed at 47V, 60°C for 16 h, stained with SYBR Green I and images were obtained using a Foto/Analyst Imaging System (Fotodyne Inc).

Cloning and sequence analysis of mcrA gene

Since co-disposal (MSW + ash + biosolids) is a widespread method of waste disposal, one of the two co-disposal bioreactors was selected for the study of methanogen populations. An early sample of leachate (day 50) and a late sample (day 218) were selected for methanogen population analysis. DNA extracted from the two samples was amplified using the ME1 and ME2 primers (Hales et al. 1996; Nercessian et al. 1999) that target the mcrA gene. PCR conditions were as described previously (Hales et al. 1996). *Methanosarcina acetivorans* strain C2A (DSM 2834) was used as the positive control.

The day 50 and day 218 amplicon bands (760 bp) were excised from the gel using a QIAQuick Gel Extraction Kit (Qiagen, CA), as per manufacturer’s instructions. The TOPO TA Cloning Kit for Sequencing (Invitrogen, CA) was used for both cloning and transformation (as per manufacturer’s instructions). The vectors (plasmids) were subsequently transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, CA). Cells were gently plated onto Luria broth (LB) agar plates amended with 100 µg ml⁻¹ ampicillin and individual colonies were re-streaked on new plates. Plasmids were extracted using the FastPlasmid Mini kit (Eppendorf, Hamburg, Germany) per manufacturer’s instructions. Insert DNA from the extracted plasmids was
amplified using the ME primer set and confirmed by agarose gel electrophoresis. PCR reactions were purified using QIAQuick PCR Purification Kit (Qiagen, Valencia, CA). The Genome Lab DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA) was used for the final sequencing PCR reaction. The amplified DNA was purified, concentrated by ethanol precipitation and sequenced using a Beckman CEQ™ 8000 Genetic Analysis System (Beckman Coulter). Sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their identities as methanogens. Possible methanogen sequences were designated JME for clones from the day 50 sample, and DME for clones from the day 218 sample.

**Statistical analysis**

Gel images were imported into Bionumerics (Version 3.0, Applied Maths, Belgium) and analyzed using the Dice similarity coefficient by constructing unweighted pair group method with arithmetic mean (UPGMA) dendrograms (optimization 1.0%, tolerance 0.5%). Principal components analysis (PCA) was performed using SPSS (SPSS Inc., Chicago, IL) to obtain two-dimensional plots showing relatedness of populations. PCA is a data reduction technique which takes into account all the variables in a given set, determines the patterns of similarities and differences between the variables and expresses the results as a two or three-dimensional plot. For fingerprint patterns such as those obtained by DGGE or RFLP, bands are classified as present or absent (binary) and compared by constructing a band-matching table (Boon et al. 2002; Caddick et al. 2006). Microbial concentrations (direct microscopic cell counts) were compared by paired t-tests (GraphPad Instat). Shannon diversity index is a measure of the richness (number of
species present in a community) and abundance of any ecological habitat. Shannon diversity index \( (H) \) was calculated by using the formula:

\[
H = - \sum p_i \log p_i
\]

calculated as \( p_i = n_i/N \) where \( n_i \) is the height of a peak and \( N \) is the sum of the peak heights of all bands in the densitometric curve (Eichner et al. 1999; Ogino et al. 2001; Haack et al. 2004).

Shannon indices were calculated for the community profile of each given time point of individual bioreactors and an average of these was reported.

**Results**

*Total cell concentrations*

Total cell concentrations (measured by epifluorescence microscopy) in the first month of bioreactor operation increased about an order of magnitude, from \( 3 \times 10^8 \) cells ml\(^{-1} \) to \( 3 \times 10^9 \) cells ml\(^{-1} \) in MSW bioreactors and co-disposal bioreactors. Cell concentrations dropped after 50 days, then stabilized in all bioreactors except MSW1. Cell concentrations varied only about four-fold from bioreactor start-up to the termination of the experiment. There was a significant difference between the mean cell concentrations of the two MSW bioreactors calculated over the course of the study (paired t-test; \( P < 0.0001 \)). The difference was attributable largely to the increased cell concentrations in the second half of the experiment in MSW1. In contrast, the difference in cell concentrations for the two co-disposal bioreactors was not significant (\( P > 0.95 \)). Cell concentrations in leachate from MSW bioreactors were significantly greater than those from co-disposal bioreactors (\( P = 0.048 \)).
**pH of leachate**

The initial increase in microbial numbers in all bioreactors corresponded with an initial decrease in pH from neutral to 5.5 by day 25. The pH returned to neutral by day 50 and remained neutral till the conclusion of the experiment.

**DGGE analysis of microbial communities**

An initial experiment was performed to test the reproducibility of DGGE in the complex matrix of the leachate. Triplicate leachate samples taken within several minutes of one another were analyzed from a selected bioreactor (Co-disposal 2). The similarity of the DGGE patterns for triplicate analyses of both archaeal and bacterial community structure based on 16S rRNA genes were greater than 95%, showing high reproducibility of the method (data not shown).

Leachate samples collected on 12 to 15 dates over a period of eight months were subjected to DGGE. The first incidence of detection of *Archaea* by PCR using 16S rRNA genes corresponded to the occurrence of negative oxidation-reduction potentials and the presence of volatile acids, indicating anaerobic conditions (Cardoso et al. 2006), on day 25. Inhibition of the PCR was not responsible for the absence of archaeal PCR products in the early leachate samples, as positive control DNA spiked into these leachates was amplified.

**Archaeal community structure**

Analysis of archaeal DGGE patterns indicates that in all the bioreactors, the initial population changed substantially between start-up (day 25) and maturation (day 50) (Figure 1). The data from a representative bioreactor for each treatment (MSW or co-
disposal) are shown in Figure 1a and 1b, respectively. In the MSW bioreactors archaeal community fingerprints over a two-month period (day 50 to day 78, designated cluster I) clustered together followed by a substantial change in the community at day 99 (Figure 1a). Cluster II, which includes patterns from day 120 to day 218, denotes a cluster of comparatively similar patterns towards the end of the study. In the co-disposal bioreactors, archaeal DGGE patterns after bioreactor start-up indicated a more gradual shift in the community structure with less well-defined clusters (Figure 1b). Principal components analysis (PCA) of the DGGE patterns also demonstrated temporal shifts in communities (Figure 1a and 1b).

Bacterial community structure

Analysis of bacterial DGGE patterns revealed that the patterns in MSW bioreactors were clustered in discrete groups of high (>75%) similarity. Cluster I included day 1 to day 25, cluster II day 50 to 99 and cluster III day 169 to 218 (Figure 2a). In contrast, the patterns in co-disposal bioreactors shifted in a more gradual manner (Figure 2b). The data from one bioreactor per treatment are shown in Figure 2; relationships among the patterns of duplicate bioreactors were similar for both MSW and co-disposal treatments. PCA results for bacterial community structure also corresponded to the results obtained from the UPGMA dendrograms (Figure 2a and 2b).

Microbial community structure was more similar within bioreactors than between bioreactors of the same treatment (data not shown). This grouping was consistently observed for Archaea and Bacteria in MSW and co-disposal bioreactors. Comparison of communities in MSW vs. co-disposal bioreactors did not reveal grouping by treatment
(waste type); thus, factors other than the waste composition were most instrumental in determining community structure.

Calculation of the Shannon diversity indices revealed a higher apparent diversity of observed archaeal populations (average $H = 1.37$) in the bioreactors as compared to the bacterial populations (average $H = 1.24$) (Table 1).

**Sequence analysis of methanogens**

Seventeen unique *mcrA* gene sequences were found out of the thirty-seven clones analyzed for the day 50 leachate sample of the co-disposal bioreactor (Table 2). The most numerically dominant clone was closely related to the uncultured methanogen clone RS-ME43 isolated from rice field soil (Lueders *et al.* 2001). Methanogen clones from the day 50 leachate sample were closely related to the members of *Methanosarcinales*, *Methanobacteriales* and *Methanomicrélales*. Twelve unique *mcrA* gene sequences were found out of the thirty-two clones analyzed for the day 218 leachate sample (Table 2). The most dominant clone was closely related to the uncultured methanogen clone MidMcrA114 isolated from the sediment of the Pearl River Estuary (Jiang *et al.* 2008, unpublished material). All the methanogen clone sequences from the day 218 leachate sample were related to members of the Order *Methanomicrélales*. 
Table 1. Shannon diversity indices calculated for the DGGE patterns of *Archaea* and *Bacteria*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Archaea</th>
<th>Bacteria</th>
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</thead>
<tbody>
<tr>
<td>MSW1</td>
<td>1.374</td>
<td>1.195</td>
</tr>
<tr>
<td>MSW2</td>
<td>1.384</td>
<td>1.218</td>
</tr>
<tr>
<td>Co-disposal1</td>
<td>1.361</td>
<td>1.241</td>
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<tr>
<td>Co-disposal2</td>
<td>1.367</td>
<td>1.296</td>
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</table>

Shannon Diversity index ($H$)
Figure 1. Dendrograms and corresponding principal components analysis of archaeal population structure in (a) MSW and (b) co-disposal bioreactors. I, II, III and IV denote clusters of similar patterns (>75% similarity). Note that *Archaea* were not detected in the leachate before day 25. (Clustering patterns of duplicate bioreactors for both MSW and co-disposal were similar). Arrow indicates increasing direction of denaturant and acrylamide gradient from lower to higher concentration.
Figure 2. Dendrograms and corresponding principal components analysis of bacterial population structure in (a) MSW and (b) co-disposal bioreactors. I, II, III, IV and V denote clusters of similar patterns (>75% similarity). (Clustering patterns of duplicate bioreactors for both MSW and co-disposal were similar). Arrow indicates increasing direction of denaturant and acrylamide gradient from lower to higher concentration.
Table 2. Frequency of methanogen clones observed in the day 50 and day 218 samples obtained from the co-disposal bioreactor.

<table>
<thead>
<tr>
<th>Clone a,b</th>
<th>Putative Group (Order)</th>
<th>Closest Relatives</th>
<th>% of clone library</th>
</tr>
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<tbody>
<tr>
<td>JME1 (FJ435818)</td>
<td><em>Methanomicrobiales</em></td>
<td><em>Methanobacterium</em> sp. MB4 (DQ677519)</td>
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<tr>
<td>JME2, 5-7 (FJ435819, FJ435822, FJ435823, FJ435824)</td>
<td><em>Methanomicrobiales</em></td>
<td><em>Methanocorpusculum parvum</em> (AY260445)</td>
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<td>JME3 (FJ435820)</td>
<td><em>Methanosarcinales</em></td>
<td>Rice field soil clone RS-ME28 (AF313863)</td>
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<td>JME4,8 (FJ435821, FJ435825)</td>
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<td>Biogas plant clone ATB-EN-5737-M022 (FJ226633)</td>
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<td>JME11-13,15,36 (FJ435826, FJ435827, FJ435828, FJ435830, FJ435849)</td>
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<td><em>Methanosarcina mazei</em> strain TMA (AB300778)</td>
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<td>JME14,22-27,30,37,43 (FJ435829, FJ435836, FJ435837, FJ435838, FJ435839, FJ435840, FJ435841, FJ435844, FJ435850, FJ435854)</td>
<td><em>Methanosarcinales</em></td>
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<td>Clone&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>Closest Relatives</td>
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<td>JME38 (FJ435851)</td>
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<td>Human fecal sample clone DC&lt;sub&gt;_c&lt;/sub&gt;lone-mcrA&lt;sub&gt;-2&lt;/sub&gt; (AM921682)</td>
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<td>Methanosarcina mazes strain LYC (AB300782)</td>
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<td>DME4,5,7 (FJ435858, FJ435859, FJ435861)</td>
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<td>Methanocorpusculum parvum (AY260445)</td>
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<td>Biogas plant clone ATB-EN-5737-M022 (FJ226633)</td>
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<td>DME9 (FJ435863)</td>
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<td>Biogas plant clone MARMC548 (DQ260615)</td>
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<td></td>
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<td>Clone(^a,b)</td>
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<td>DME10,20-22</td>
<td>\textit{Methanomicrobiales}</td>
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<td>Gas condensate-contaminated aquifer clone L44B (EU364876)</td>
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<td>Biogas plant clone G8RTCR50 (DQ260503)</td>
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</table>

\(^a\)All isolates labeled JME denote clones obtained from day 50 sample

\(^b\)All isolates labeled DME denote clones obtained from day 218 sample

**Discussion**

The various cells of a landfill generally contain waste that is at different stages of decomposition depending upon waste composition, residence time, moisture, etc., making it difficult to study the progression of microbial population development in the highly
heterogeneous landfill environment. Compared to landfills, bioreactors are simpler systems that allow better control over the variables that play a role in waste degradation. As a simplified system, bioreactors have been employed in other studies to study microbial community dynamics (Barlaz et al. 1989a), cellulose degrading Clostridium populations (Burrell et al. 2004), methanogen communities (Griffin et al. 1997) and chemical composition of leachate (Pohland and Kim 2000; Ledakowicz and Kaczorek 2004).

In spite of the attempt to construct parallel (duplicate) bioreactors in this study, cell concentrations in duplicate MSW bioreactors varied significantly over the course of the experiment, while these values in co-disposal bioreactors, which included biosolids, were similar. The variable cell concentrations between duplicate MSW bioreactors may be attributable to the heterogeneous nature of the waste, which was shredded municipal waste obtained from a landfill in Palm Beach County, FL. This waste contained paper, plastic, food, and other common components of garbage, and was not standardized. This heterogeneity probably also contributed to the dissimilarity of community profiles in duplicate bioreactors. These results underscore the complexity of determining the factors that influence processes such as clogging, which can lead to landfill failures and management problems (Rohde and Gribb 1990; Fleming et al. 1999).

A common trend seen in all bioreactors was a decrease in pH, which was consistently accompanied by a rise in cell concentrations over the first 25-30 days of the study. This trend reflects the successive processes that typically occur during solid waste degradation. During the early stages, aerobic and facultative anaerobic heterotrophs decompose organic substrates and quickly exhaust oxygen and nitrate (Barlaz et al. ...
1989a). Cellulose and hemicellulose comprise a high percentage (45% – 60%) of landfill material and are easily biodegradable (Barlaz et al. 1989b). Cellulose degradation is performed by hydrolytic and fermentative bacteria and fungi in the primarily anaerobic conditions of the landfill (Pourcher et al. 2001; Van Dyke and McCarthy 2002; Burrell et al. 2004; Lockhart et al. 2006; McDonald et al. 2008). Fermentative bacteria use monosaccharides and amino acids to produce alcohols, organic acids, carbon dioxide and hydrogen (Barlaz et al. 1989a), which causes more acidic conditions (lower pH). Methanogens utilize carbon dioxide, hydrogen, acetate, and formate resulting in an increase in pH (Mormile et al. 1996). Removal of acetate by iron reducing bacteria (Frenzel et al. 1999; Lin et al. 2007) and bicarbonates produced by sulfate-reducing bacteria (SRBs) (Elliott et al. 1998) could also cause increase in pH of leachates After the initial decrease in pH at day 25, pH values increased to near neutral and remained at that level through the conclusion of the study.

Another common trend among bioreactors was that, irrespective of waste composition (MSW vs. co-disposal), archaeal populations exhibited higher apparent diversity as assessed by DGGE than the bacterial populations. To the best of our knowledge, no previous study has compared the diversity in 16S rRNA sequences of archaeal vs. bacterial populations in decomposing solid waste. The emergence of a diverse population of Archaea about 25 days after inoculation and its maintenance throughout the study reflects the process of succession and ultimately maturation of the microbial community in the bioreactors. Although DGGE reflects a broad community structure, like any other molecular technique, it is subject to biases and errors such as selective amplification, heteroduplex formation and co-migration of DNA fragments (Muyzer and Smalla 1998).
However, DGGE is widely employed in ecological studies because it enables quick and convenient comparison of temporal and spatial distributions of the predominant members in a population as compared to other molecular methods such as cloning and sequencing.

Analysis of microbial community structure in the laboratory bioreactors revealed temporal shifts of archaeal and bacterial populations in bioreactors regardless of the waste content, suggesting a succession process from an immature to a mature community. These results concur with other studies that demonstrated succession during solid waste decomposition. For example, a study on composting of MSW using phospholipid fatty acid analysis (PLFA) to identify operational taxonomic units suggested four stages of waste degradation (Herrmann and Shann 1997). Two studies using culture-based methods of microbial identification also found population shifts that suggested succession processes (Barlaz et al. 1989a; Boothe et al. 2001). Interestingly, microbial populations in MSW bioreactors were clustered into groups with high similarity, whereas a gradual change was detected in microbial populations from co-disposal bioreactors. The diverse inoculum provided by the biosolids may have contributed to the gradual change in community structure for the co-disposal bioreactors.

Methanogens belong to the archaean kingdom *Euryarchaeota* (Winker and Woese 1991; Barns et al. 1996), and are divided into five orders: *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteria*, *Methanococcales* and *Methanopyrales* (Bapteste et al. 2005). Our study found representatives from the *Methanosarcinales*, *Methanobacteria* and *Methanococcales* in the initial stages of waste degradation (day 50 sample). Interestingly, the later stages (day 218 sample) were exclusively dominated by members of the *Methanomicrobiales*, which include genera such as
*Methanocorpusculum* and *Methanoculleus*. Several of the sequences obtained in this study were most similar to cultured methanogens, e.g. *Methanobacterium* sp. MB4, *Methanosarcina mazei* and *Methanocorpusculum parvum* while others were most similar to sequences obtained from uncultured organisms found in a variety of environments, including biogas plants, rice field soil, subsurface sediments and the bovine rumen. Previous studies of methanogens in leachate from bioreactors or landfills identified a number of phylogenetic groups including *Methanosaeta* and *Methanobacteriaceae* (Calli *et al.* 2003), *Methanomicrobiales* (*Methanoculleus* and *Methanofollis*) and *Methanosarcinales* (*Methanosaeta* and *Methanosarcina*) (Uz *et al.* 2003). *Methanosarcina* sp., *Methanobacterium* sp. and *Methanocorpusculum* sp. were isolated from anaerobic sewage sludge digestors (Bryant and Boone 1987; Raskin *et al.* 1994; Griffin *et al.* 1997; Whitehead and Cotta 1999).

Despite the great variability in leachate microbial community profiles, the temporal trend in microbial community structure was consistently observed for archaeal and bacterial populations in the simulated solid waste bioreactors. Gaining an understanding of the environmental factors that influence these high-diversity communities will require extensive research, but this effort is justified by the potential for using this knowledge to improving landfill management practices.

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Abstract

Landfill waste contains microbial pathogens and fecal indicator organisms (IOs). Solid waste degradation is expected to reduce their populations, yet little is known about the factors that influence their persistence in landfills. This study was designed to follow changes in IO concentrations during the process of waste degradation (“hydrated” phase) and to determine the fate of IOs in the waste after an extended period of moisture deficiency (“dehydrated” phase). Landfill conditions were simulated by constructing bioreactors packed with municipal solid waste (MSW), combustion residues and biosolids from wastewater treatment facilities. Leachate generated by moistening MSW was sampled weekly for seven months and analyzed for total cells by fluorescence microscopy and for culturable IO concentrations. Total live cell concentrations in leachate were measured by BacLight staining. They decreased from $1.9 \times 10^9$ cells/ml to $4.9 \times 10^8$ cells/ml after seven months. Fecal coliform concentrations decreased from $6.3 \times 10^4$ CFU/ml to below detection limits while Enterococcus concentrations decreased from $1 \times 10^6$ CFU/ml to below detection limits by the end of the second month of bioreactor operation and remained undetectable for the rest of the hydrated phase. Decay rates for fecal coliforms were not significantly different from the decay rates for enterococci in both bioreactors. A one-year drying period was followed by rehydration and a 24-h sampling regimen. Total cell concentrations increased from ~$10^7$ cells ml$^{-1}$ immediately after rehydration to ~$10^8$ cells ml$^{-1}$ after 24 hours. Hourly changes in the bacterial community structure analyzed by denaturing gradient gel electrophoresis (DGGE)
analysis following rehydration revealed a fairly stable, low-diversity community. Fecal coliforms and enterococci were detected at low concentrations (≤20 or 30 CFU/100 ml, respectively) in the leachate even after the prolonged dry period. The recovery of IOs after prolonged dehydration was unexpected, and raises questions about the fate of pathogens during the process of waste degradation.

**Introduction**

The survival and composition of microbial communities in landfills depends on several factors such as waste composition, pH, temperature, toxic metal levels and moisture availability (Boothe *et al.* 2001). The amount of moisture available to support microbial activity depends on local climatic conditions, i.e. rainfall and temperature, the composition of waste deposited in the landfill, and the frequency of leachate removal and/or recirculation (El-Fadel 1999). The majority of the waste deposited in landfills comprises municipal solid waste (MSW) which includes household waste, newspapers, glass, and metals (Kjeldsen *et al.* 2002). Co-disposal of ash from waste-to-energy (WTE) processes and biosolids from wastewater treatment is also practiced in Class I landfills (Reinhart 2003).

Biosolids are nutrient and moisture rich and add to the microbial load of the waste. One major concern associated with deposition of biosolids in landfills is the possible presence of pathogens that have survived the treatment process. Pathogens can also be introduced in landfills from a variety of other sources such as soiled diapers, biomedical waste and pet feces (Peterson 1974; Mose and Reinthaler 1985; Trost and Filip 1985; Gerba *et al.* 1995). Dissemination of human pathogens into the community can occur due to groundwater contamination by landfill leachate. This potential risk is evaluated by
enumerating indicator organisms (IOs) such as fecal coliforms and enterococci in groundwater that may be contaminated by leachate (Rose 2004).

Landfills undergo periods of moisture abundance during rainy periods and moisture deprivation in most climates. Compaction of refuse reduces infiltration of water through the waste layers, thereby pooling less leachate in the collection systems (Tatsi and Zouboulis 2002) and minimizing the probability of leachate contaminating the groundwater. In contrast, heavy rainfall dilutes toxic compounds in leachate and accelerates refuse decomposition (Wreford et al. 2000) but increases the potential for groundwater contamination. This study aims to evaluate the changes in concentrations of the overall microbial population and IOs during moisture-rich and moisture-deprived conditions. We hypothesize that indicator organisms will persist well in the moisture-rich (“hydrated”) phase while being unrecoverable in the moisture-deprived (“dehydrated”) phase of waste degradation.

For the hydrated phase of the study, bioreactors were constructed and operated in duplicate and the total cell concentrations and IO concentrations in leachate were enumerated for a period of seven months. Due to practical reasons and space restrictions, only one bioreactor was operated for the dehydrated phase of the study. This bioreactor was subjected to a dry period of one year, after which it was rehydrated and the leachate was tested over a 24-hour period to determine total microbial concentrations, heterotrophic plate counts and IO concentrations. Changes in the bacterial community structure post-rehydration were also investigated by denaturing gradient gel electrophoresis (DGGE).
Materials and methods

Landfill conditions were simulated in duplicate laboratory scale bioreactors built from 150 cm tall; 20 cm diameter PVC pipes (Figure 3). They were packed with 60% municipal solid waste (MSW), 20% combustion residues (ash) from waste-to-energy processes, 10% biosolids from wastewater treatment and 10% sand (inert material) (all measurements by mass). Combustion residues were obtained from the Hillsborough County Resource Recovery Facility (Covanta Hillsborough Inc.) at Falkenburg Road, Tampa, FL. Biosolid material (dewatered sludge) was obtained from the Falkenburg Road Advanced Wastewater Treatment Plant in Tampa, FL. The waste mixtures were placed in the bioreactors and hydrated to field capacity with distilled water, which was held for 24 hours before draining. The bioreactors were rehydrated with a mixture of 2.5 liters of drained leachate and 1 liter of distilled water.

Hydrated conditions were maintained by recirculating a mixture of leachate: distilled water (2.5:1) three times a week for seven months. Leachate samples were collected and analyzed weekly during the entire hydrated phase (see below), but were collected more frequently after rehydration of the dehydrated bioreactor (see below). One bioreactor was randomly chosen for the dehydration study. After a year-long dry period, the bioreactor was rehydrated by adding distilled water (20 L) and leachate was sampled every hour for the first eight hours (time zero to time seven) and once at the 24-hour mark.
Figure 3. Diagram of bioreactor configuration.

Total microbial concentrations were determined by direct fluorescence microscopy using two staining methods. The more commonly used DAPI (4,6-diamidine-2-phenylindole) stains the DNA of all cells and does not differentiate between live and dead cells. The Live/Dead BacLight Bacterial Viability kit (Molecular Probes, Invitrogen) includes SYTO 9 that stains both live and dead cells (green fluorescence) and propidium iodide that stains cells with compromised cell membranes (red fluorescence). The advantage of BacLight in differentiating live from membrane-damaged cells is counterbalanced by the
fact that samples must be observed immediately after staining due to the potential for sample drying and loss of fluorescence, but this is not the case with DAPI-stained slides, which can be stored for later counting. Both staining methods were employed during the hydrated phase of the experiment to compare cell concentrations. DAPI staining (1 mg ml⁻¹) was performed as described previously (Nayak et al. 2009). Live versus membrane-damaged cells were estimated by staining duplicate leachate samples with the Live/Dead BacLight Bacterial Viability kit for microscopy as per manufacturer’s instructions.

IO concentrations were determined (in duplicate) by standard membrane filtration methods. The volume of leachate filtered was increased from 1 ml in the initial stages of the experiment to 100 ml (25 ml on each of four separate filteres) as the IO concentrations decreased towards the end of the experiment. Filters were placed on mFC agar for fecal coliforms and mEI agar for enterococci (American Public Health Association (APHA) 1998). After incubation for 18-22 hours (U. S. Environmental Protection Agency 1997), blue colonies (fecal coliforms) from mFC agar and colonies with blue halo (presumptive enterococci) from mEI agar were enumerated.

For the dehydrated phase, it was not feasible to perform BacLight staining due to time restrictions (samples were collected and processed every hour). Total cell concentrations were estimated by DAPI staining and IO concentrations were determined by membrane filtration of 100 ml (50 ml each filtered two times) as described above. To determine if culturable bacteria persisted in the bioreactor after prolonged dehydration, heterotrophic plate counts were obtained by spread plating 100 µl of leachate on R2A agar (Becton Dickinson, MD) for aerobic organisms and anaerobic agar for anaerobic organisms.
(Becton Dickinson, MD). R2A agar plates were incubated at room temperature under ambient conditions for 3 days whereas anaerobic agar plates were incubated in Gas-Pak chambers (H₂ + CO₂) (Becton Dickinson, MD) for 5 days. Total community DNA was extracted from leachate samples using the Ultraclean Soil DNA Kit (MoBio Laboratories, Inc.) per manufacturer’s instructions. Bacterial 16S rRNA genes were amplified using the primer set 1070f and 1392r (Ferris et al. 1996). PCR conditions and the DGGE procedure details are as described previously (Nayak et al. 2009).

Statistical analysis: Log₁₀ values of the microbial concentrations (direct microscopic cell counts) and IO concentrations were compared by paired t-tests (GraphPad Instat). The decrease in IO concentrations was biphasic; i.e. there was a steep decline in concentrations from day 1 to day 21 followed by a more gradual decline. Hence the initial decay rate was calculated from day 1 to day 21 and the overall decay rate was calculated from day 1 to the last day of IO detection.

Decay rates for IOs were calculated using the formula:

\[
\text{Decay rate (k)} = \frac{(\log_{10} N - \log_{10} N_0)}{(t + 1)}
\]

where \( N \) = concentration of organisms on day 21 (for initial decay rate) or last day of detection (for overall decay rate)

\( N_0 \) = concentration of organisms on the first day of the experiment

\( t \) = day 21 (for initial decay rate) or last day of detection (for overall decay rate)

Paired t tests were performed to compare IO concentrations in duplicate bioreactors by normalizing each concentration to the starting density. Fecal coliform concentrations were normalized to \( 10^5 \) CFU ml⁻¹ whereas Enterococcus concentrations were normalized to \( 10^6 \) CFU ml⁻¹.
Shannon diversity index \((H)\) was calculated by using the formula:

\[
H = - \sum p_i \log p_i
\]
calculated as \(p_i = n_i / N\) where \(n_i\) is the height of a peak and \(N\) is the sum of the peak heights of all bands in the densitometric curve (Eichner et al. 1999; Ogino et al. 2001; Haack et al. 2004). Shannon indices were calculated for the community profile of each given time point and an average of these was reported.

**Results**

*Hydrated phase*

In the first, hydrated phase of the experiment, total (live + dead) cell concentrations in leachate as calculated by BacLight staining increased from \(2.8 \times 10^8\) cells ml\(^{-1}\) to \(2.3 \times 10^9\) cells ml\(^{-1}\) after the first month and dropped down to \(7.0 \times 10^8\) cells ml\(^{-1}\) by the end of the experiment (Figure 4). BacLight concentrations were slightly but not significantly lower than DAPI concentrations and followed the same trends (data not shown). Live cell concentrations in bioreactors increased from \(2.3 \times 10^8\) cells ml\(^{-1}\) to \(1.9 \times 10^9\) cells ml\(^{-1}\) after the first month and dropped down to \(4.9 \times 10^8\) cells ml\(^{-1}\) at the end of the seventh month (Figure 4). There was no significant difference between the mean cell concentrations (BacLight staining) of the two bioreactors for the total counts \((P = 0.63)\) or live counts \((P = 0.58)\). On an average, 82.3% of total cells (red + green) appeared live throughout the hydrated phase. No increasing or decreasing trend was observed in the ratio of live vs. dead cells.

pH values in leachate samples from bioreactors dropped from neutral to 5.8 by day 14 of the experiment (Figure 5). The pH returned to neutral by day 28 and remained neutral till the end of the experiment. Fecal coliform concentrations decreased from \(6.3 \times 10^4\) CFU
ml\(^{-1}\) to below detection limits (<1 CFU ml\(^{-1}\)), while *Enterococcus* concentrations decreased from \(1 \times 10^6\) CFU ml\(^{-1}\) to below detection limits (<1 CFU ml\(^{-1}\)) by day 56 of the experiment (Figure 6). There was no significant difference in the fecal coliform concentrations \((P = 0.76)\) or *Enterococcus* concentrations \((P = 0.10)\) in duplicate bioreactors. Initial decay rates (day 1 to 21) for fecal coliforms in each bioreactor were \(-0.23\) and \(-0.20\) and the total decay rates were \(-0.08\) and \(-0.09\). Initial decay rates (day 1 to 21) for enterococci in each bioreactor were \(-0.23\) and \(-0.21\) and the total decay rates were \(-0.11\) and \(-0.10\). Paired t-tests were performed on normalized fecal coliform and *Enterococcus* concentrations and no significant differences were found in the concentrations of the two IOs \((P = 0.94)\).

**Figure 4.** Total cell concentrations \((x 10^9/\text{ml})\) in leachate during the hydrated phase as measured by BacLight Live/Dead staining. Results of duplicate bioreactors (B1 and B2) are presented.
Figure 5. pH values in duplicate bioreactors (B1 and B2) during the hydrated phase.

Figure 6. Mean concentrations of fecal coliforms (FC) and enterococci (ENC) in leachate sampled weekly from duplicate bioreactors in the hydrated phase of the experiment. Data shown only until culturable cells were no longer detectable in the hydrated phase.
Dehydrated phase

In the second, dehydrated phase of the experiment, total cell concentrations in the leachate increased from $1.5 \times 10^7$ cells ml$^{-1}$ at time zero to $2.4 \times 10^8$ cells ml$^{-1}$ at 5 hours. Total cell concentrations subsequently stabilized at approximately $1.1 \times 10^8$ cells ml$^{-1}$ (Figure 7). Heterotrophic plate counts for culturable aerobic organisms increased from $5.9 \times 10^5$ CFU ml$^{-1}$ at time zero to $2 \times 10^6$ CFU ml$^{-1}$ at 24 hours (Figure 8). A similar increase was noted for culturable anaerobic organisms, which increased from $6 \times 10^3$ CFU ml$^{-1}$ at time zero to $4 \times 10^4$ CFU ml$^{-1}$ at 24 hours (Figure 8). Since anaerobic chambers were not used while plating the leachate, the concentrations do not include any extremely oxygen-sensitive anaerobes that may have been present. Fecal coliforms were detected in the leachate five hours after the bioreactor was rehydrated (8 CFU 100 ml$^{-1}$), but not before that time. Twenty-four hours after rehydration, the fecal coliform concentrations increased to 20 CFU 100 ml$^{-1}$. Enterococci were detected in the leachate three hours after rehydration of the bioreactor (20 CFU 100 ml$^{-1}$). Twenty-four hours after rehydration, Enterococcus concentrations increased to 30 CFU 100 ml$^{-1}$.

DGGE was performed to observe the bacterial community structure in leachate after this prolonged dry period and to determine changes in the community during the next twenty-four hours. A shift was observed in the bacterial community structure (assessed by DGGE) between time zero and the first hour following rehydration, after which the populations were approximately 90% similar (Figure 9). There was a considerable change in the population structure again at the last measurement (24 hour). The average Shannon diversity index ($H$) for the twenty-four period was calculated at 1.04.
Figure 7. Total cell concentrations (x 10^7/ml) in leachate from time zero (T<sub>0</sub>) to twenty four (T<sub>24</sub>) after rehydration of the bioreactor. The line between T<sub>7</sub> and T<sub>24</sub> denotes a time break.

Figure 8. Heterotrophic plate counts on R2A agar (aerobic organisms) and anaerobic agar (anaerobic organisms) after bioreactor rehydration. The line between T<sub>7</sub> and T<sub>24</sub> denotes a time break.
Figure 9. Dendrogram showing the percent similarity of bacterial DGGE patterns from time zero ($T_0$) to twenty four ($T_{24}$) after rehydration of the bioreactor. The circle denotes a previously undetectable, relatively low GC content band in the 24th hour sample. Arrow indicates increasing direction of denaturant and acrylamide gradient from lower to higher concentration.

Discussion

The effect of moisture on the fate of IOs was studied by determining culturable fecal coliform and *Enterococcus* spp. concentrations in the leachate from laboratory-scale bioreactors subjected to moisture-rich and moisture-deprived conditions. Fecal coliforms and enterococci remained detectable for the first 50 days of the hydrated phase, after which they were undetectable till the end of the hydrated phase. The transition of fecal coliforms and enterococci to a viable but non-culturable (VBNC) state could explain the drop in the concentrations below detection level after the first 50 days. During the initial stages of waste degradation cellulose degrading bacteria such as *Clostridium* spp. and *Eubacterium* spp. hydrolyze cellulose and hemicellulose into monosaccharides that are further fermented to produce alcohols and carboxylic acids (Van Dyke and McCarthy 2002; Burrell et al. 2004). Accumulation of these metabolic products results in a drop in pH, which could be the stressors driving IOs into the VBNC state (Oliver 1993; Higgins...
The decline in the pH of the leachate from neutral to acidic by day 14 corresponds with the initial linear decline in IO concentrations.

Fecal coliform decay rates were not significantly different from Enterococcus decay rates and a similar decreasing trend in IO concentrations was observed over a period of time. The decay rates of both IOs demonstrated a biphasic trend wherein a linear decay in concentrations was observed initially and subsequently leveled off until culturable cells were no longer detectable. The IO decay rates observed in our study are comparable to those observed by other studies, although they were measured in different environments (Anderson et al. 2005; Badgley 2009). Enterococcus populations in freshwater mesocosms demonstrated an initial decay rate of –0.63 and an overall decay rate of –0.02 over a period of two weeks (Badgley 2009). This is in agreement with the biphasic trend of the Enterococcus decay rates observed in our study. In another study, Anderson et al documented decay rates of –0.27 and –0.31 for fecal coliforms and enterococci respectively in freshwater mesocosms inoculated with wastewater (Anderson et al. 2005). The IOs persisted for two weeks and four weeks in the water and sediment columns of the mesocosms, respectively. Both these studies used freshwater as the matrix, which is low in nutrients, and hence the IOs did not persist for long. In comparison, waste is high in nutrients and hence IOs were detected in the leachate samples for about seven weeks.

The ratio of live to dead cells remained constant throughout the hydrated phase of the study. We hypothesize that the consistent survival of cells over time in the mesocosms is due in part to the high nutrient concentration in the leachate, where one microbial population is constantly replaced by another (succession) resulting in no net increase or decrease in the concentration of live or dead cells.
Few studies have investigated the survival of indicator organisms during the process of waste decomposition. Cameron and McDonald reported the early die-off of total and fecal coliforms when raw wastewater was mixed in a 9:1 and 1:1 ratio with leachate produced in bioreactors (Cameron and Mcdonald 1977). Since IO survival was determined by mixing wastewater in leachate and not within the bioreactors as part of the waste degradation process, a direct comparison cannot be made with the current study.

Deportes et al documented an initial increase in fecal coliform concentrations in raw waste followed by a steady decline (two log decrease per week) within twenty days in an MSW composting plant (Deportes et al. 1998). The decrease in pathogen concentrations (Salmonella, Shigella and Ascaris eggs) correlated with the decrease in fecal coliform concentrations but not with the concentrations of total and fecal streptococci. They concluded that the combined monitoring of indicators and pathogens provides a better measure of the sanitization of waste. In comparison, we did not see an initial increase in fecal coliform concentrations but the decrease in concentrations was comparable with the initial decline (day 1 to day 21) observed in our study.

The detection of fecal coliforms and enterococci after one year of dehydration was remarkable and completely unexpected. The recovery of these cells in a culturable state after a prolonged moisture-deprived phase indicates that they can survive, possibly within localized “pockets” of moisture within the waste (Lleo et al. 1998). Zaleski et al reported a decrease in fecal coliform and E. coli concentrations in biosolids even when abundant moisture conditions were maintained (Zaleski 2005). They documented culturable fecal coliforms in biosolids that were moistened (rainfall) after a desiccation period and attributed it to regrowth due to fecal contamination from birds. The decrease in fecal
coliform concentrations during moisture-abundant conditions is in agreement with that observed in our study. In our study, the detection of fecal coliforms after bioreactor rehydration in our study cannot be attributed to any external influence since the bioreactor was sealed and maintained in the laboratory.

Even after a prolonged dry period, the total cell concentrations in the leachate were lower by just one log as compared to total cell concentrations measured during the hydrated phase. The recovery of culturable aerobic and anaerobic heterotrophs immediately after rehydration is also noteworthy. Changes occurring in the bacterial population structure in leachate were followed every hour for the first eight hours after rehydration using DGGE to observe the dominant and transient communities and to determine the extent of diversity in the bioreactor community after an extended dry period. Bacterial populations in the leachate in the twenty-four hour time period after rehydration were highly similar. The emergence of a previously undetectable, lower GC content band in the 24th hour sample (circled in Figure 9) suggests a reviving population in a succession process that will probably continue over time (Nayak et al. 2009).

In our previous study, we used DGGE to track the bimonthly changes in microbial populations in leachate sampled from frequently hydrated bioreactors (Nayak et al. 2009). Bacterial populations exhibited higher diversity (1.37) than those in the current study (1.04) and considerable shifts were observed in the dominant species in the two-week period between samples. Here, dehydration of the bioreactor resulted in a reduction of microbial concentrations, which may be coupled with a reduction in population diversity. However, since we did not measure population diversity in the hydrated phase, the change in diversity between the two phases cannot be compared.
Other population studies have employed the Shannon diversity index to calculate the diversity of bacterial communities. For example, sulfate-reducing bacteria (SRBs) in oxic sediment layers of an oligotrophic lake exhibited lower diversity ($H = 1.83$) as compared to anoxic layers ($H = 2.59$) (Sass et al. 1998). In another study, high diversity of microbial communities was observed in mercury-contaminated soil ($H = 3.48$) and was comparable to the uncontaminated control ($H = 3.83$) (Muller et al. 2002). In comparison, we observed lower bacterial community diversity in our previous and current bioreactor studies. Landfill waste is high in nutrients but also carries concentrated amounts of toxic metabolic products, heavy metals and pharmaceuticals, which could result in lower population diversity.

This study demonstrated the survival of IOs in a landfill bioreactor even after a prolonged dry period. The implications of these results with respect to the fate of pathogens in the waste should be further explored, as accurate assessment of the threat posed by the release of pathogens into groundwater is desirable from the public health perspective. Further research should be conducted to investigate the survival of pathogens during waste degradation and its correlation to the survival of IOs to determine if current monitoring practices are appropriate to protect public health.

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RESEARCH SIGNIFICANCE

Landfills are massive, subterranean areas where waste is deposited in compact cells over a period of time. Each lateral and vertical section of the landfill is at a different stage of the waste degradation process. The heterogeneous nature of the waste and the variability in the “age” of different sections of landfills makes it difficult to design sampling strategies for investigating the microbial communities in the waste. Leachates collected from landfills carry a representative sample of the numerous microorganisms involved in degradation of that waste. The physical, chemical and microbial characteristics of leachate have been studied in detail by field experiments as well as the construction of laboratory-scale bioreactors or lysimeters.

Microbial community structure

Bioreactors have been used in several studies to mimic waste degradation conditions in landfills (Pohland and Kim 2000; Cooke 2001; Youcai et al. 2002; Ledakowicz and Kaczorek 2004). Bioreactors were used in this study to simulate MSW-only and co-disposal landfill conditions. Changes in the microbial community structures, influenced by the presence or absence of biosolids, were investigated by sampling leachate over a period of time. To date, this is the only study that has followed the population changes in both prokaryotic domains (Archaea and Bacteria) for such an extended period of time.
using denaturing gradient gel electrophoresis (DGGE). Temporal shifts were observed in the archaeal and bacterial populations in bioreactors regardless of the waste content, suggesting a succession process from an immature to a mature microbial community. This study advances the basic understanding of changes in the microbial community structure during solid waste decomposition. Future research efforts could focus on identifying the dominant microbial species during specific time intervals with the progression of waste degradation. A better understanding of the microbial community structure in solid waste disposal facilities will allow more effective degradation of waste and management of the infrastructure.

**Methanogen populations**

Methanogenic *Archaea* are responsible for the generation of methane in landfills. Methanogens are strictly anaerobic and sensitive to changes in pH, temperature, moisture levels etc (Gurijala and Suflita 1993; Mormile *et al.* 1996; Shen *et al.* 2001; Mori *et al.* 2003). Complete anaerobic decomposition of waste is achieved when most of the organic carbon is transformed to methane and carbon dioxide. In this study, methanogen sequences were retrieved from the leachate during the early and late phases of bioreactor operation. Methanogen clones from the early phase were closely related to the members of *Methanosarcinales, Methanobacteriales* and *Methanomicrobiales* while methanogen clone sequences from the late phase were related to members of the Order *Methanomicrobiales*. Gaining an understanding of the environmental factors that influence the methane production by methanogens will require extensive research, but this effort is justified by the potential for using this knowledge to improving methane management practices.
Survival of indicator organisms during waste degradation

Compromised leachate collection systems, excessive rainfall or the release of improperly treated leachate into surface waters are some of the ways in which pathogens from landfill leachate can come into contact with the community in general. It is not feasible to analyze treated leachate or groundwater suspected of leachate contamination for all of the various pathogens that could be present. Hence, many studies evaluate the threat posed by leachate-borne pathogens by enumerating the indicator organisms in treated leachate (Wreford et al. 2000; Tatsi and Zouboulis 2002; Manios and Stentiford 2004).

I observed a decline in the culturable concentrations of indicator organisms in bioreactor leachate during moisture-rich conditions, which is supported by other such studies (Cameron and Mcdonald 1977; Deportes et al. 1998). Interestingly, I could detect fecal coliforms and enterococci in the leachate even after a prolonged moisture-deprived stage. The persistence of indicator organisms in the leachate signifies the potential for pathogen survival and subsequent dissemination into the community in the event of a failure in leachate management and disposal practices.

Waste degradation studies

The majority of the landfill-related studies are conducted on the European continent, where land availability is scarce due to the small land areas of countries, or in Asia, where increasing human population leads to production of vast amounts of waste. Although the land to human ratio in the U.S. is large, it is imperative to establish better landfilling practices in order to control problems such as clogging of leachate collection
systems due to mineral deposits and prevent unnecessary waste of land space. This study has added to the existing knowledge of microbial populations in solid waste by tracking the changes in archaeal and bacterial community structures, which exhibited high diversity and distinct temporal trends. Methanogens actively involved in the early and late stages of waste degradation were sequenced and identified, extending our knowledge of these important fuel-producers and agents of greenhouse gas production. The effect of moisture on the fate of indicator organisms was also studied. This research was funded by the Hinkley Center for Solid and Hazardous Waste Management, FL and the information obtained during the study was submitted as a report that can be used to improve landfill management practices.

References


EVALUATION OF GENETIC RELATIONSHIPS AND PREVALENCE OF VANCOMYCIN RESISTANCE IN ENVIRONMENTAL ENTEROCOCCI

BACKGROUND

Enterococci are ubiquitous in nature and are particularly challenging to study due to the difficulty in differentiating among certain species and strains (Devriese et al. 1993; Donabedian et al. 1995; Patel et al. 1998). Members of the genus Enterococcus are normal inhabitants of the gastrointestinal (GI) tract of mammals and birds. Enterococcus faecalis and Enterococcus faecium are the predominant species of enterococci colonizing the human GI tract. As many as $10^8$ colony forming units (CFU) of enterococci can be found per gram of human feces (Noble 1978). Enterococci are used as regulatory tools (U.S. Environmental Protection Agency 1986) and in microbial source tracking (MST) methods (Harwood et al. 2004; Scott et al. 2005) as indicators of water quality in fresh and saline waters. Enterococci have been known to persist in environmental waters and have been isolated from food and clinical samples (Eaton and Gasson 2001; Cupakova and Lukasova 2003; Giraffa 2003; Harwood et al. 2004). Some enterococci have the potential to cause infections, especially in immunocompromised humans (Maki and Agger 1988; Svec and Sedlacek 1999). Possession of virulence factors, pathogenicity islands and the capacity to acquire antibiotic resistance genes make Enterococcus species a formidable group from the public health perspective.
Rapid identification of *Enterococcus* species in clinical isolates is essential for treatment and prevention of the nosocomial spread of the pathogen. Phenotypic methods are time consuming and cannot discriminate between some *Enterococcus* species (Devriese *et al.* 1993). Therefore, DNA-based analyses such as genotyping, genetic sequencing and targeting particular genes with specific probes and primers are employed (Malathum *et al.* 1998; Harwood *et al.* 2004; Jackson *et al.* 2004). Methods used to genotype organisms include, but are not limited to, restriction digestion of the chromosomal DNA or targeting repetitive DNA sequences to generate a genomic “fingerprint” of the organism.

Genotyping facilitates identification of variant strains and epidemiological tracking of virulent enterococci to determine their geographic distribution. From an environmental standpoint, enterococcal diversity and prevalence in different habitats can be elucidated by comparison of genotypes. This study was designed with both the environmental and clinical perspective of determining the strain distribution and vancomycin resistance of enterococci in local water bodies and wastewater by genotyping, phylogenetic identification and antibiotic susceptibility testing.

**Identification of enterococci**

Enterococci are facultatively anaerobic, gram-positive, catalase-negative cocci. In general, members of the genus are capable of growth at temperatures between 10 to 45°C, with an optimum growth temperature of 35°C for most species. Some species of the genus *Enterococcus* are motile. Growth in broth containing 6.5% NaCl and hydrolysis of esculin in the presence of bile salts are two important phenotypic characteristics used to identify enterococci at the genus level (Facklam *et al.* 1974). Other tests such as production of leucine aminopeptidase (LAP) and hydrolysis of pyrrolidonyl-β-
naphthylamide (PYR) are used to differentiate enterococci from other gram-positive, catalase-negative cocci such as those belonging to the genera *Leuconostoc/ Weissella* and *Pediococcus* (Facklam and Elliott 1995). They can be differentiated at the species level by phenotypic methods using commercially available kits such as API 20 Strep biochemical test kits (Sader *et al.* 1995; Manero and Blanch 1999), MicroScan gram-positive identification panel (Iwen *et al.* 1999) and Biolog microbial ID/ characterization systems (Moore *et al.* 2006; Graves *et al.* 2007). Yet, phenotypic identification and classification of enterococci is difficult due to the phenotypic similarity of certain species such as *E. gallinarum* and *E. casseliflavus, E. cecorum* and *E. columbae, and E. hirae* and *E. durans* (Devriese *et al.* 1993).

Molecular characterization to the species level has been achieved using DNA-DNA reassociation, 16S rRNA gene sequencing and whole-cell protein (WCP) analysis (Farrow *et al.* 1983; Williams *et al.* 1991; Merquior *et al.* 1994). Presently, twenty-three distinct *Enterococcus* species have been identified (Jackson *et al.* 2004). Molecular typing methods such as fluorescent internally transcribed spacer region PCR (ITS-PCR) (Tyrrell *et al.* 1997), AFLP typing (Ulrich and Muller 1998) and repetitive extragenic palindrome-PCR (REP-PCR) (Svec *et al.* 2005) have also been used in conjunction with phenotypic methods for the identification and typing of *Enterococcus* isolates (Pangallo *et al.* 2008). Several other molecular techniques used for typing and speciation of enterococci include tRNA intergenic spacer PCR (t-DNA PCR) (Baele *et al.* 2000), broad-range amplification of the 16S rDNA gene (Monstein *et al.* 1998), randomly amplified polymorphic DNA analysis (RAPD) (Monstein *et al.* 1998; Quednau *et al.*...
Genetic “fingerprinting” techniques used to type enterococci

Typing of bacterial isolates generates a unique “molecular fingerprint” for each isolate that can be used for strain differentiation. Genotyping of enterococci facilitates discrimination of closely related species and determination of dominant strains in ecological studies (Brownell et al. 2007; Hassan et al. 2007). Supplementing genotype studies with phylogenetic analysis will provide additional information about the relationships among species and strains of enterococci. However, no study has demonstrated that the discrete clusters formed by enterococcal BOX-PCR patterns are phylogenetically related species/strains. This study aims to shed light on the relationships between enterococcal species and strains using both genotypic and phylogenetic data.

Repetitive DNA sequences have been identified and their presence demonstrated in the genomes of several prokaryotes as well as eukaryotes. DNA repeat sequences have been useful in typing certain eukaryotes including protozoan parasites such as *Trichomonas vaginalis*, *Giardia lamblia*, *Trypanosoma* spp. and *Leishmania donovani* and non-pathogenic organisms such as *Paramecium tetraurelia* and *Saccharomyces cerevisiae* (Riley et al. 1991). Palindromic units (PU) or repetitive extragenic palindromes (REP) (Higgins et al. 1982; Gilson et al. 1984; Dimri et al. 1992) and the enterobacterial repetitive intergenic consensus sequences (ERIC) (Sharples and Lloyd 1990; Hulton et al. 1991) are two of the most well studied DNA repeat sequences in bacteria. These repetitive sequences have been identified in *Escherichia coli* (Gilson et al. 1984; Hulton

The presence of repeat DNA elements in prokaryotes is especially intriguing considering the fact that prokaryotic genomes are small and it is generally thought that they cannot afford any waste of coding space. These repeat sequences are postulated to preserve themselves as “selfish DNA” and their proposed functions include regulation of gene expression (Newbury et al. 1987; Stern et al. 1988), a structural role in chromosomal rearrangements (Stern et al. 1984; Gilson et al. 1986; Gilson et al. 1987) and a role in bacterial virulence (Haas and Meyer 1986). PCR-based DNA typing of organisms using primers targeting repetitive DNA elements (known as rep-PCR) has proved valuable in differentiating them at the sub-species level (Versalovic et al. 1991). The highly reproducible and discriminatory nature of this typing technique has resulted in its utilization in epidemiological, agricultural and industrial applications (de Bruijn 1992).

The first group of repetitive sequences identified in gram positive bacteria was designated BOX elements (Martin et al. 1992), which were initially discovered in *Streptococcus pneumoniae*. The sequence is highly conserved within its chromosomal intergenic regions. There are approximately 25 copies of BOX elements in the genome of *S. pneumoniae*. Based on their proximity to the competence-specific and virulence-related genes, it is proposed that these elements play a functional role in regulation of genetic transformation and virulence. BOX elements are comprised of boxA, boxB and boxC subunits, which are 59, 45 and 50 base pairs long, respectively and have very low sequence similarity to one another. The boxB subunit can exist alone or as a part of the
BOX group of elements. More than one repeat of the boxB subunit has also been reported in some BOX elements (Martin et al. 1992). The boxA and boxC subunits have been observed to form stable stem-loop structures. Among the three subunits, the boxA subunit appears to be the most conserved among different bacterial species (Koeuth et al. 1995).

In bacteria other than *S. pneumoniae*, boxA-like elements were found to exist independently of boxB-like and boxC-like subunits. The complete set of BOX elements (boxA, boxB and boxC) have been found only in the genomes of *S. pneumoniae* and *S. agalactiae* (Koeuth et al. 1995). About 25 copies of BOX elements have been detected in these genomes.

Several studies have generated BOX-PCR patterns from other gram positive bacteria such as *Enterococcus* spp. (Malatham et al. 1998; Brownell et al. 2007; Pangallo et al. 2008) and *Lactobacillus* spp. (Gevers et al. 2001). Recent studies have used BOX-PCR to produce genotypic dendrograms of enterococcal isolates since fingerprints generated by BOX-PCR are reproducible and can differentiate enterococci to the species (Pangallo et al. 2008) and strain (Malatham et al. 1998; Proudy et al. 2008) levels. BOX-PCR fingerprinting has been used to type enterococci in ecological studies (Brownell et al. 2007) and MST studies (Brownell et al. 2007; Hassan et al. 2007). These studies include dendrograms that display population similarities based solely on BOX-PCR genotyping. However, no study has demonstrated that BOX-PCR patterns of various strains of a particular enterococcal species are more closely related than strains from different species.

To increase the precision of the genotyping technique, Johnson et al developed a method using fluorescently labeled primers and internal fluorophore-tagged markers that enable
precise alignment of bands within each gel and better normalization of data from
different gels (Johnson et al. 2004). This novel method, called the horizontal,
fluorophore-enhanced, repetitive extragenic palindromic-PCR (HFERP) technique, has
been used to fingerprint E. coli strains from different sources such as animal feces, soils
and environmental waters (Byappanahalli et al. 2006; Hamilton et al. 2006; Ishii et al.
2006; Ksoll et al. 2007). To date, this technique has not been applied to typing of
enterococcal isolates. Due to the increased precision of the method compared to
conventional BOX-PCR typing, it was chosen in the current study to type enterococci.

**Virulence in enterococci**

Enterococci are commensals that inhabit the gastrointestinal tracts of humans and other
mammals. It is postulated that commensal enterococci can acquire additional genes on
mobile genetic elements, which enable them to cause disease (Gilmore and Ferretti
2003). The virulence traits of E. faecalis strains are more extensively studied compared to
the virulence traits of other pathogenic Enterococcus species. E. faecalis strains capable
of causing disease frequently possess adhesins that mediate attachment to host cell
surfaces (Lowe et al. 1995; Archimbaud et al. 2002). Aggregation substance, a surface
protein, plays a role in conjugative transfer of a sex pheromone plasmid by binding donor
and recipient bacterial cells, but its similarity to eukaryotic fibronectin enables it to bind
to integrins on host epithelial cells (Galli 1990, Olmsted 1994). Furthermore, it prevents
respiratory burst after phagocytosis, making the organism resistant to the host immune
response (Ratika 1999, Sussmuth 2000). It is also known to activate the quorum sensing
mechanism that regulates cytolysin production by Enterococcus faecalis, thereby causing
further tissue damage (Chow 1993).
Another cell surface protein expressed by *E. faecalis* and *E. faecium* is the enterococcal surface protein (esp). The *esp* gene, which was initially detected in *E. faecalis*, encodes a protein that is believed to facilitate colonization of the urinary tract and biofilm formation (Shankar *et al*. 1999; Shankar *et al*. 2001; Toledo-Arana *et al*. 2001). A variant of the *E. faecalis esp* gene was later described in clinical isolates of *E. faecium* (Willems *et al*. 2001; Woodford *et al*. 2001; Leavis *et al*. 2004). The variant *esp* gene of *E. faecium* was proposed as a marker of human fecal pollution in environmental waters (Scott *et al*. 2005). Several microbial source tracking (MST) studies have documented the presence of the *esp* gene in *E. faecium* isolated from environmental sources and municipal wastewater (McDonald *et al*. 2006; McQuaig *et al*. 2006; Whitman *et al*. 2007; Ahmed *et al*. 2008a; Ahmed *et al*. 2008b).

Some *E. faecalis* strains secrete a toxin called cytolysin that is both hemolytic and bactericidal (Gilmore 1991). The dual action of the cytolysin provides a competitive edge for proliferation of the organism in the intestinal epithelium. The enterococcal polysaccharide antigen (Epa) and other capsular carbohydrates may also contribute to evasion of the host immune response (Arduino *et al*. 1994; Teng *et al*. 2002). Most *E. faecalis* strains and some *E. faecium* strains produce extracellular superoxide, which results in destruction of epithelial cells and deeper tissue invasion (Huycke *et al*. 1996).

**Antibiotic resistance in enterococci**

Enterococci can be resistant to a wide range of antimicrobial agents such as β-lactams (penicillins, carbapenems and cephalosporins), aminoglycosides (gentamicin, tobramycin and kanamycin) and glycopeptides (vancomycin and teicoplanin). Both vancomycin and teicoplanin are glycopeptide antibiotics used to treat infections caused by gram-positive
organisms. Glycopeptides inhibit cell wall biosynthesis in gram-positive organisms by complexing with the D-alanine-D-alanine (D-Ala-D-Ala) terminus of the pentapeptide, thereby blocking the subsequent transglycosylation and transpeptidation steps.

Vancomycin resistance is achieved by modifying the pentapeptide ending in D-Ala-D-Ala to one ending in a different amino acid such as D-Ala-D-lactate or D-Ala-D-serine (Bugg et al. 1991; Billot-Klein et al. 1994).

Vancomycin resistance in enterococci is the result of either intrinsic or acquired genes (Rice et al. 1995). The different known genotypes of vancomycin resistance include vanA (main reservoir: E. faecium, also found in E. faecalis and other species), vanB (E. faecium, E. faecalis) and vanC (E. gallinarum, E. casseliflavus) (Arthur and Courvalin 1993). The vanA type of resistance is clinically very important because enterococci possessing this genotype exhibit a high level of resistance to teicoplanin (MIC: 16 - 512 µg/ml) in addition to vancomycin (MIC: 64 - 1000 µg/ml) (Klare et al. 2003). The vanA operon can be located on a plasmid or on the chromosome as a transferable element (usually Tn1546 or a member of Tn3 family) (Arthur et al. 1993). Approximately sixty percent of the vancomycin resistant enterococci (VRE) isolated in the United States possess the vanA genotype (Clark et al. 1993; Deshpande et al. 2007). Apart from resistance to multiple antibiotics, there is a possibility of gene transfer from VREs to non-resistant strains of enterococci and other organisms (such as Staphylococcus aureus) (Noble et al. 1992; Rotun et al. 1999; Smith et al. 1999). Vancomycin resistant Staphylococcus aureus (VRSA) strains studied to date have been shown to possess the vanA gene on a Tn1546-like element (Weigel et al. 2003; Clark et al. 2005).
vanB is the second most clinically important genotype, conferring moderate to high level resistance to vancomycin (MIC: 4 - 1000 µg/ml) but not teicoplanin. Similar to vanA, the vanB operon can be plasmid-borne or chromosomally encoded as a transferable element (Tn1547) (Quintiliani et al. 1993; Evers et al. 1994). vanC is the only intrinsic, chromosomally encoded low-level type of resistance (MIC: 2 - 32 µg/ml), which can occur as either vanC1 or vanC2/3 operons (Klare et al. 2003). The vanC genotype is only found in certain species such as *E. gallinarum* and *E. casseliflavus* that are generally non-pathogenic. Other lesser-known genotypes of vancomycin resistance include the vanD, vanE and vanG types that are suspected to be chromosomally encoded and non-transferable (Cetinkaya et al. 2000). The emergence of multi-drug resistant (MDR) enterococci in clinical settings has amplified the importance of these organisms as nosocomial pathogens. Genetic exchange can lead to the transfer of genes conferring resistance to a wide range of antibiotics such as aminoglycosides, macrolides, streptogramins, chloramphenicol and vancomycin.

**Emergence of VRE**

Commercial production of vancomycin derived from the actinomycete *Amycolatopsis orientalis* began in the late 1950s (McCormick 1956). It was initially used for the treatment of all staphylococcal infections but by the mid-1970s it was the drug of choice for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Sande and Johnson 1975). The emergence of vancomycin resistant enterococci (VRE) in the U.S. and Europe are believed to be the result of different selective pressures. The first clinical incidence of infections caused by VRE were reported from the UK and France in 1986 (Leclercq et al. 1988; Uttley et al. 1989). The use of the
glycopeptide avoparcin as a growth promoter in animal feed is suspected to be the primary cause of the emergence of VRE in Europe (McDonald et al. 1997). Several studies have shown that the selective pressure exerted by avoparcin results in the development of resistance to vancomycin in enterococcal strains (Aarestrup 1995; Bager et al. 1997). When the association between the use of avoparcin as feed additive and the growing incidence of VRE was recognized, the practice of using avoparcin in animal feed was discontinued by the European Union in 1997 (Commission Directive 97/6 EC). After the ban, the prevalence of VRE in the fecal flora of food animals and healthy humans has decreased markedly (Bager et al. 1999; Klare et al. 1999; Pantosti et al. 1999; Hammerum et al. 2007).

In 1987, one year after the emergence of VRE in Europe, clinical E. faecalis isolates possessing the vanB gene were isolated in a hospital in St. Louis, MO (Uttley et al. 1988). Use of vancomycin to treat infections in US hospitals had increased from approximately 2000 kg in 1984 to 10,000 kg in 1996 (Kirst et al. 1998). The increase in the use of vancomycin to treat infections caused by Clostridium difficile and MRSA probably provided the selective pressure for emergence of vancomycin resistant Enterococcus strains (Tenover 2001). According to the National Nosocomial Infections Surveillance System, the percentage of all nosocomial enterococcal isolates resistant to vancomycin increased from 0.3% to 7.9% between 1989 and 1993 (NNIS 1994). By 1999, the percentage of VRE isolates went up to 25.9% (NNIS 2001).

In the 1990s, E. faecalis and E. faecium were the most frequently isolated enterococcal species from clinical samples worldwide (Mutnick et al. 2003). E. faecalis was more commonly recovered in comparison to E. faecium at a ratio of 5:1. Recent years have
seen a reversal of this trend, as recovery of *E. faecium* isolates has been tenfold higher than that of *E. faecalis* (Deshpande *et al.* 2007; Top *et al.* 2007). The increase in the prevalence of vancomycin resistant *E. faecium* (VREF) in clinical samples is attributed to the worldwide dissemination of a highly virulent, hospital-adapted cluster designated clonal complex (CC)-17 (Klare *et al.* 2005; Treitman *et al.* 2005; Willems *et al.* 2005; Leavis *et al.* 2006; Top *et al.* 2008; Valdezate *et al.* 2009). Emergence of this pathogenic lineage of VREF possibly resulted from acquisition of antibiotic resistance genes, virulence factors and pathogenicity islands through horizontal gene transfer via plasmids, transposons or chromosomal exchange (Rice *et al.* 1998; Woodford *et al.* 2001).

The CC-17 cluster is characterized by resistance to ampicillin and ciprofloxacin and the presence of a variant *esp* gene (Willems *et al.* 2005; Deshpande *et al.* 2007). Many of these isolates have acquired the variant *esp* gene on a putative pathogenicity island that has been predominantly observed in VREF clones associated with disease and/or epidemics and infrequently found in community VREF isolates (Willems *et al.* 2001; Leavis *et al.* 2004). Genotyping methods such as multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) or multi locus sequence typing (MLST) have been used for epidemiological surveillance of this cluster and databases of patterns exist for comparison of these patterns worldwide (Klare *et al.* 2005; Werner *et al.* 2007). Recently, some of these strains have demonstrated resistance to chloramphenicol and linezolid; two antibiotics used in the treatment of infections caused by VREF (Potoski *et al.* 2002; Lautenbach *et al.* 2004). A combination of quinupristin and dalfopristin is the current drug of choice although some studies have documented resistance against these antibiotics as well (Baysallar *et al.* 2004; Lewis *et al.* 2005).
**VRE in the environment**

Antibiotic resistant enterococci have been detected in environmental waters, sewage, agricultural runoff, animal feces and feces of healthy human hosts in parts of Europe (Devriese *et al.* 1996; VanderAuwera *et al.* 1996; Aarestrup *et al.* 1998; Stobberingh *et al.* 1999; Gambarotto *et al.* 2000; Dicuonzo *et al.* 2001; Guardabassi and Dalsgaard 2004). One study found VanA (high level) VRE in turkeys, turkey farmers, turkey slaughterers and suburban residents in the Netherlands (Stobberingh *et al.* 1999). The pulsed-field gel electrophoresis (PFGE) patterns of VRE isolates from human and animal origin were different but in some cases, sequences of the *vanA*-containing transposon in both groups were similar, suggesting that the *vanA* gene is transferable from animal to human associated enterococci or that some VRE strains can colonize animal and human gastrointestinal tracts. VRE exhibiting the VanA phenotype were isolated from seawater, nonagricultural soils and blue mussels in Denmark (Guardabassi and Dalsgaard 2004). High-level VRE were readily isolated from the non-hospital associated community in European countries including the Netherlands, UK, Germany and Belgium (Aarestrup and Wegener 1999; Wegener *et al.* 1999). The high frequency of VRE isolation from the general community indicates that consumption of food products harboring VRE could be a mechanism for VRE dissemination (Bates *et al.* 1994; Jensen *et al.* 1999). VRE have been isolated from pork samples, minced beef and poultry products in Europe (Klein *et al.* 1998; van den Braak *et al.* 1998). Comparison of Tn1546-like elements of enterococci isolated from hospital patients and animal feces demonstrated high similarity, indicating the possibility of horizontal gene transfer between isolates from different origins and/or a common reservoir of resistance genes (Jensen *et al.* 1998; Woodford *et al.* 1998).
Avoparcin was never approved for use in animal feed in the US and high-level VRE are not commonly isolated from environmental waters or non-hospital related sources here. Low level VanC VRE have been isolated from chicken (Harwood et al. 2001) and horse (Thal et al. 1995) fecal samples and freshly slaughtered chickens and turkeys (Coque et al. 1996) A recent study found vanA and vanB VRE in marine waters from Washington and California (Roberts et al. 2009). In the US, high-level VRE are most commonly isolated from clinical sources (Harwood et al. 2001; Harwood et al. 2004; Treitman et al. 2005).

**Nosocomial VRE**

Vancomycin resistant enterococci (VRE), particularly *Enterococcus faecalis* and *Enterococcus faecium*, are notorious for causing nosocomial bacteremia (Noskin et al. 1995; Stosor et al. 1998), endocarditis (Megran 1992) and urinary tract infections (Gross et al. 1976). According to the Centers for Disease Control (CDC), in 2004, one of every three infections in hospital intensive care units were caused by VRE (Cardo et al. 2004). VRE colonization of patients occurs due to prolonged antibiotic usage or exposure to other infected patients (Calfee et al. 2003). Such patients can themselves be at risk or may act as a reservoir for the transmission of VRE to other patients and healthcare workers (Crossley 2001; Duckro et al. 2005). Immunocompromised, geriatric and organ transplant patients have a higher risk of developing VRE infections than other patients (Bonomo 2000). The emergence of multi-drug resistant (MDR) enterococci in clinical settings has amplified the importance of these organisms as nosocomial pathogens.
Research goals

Enterococci isolated from water, sediments and vegetation of a lake (Lake Carroll), a river (Hillsborough River) and an estuary (Ben T. Davis beach, Tampa Bay) in Florida during one sampling event in summer were typed using the HFERP method and their 16S rRNA genes were sequenced. This process was repeated for one sampling event in the winter season. Dendrograms illustrating the relatedness of the isolates by BOX-PCR and by 16S rRNA sequencing were generated. The purpose of this portion of the study was to compare the ability of BOX-PCR to determine genetic relatedness with that of the “gold standard” method, 16S rRNA gene sequencing. BOX-PCR typing is a high throughput and cost-effective technique as compared to sequencing analysis for processing a large number of isolates. If the typing results are comparable to the results obtained by sequencing, studies involving greater sampling effort can rely on BOX-PCR typing to produce reliable estimates of the presence of specific Enterococcus species and the population diversity of the enterococci.

Hypothesis: Relationships projected by the genotypic BOX-PCR dendrograms will be similar to those obtained by phylogenetic analysis.

Survival studies have demonstrated increased persistence of enterococci in sediments as compared to environmental waters, indicating that sediments may play a role in protecting the organisms from stressors such as elevated temperatures and ultraviolet radiation from the sun (Sherer et al. 1992; Howell et al. 1996; Anderson et al. 2005). Vegetation may possibly play a similar role in providing protection and acting as a reservoir for these organisms. This study aims to investigate the incidence of VRE in environmental matrices with particular emphasis on the clinically important VanA and
VanB phenotypes. Water, sediment and vegetation samples from two fresh water sites (Lake Carroll and Hillsborough River) and one estuarine site (Ben T Davis beach) and wastewater samples from a treatment plant, septic tanks and a hospital sewer line were subjected to VRE detection and identification. Vancomycin resistance was determined using the agar dilution method by inoculating known concentrations of enterococcal isolates on vancomycin-amended media. These results were supplemented by molecular methods such as the detection of vanA, vanB, vanC1 and vanC2/3 genes using PCR.

Hypothesis: Enterococcus spp. that are resistant to high levels of vancomycin (VanA and VanB phenotype) will be isolated from hospital wastewater. The majority of the enterococci isolated from environmental samples and residential wastewater will prove susceptible to vancomycin.

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COMPARISON OF GENOTYPIC AND PHYLOGENETIC RELATIONSHIPS OF ENVIRONMENTAL ENTEROCOCCUS ISOLATES BY BOX-PCR TYPING AND 16S rRNA SEQUENCING

Introduction

Enterococci are facultatively anaerobic, gram-positive, catalase-negative cocci that are commonly found in the gastrointestinal (GI) tract of mammals and birds. Members of the genus Enterococcus are also readily isolated from soil, surface waters, sediments and vegetation associated with surface waters, and sometimes food (Fujioka 1999; Bordalo et al. 2002; Giraffa 2003; Anderson et al. 2005). Enterococci are used as regulatory tools to assess water quality in fresh and saline waters (U.S. Environmental Protection Agency 1986). Some Enterococcus species possess virulence factors and antibiotic resistance genes and are capable of causing disease (Shankar et al. 2001; Teng et al. 2002; Rice et al. 2003). Vancomycin resistant enterococci (VRE) are important nosocomial pathogens that have been isolated from approximately 30% of the patients in intensive care units in US hospitals (NNIS 2004; Rice et al. 2004).

Accurate identification and classification of the different species belonging to the genus Enterococcus is important for both environmental and clinical studies. Phenotypic methods used to identify Enterococcus species are not very discriminatory or accurate due to the phenotypic similarity of certain species such as E. gallinarum and E. casseliflavus, E. cecorum and E. columbae, and E. hirae and E. durans (Devriese et al. 1993). Therefore, DNA-based analyses such as genotyping, genetic sequencing and targeting particular genes with specific probes and primers are also employed for the

Sequencing of the 16S rRNA gene is considered the “gold standard” for microbial identification (Weisburg et al. 1991; Claridge 2004; Harmsen and Karch 2004). The 16S rRNA gene is highly conserved within species and among different species belonging to the same genus (Woese 1987). Genotyping methods are high throughput and cost-effective as compared to sequencing analysis for processing a large number of isolates. Typing of bacterial isolates generates a unique “molecular fingerprint” for each isolate that can be used for species and strain differentiation. Environmental studies employ genotyping methods for determination of enterococcal diversity and prevalence in different habitats (Seurinck et al. 2003; Anderson et al. 2005; Brownell et al. 2007; Hassan et al. 2007; Pangallo et al. 2008). In clinical studies, genotyping facilitates identification of variant strains and epidemiological tracking of virulent enterococci (Malathum et al. 1998; Dicuonzo et al. 2001; Coque et al. 2005; Werner et al. 2007).

BOX-PCR is a genotyping method that amplifies the DNA sequences between highly conserved repetitive sequences called BOX elements (Martin et al. 1992). BOX elements are comprised of boxA, boxB and boxC subunits, which are 59, 45 and 50 base pairs long, respectively and have very low sequence similarity to one another. Among the three subunits, the boxA subunit appears to be the most conserved in different bacterial species (Koeuth et al. 1995). The BOXA2R primer sequence is 22 bp long and was originally derived from the boxA subunit of Streptococcus pneumoniae (Martin et al. 1992; Koeuth et al. 1995). When BOXA2R sequences in the genome of a bacterial species are targeted in PCR, it results in differently sized amplicons of the DNA sequences between the
interspersed repeats. Separation of these amplicons using gel electrophoresis leads to the development of species- or strain- specific fingerprint patterns.

Diverse BOX-PCR patterns are generated due to distinct differences in the genome organization of bacterial species. Better resolution of inter- and intra-species differences can be achieved by BOX-PCR typing than 16S rRNA sequencing since BOX-PCR targets DNA sequences in the entire genome while 16S rRNA sequencing targets a small portion (~1500 bp) of the genome. The evolutionary conservation of BOX elements (similar to that of the 16S rRNA sequences) enables the comparison of genotypic relatedness of bacterial species with their phylogenetic relationships.

Several studies have used BOX-PCR typing in environmental studies to determine genotypic relationships of enterococcal isolates (Brownell et al. 2007; Hassan et al. 2007). These studies include dendrograms that display population similarities based solely on BOX-PCR genotyping. However, no study has demonstrated that the BOX-PCR patterns of various strains of a particular enterococcal species are more similar than strains from different species. Supplementing genotype studies with phylogenetic analysis will provide additional comparative information about the relationships among species and strains of enterococci.

The purpose of this study was to compare the ability of BOX-PCR to determine the genetic relatedness of *Enterococcus* species and strains with that of the “gold standard” 16S rRNA gene sequencing. To this aim, enterococci were isolated from different matrices (water, sediments and vegetation) of two freshwater sites and one estuarine site in Florida during two separate sampling events in an effort to maximize sampled strain diversity. These isolates were typed using BOX-PCR and their 16S rRNA genes were
sequenced. We hypothesized that the relationships projected by the genotypic BOX-PCR dendrograms will be similar to those obtained by phylogenetic analysis.

Materials and methods

Sample collection and processing

Water, sediment and vegetation samples were collected during two separate sampling events from Lake Carroll, Hillsborough River and Ben T Davis beach (Tampa Bay). A list of isolates used in this study according to site and isolation matrix is compiled in Table 3. Samples were collected in sterile bottles, maintained at 4°C and processed within 4 hours of collection. Sediment and vegetation samples were diluted 1:10 with phosphate buffered dilution water (0.0425 g · L⁻¹ KH₂PO₄ and 0.4055 g · L⁻¹ MgCl₂; pH 7.2) (American Public Health Association (APHA) 1998) and particle-associated bacteria were dislodged using sonication (Anderson et al. 2005). Microorganisms in water (10ml, 100ml), sediment (2ml, 20ml) and vegetation (2ml, 20ml) samples were concentrated by membrane filtration through 0.45 µm pore size filters and the filters were incubated on mEI agar at 41°C for 18-22 hours (U. S. Environmental Protection Agency 1997). Individual colonies with a blue halo (presumptive enterococci) were picked using sterile toothpicks and inoculated into Enterococcosel broth (EB) in 96-well microtitre plates. After incubation at 37°C for approximately 22 hours, glycerol was added to each well and the EB plate was frozen at -80°C until cultures were reanimated. Individual isolates grown in EB were streaked on TSA for further isolation and incubated overnight at 37°C. Individual colonies were picked, inoculated in 2ml of BHI and grown overnight at 37°C. DNA was extracted from the broth cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) as per manufacturer’s instructions.
**Table 3.** Isolates used in this study listed according to site (Lake Carroll, Hillsborough River and Ben T. Davis beach) and environmental matrix (water, sediment and vegetation).

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Site</th>
<th>Source (matrix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC12W08</td>
<td>Lake Carroll</td>
<td>water</td>
</tr>
<tr>
<td>LC17W08</td>
<td>Lake Carroll</td>
<td>water</td>
</tr>
<tr>
<td>LC1W08</td>
<td>Lake Carroll</td>
<td>water</td>
</tr>
<tr>
<td>LC3W08</td>
<td>Lake Carroll</td>
<td>water</td>
</tr>
<tr>
<td>LC15W07</td>
<td>Lake Carroll</td>
<td>water</td>
</tr>
<tr>
<td>LC28S07</td>
<td>Lake Carroll</td>
<td>sediment</td>
</tr>
<tr>
<td>LC1S07</td>
<td>Lake Carroll</td>
<td>sediment</td>
</tr>
<tr>
<td>LC11S07</td>
<td>Lake Carroll</td>
<td>sediment</td>
</tr>
<tr>
<td>LC24S08</td>
<td>Lake Carroll</td>
<td>sediment</td>
</tr>
<tr>
<td>LC8S07</td>
<td>Lake Carroll</td>
<td>sediment</td>
</tr>
<tr>
<td>LC8V08</td>
<td>Lake Carroll</td>
<td>vegetation</td>
</tr>
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</tr>
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<td>water</td>
</tr>
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<td>Hillsborough River</td>
<td>water</td>
</tr>
<tr>
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<td>Hillsborough River</td>
<td>water</td>
</tr>
<tr>
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</tr>
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<td>water</td>
</tr>
<tr>
<td>HR11W07</td>
<td>Hillsborough River</td>
<td>water</td>
</tr>
<tr>
<td>HR3W07</td>
<td>Hillsborough River</td>
<td>water</td>
</tr>
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<td>HR5W07</td>
<td>Hillsborough River</td>
<td>water</td>
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<td>HR8W07</td>
<td>Hillsborough River</td>
<td>water</td>
</tr>
<tr>
<td>HR31S07</td>
<td>Hillsborough River</td>
<td>sediment</td>
</tr>
<tr>
<td>HR26S08</td>
<td>Hillsborough River</td>
<td>sediment</td>
</tr>
<tr>
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<td>Hillsborough River</td>
<td>vegetation</td>
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<tr>
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<td>Hillsborough River</td>
<td>vegetation</td>
</tr>
<tr>
<td>HR27V08</td>
<td>Hillsborough River</td>
<td>vegetation</td>
</tr>
<tr>
<td>HR12V07</td>
<td>Hillsborough River</td>
<td>vegetation</td>
</tr>
</tbody>
</table>
Isolate ID | Site                      | Source (matrix)
--- | -------------------------- |------------------------
BD1W07   | Ben T Davis beach         | water                  
BD5W07   | Ben T Davis beach         | water                  
BD8W08   | Ben T Davis beach         | water                  
BD13W08  | Ben T Davis beach         | water                  
BD17W07  | Ben T Davis beach         | water                  
BD20W08  | Ben T Davis beach         | water                  
BD11S08  | Ben T Davis beach         | sediment               
BD31S07  | Ben T Davis beach         | sediment               
BD8S08   | Ben T Davis beach         | sediment               
BD28S07  | Ben T Davis beach         | sediment               
BD5S07   | Ben T Davis beach         | sediment               
BD9S08   | Ben T Davis beach         | sediment               
BD29S08  | Ben T Davis beach         | sediment               
BD25S07  | Ben T Davis beach         | sediment               
BD26V08  | Ben T Davis beach         | vegetation             
BD22V08  | Ben T Davis beach         | vegetation             
BD1V08   | Ben T Davis beach         | vegetation             
BD21V08  | Ben T Davis beach         | vegetation             
BD11V08  | Ben T Davis beach         | vegetation             
BD9V08   | Ben T Davis beach         | vegetation             
BD19V08  | Ben T Davis beach         | vegetation             

*Sequencing the 16S rRNA gene*

DNA extracted from individual isolates was amplified using the bacterial universal primers 8f (5'–AGA GTT TGA TCM TGG CTC AG-3’) and 1492r (5’-GGT TAC CTT GGT TAC CTT T3’) (Lane 1991). The PCR master mix was prepared using 13.5µl of Jumpstart ReadyMix Taq (Sigma, St. Louis, Missouri), 8.5 µl sterile water, 1µl of each primer (10 µM) and 1µl of extracted DNA (5 to 15 µg ml⁻¹ adding up to a total volume of 25µl). PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 10 min. PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen, Valencia, CA), and were shipped to Macrogen Corp. (Rockville, MD). Each sample was sequenced in duplicate
using the forward primer 8f and approximately 900 bp of clean sequences were obtained. Sequences were assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI) and analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their identities. Phylogenetic dendrograms were created using the neighbor-joining method and the bootstrap test was carried out with 500 replications (MEGA 4.0, Tempe, AZ).

**BOX-PCR genotyping of enterococci**

*Enterococcus* isolates were typed using the horizontal, fluorophore-enhanced, repetitive extragenic palindromic-PCR (HFERP) technique (Johnson *et al.* 2004). Working primer stock was prepared by mixing 0.09 µg of unlabeled BOX A2R primer (Malathum *et al.* 1998) (0.68 µg µl⁻¹) per µl and 0.03 µg of 6-FAM (fluorescein) labeled BOX A2R primer (0.74 µg µl⁻¹) per µl. The PCR master mix was prepared using 11.6 µl of sterile water, 5x Gitschier buffer (Kogan *et al.* 1987), 2.5µl of 10% DMSO, 1.5 µl BOX A2R working primer, 0.4µl of 2% BSA, 1mM dNTP mixture, 1µl of Taq DNA polymerase and 1µl of extracted DNA, all adding up to a total volume of 25µl. The following PCR conditions were used: an initial denaturation at 95°C for 7 min, followed by 35 cycles of 90°C for 30 s, 40°C for 60 s, 65°C for 8 min. and a final extension at 65°C for 16 min. *Enterococcus faecium* C68 was used as the control strain in PCR and loaded onto individual gels to determine inter gel variability.

A ladder plus non-migrating loading dye mixture was prepared by mixing 5 µl of Genescan-2500 ROX internal lane standard (Applied Biosystems, Foster City, CA) and 20 µl dye (150 mg Ficoll 400 per ml, and 25 mg blue dextran per ml). 12.5 µl of individual PCR products was mixed with 3.3 µl of the ROX-dye mixture, loaded in a 1.5% agarose gel and electrophoresced at 90V for 4 hours. Gel images were scanned
using a Typhoon 8600 Variable Mode Imager (GE Healthcare). BOX-PCR gel images were subsequently imported into Bionumerics (Applied Maths, Belgium) and analyzed using the Pearson similarity coefficient by constructing dendrograms using the unweighted pair group method with arithmetic mean (UPGMA) (optimization 1.0%, tolerance 0.5%). BOX-PCR patterns were also compared visually to confirm results.

Known Enterococcus species, including Enterococcus faecalis ATCC 19433, Enterococcus faecalis ATCC 29212, Enterococcus faecalis ATCC 49383, Enterococcus faecalis ATCC 700802, Enterococcus faecium C68, Enterococcus faecium ATCC 49224 and Enterococcus casseliflavus ATCC 700327 were also sequenced and typed for comparison purposes.

Results and Discussion

BOX-PCR dendrograms were in good agreement (77%) with the 16S rRNA phylogenetic tree as hypothesized. 16S rRNA sequencing was incapable of differentiating among the known strains of E. faecalis and also among the known strains of E. faecium (Figure 10); however it could discriminate between the two species. The BOXA2R patterns of three known strains of E. faecalis (ATCC 19433, 29212 and 700802) were 95% similar and identical when examined by eye (Figure 11). Minor differences (90%) were observed in the BOXA2R patterns of the two known strains of E. faecium. Since BOXA2R patterns of the control strain E. faecium C68 from different gels were 89% similar, patterns with similarity values greater than 89% were considered indistinguishable.

BOX-PCR typing enabled better differentiation of strains within individual environmental Enterococcus species as compared to 16S rRNA gene sequencing (Figures 10 and 11). This can be explained in part by the difference in methodology between 16S
rRNA gene sequencing and BOX-PCR typing. The 16S rRNA gene sequence (~1500 bp) has both highly conserved and variable regions and is widely used for determination of taxonomical evolution (Woese 1987). However, the resolution capacity of the 16S rRNA gene is limited when identifying closely related organisms (Fox et al. 1992; Stackebrandt and Goebel 1994). 16S rRNA gene sequencing concentrates on a much smaller portion of the genome as compared to BOX-PCR. BOX-PCR typing targets sequences located between interspersed repetitive DNA elements resulting in amplification products of different sizes that generate a unique genomic fingerprint of individual bacterial strains. The number and location of bands in the fingerprint depend upon the size of the genome and the number of primer binding sites. Variation in genome sizes among different strains of a particular species leads to generation of multiple strain-specific fingerprint patterns. For example, Oana et al mapped the genomes of four strains of *E. faecium* and reported genome sizes that varied from 2550 to 2995 kb (Oana et al. 2002). Therefore, BOX-PCR typing can differentiate between different strains of the same species better than 16S rRNA sequencing.

The 16S rRNA gene sequences of *E. faecium* and *E. mundtii* were approximately 98% identical and formed a single cluster in the phylogenetic tree. In contrast, the BOX-PCR patterns of *E. faecium* and *E. mundtii* demonstrated less than 60% similarity. This demonstrates the ability of BOX-PCR genotyping to differentiate between closely related species of enterococci. In a similar study, Rademaker et al compared genotypic relationships of *Xanthomonas* species and strains by BOX-PCR with DNA-DNA hybridization studies and observed a high correlation between the two methods (Rademaker et al. 2000). This observation is in agreement with the high correlation
between the genotypic and phylogenetic relationships of Enterococcus spp. observed in our study. In contrast, other studies have found poor correlation between genotypic and phylogenetic methods used to project species/strain relationships (Tacao et al. 2005; Binde et al. 2009).

The 16S rRNA gene sequence of an environmental strain of Lactococcus garvieae served as an outgroup while constructing the phylogenetic tree (Figure 10). The 16S rRNA sequence of Lactococcus garvieae is 11.4 to 11.8% different from that of Enterococcus species (Patel et al. 1998). The BOX-PCR pattern of Lactococcus garvieae clustered together with other species of enterococci and did not form an outgroup in the BOX-PCR dendrogram (Figure 11). This indicates that certain closely related genera might not be as distinguishable using BOX-PCR typing as they are by 16S rRNA sequencing.

Of the 61 isolates sequenced in this study, only one isolate was identified as a non-Enterococcus. This finding demonstrates the specificity of mEI agar, the medium used for isolation of enterococci from surface waters. In other studies, mEI agar was found to be less specific since organisms belonging to other genera were isolated from environmental samples (Moore et al. 2006), biosolids (Viau and Peccia 2009) and clinical samples (Goh et al. 2000) along with Enterococcus spp. According to the US Environmental Protection Agency (USEPA), the false-positive rate for isolation of non-enterococci from environmental samples on mEI agar is 6% (U. S. Environmental Protection Agency 1997; Messer and Dufour 1998). In comparison, we observed a very low false-positive rate (1.6%) for isolation of non-enterococci from environmental matrices in our study.
A BLAST query was performed on the entire genomes of *E. faecium* C68 (ACJQ00000000) and *E. faecalis* ATCC 700802 (AE016830) with the BOXA2R primer (5’- ACG TGG TTT GAA GAG ATT TTC G -3’) as the target sequence. The BOXA2R primer sequence appeared 33 times within the genome of *E. faecium* C68 and 36 times within the genome of *E. faecalis* ATCC 700802. In the first documentation of BOX elements in *Streptococcus pneumoniae*, the authors noted the presence of 25 BOX sequences in the genome of the organism. Further analysis of the distance between the BOXA2R sequence segments might be useful in predicting the length of amplicons produced during BOX-PCR typing.

Genotypic and phylogenetic relationships between *Enterococcus* species and strains were compared using BOX-PCR typing and 16S rRNA sequencing, respectively. Although BOX-PCR genotyping was found to be more discriminatory at the strain level than 16S rRNA sequencing, the incorrect grouping of some strains with strains belonging to a different species instead of its own species group raises doubts about the ability of the method to correctly project relatedness of all *Enterococcus* strains. While studies relying solely on BOX-PCR typing should exercise caution while interpreting phylogenetic relationships projected by BOX-PCR dendrograms, the method does provide a useful approximation of phylogeny. BOX-PCR typing may be an excellent tool for investigating strain diversity but this method should not be employed exclusively for species identification and association.
Figure 10. Phylogenetic tree constructed using the neighbor-joining algorithm to evaluate the distance between 16S rRNA gene sequences of environmental enterococci.
Figure 10. continued
Figure 10. continued
Figure 11. Dendrogram demonstrating the similarity of BOX-PCR patterns of Enterococcus species isolated from environmental matrices.
Figure 11. continued
References


Vancomycin resistant enterococci (VRE) are important nosocomial pathogens whose prevalence in environmental waters is infrequently investigated. Environmental and wastewater samples from Florida (USA) were screened for VRE. Low-level VRE (< 32 µg ml⁻¹) were a proportionally greater fraction of enterococci at freshwater vs. estuarine sites, while high-level VRE (≥ 32 µg ml⁻¹) were not detected. Between 20% and 61% of the total enterococci isolated from surface water sites were low-level VRE. Genotype \(\text{vanC2/3 Enterococcus casseliflavus-flavescens}\) dominated environmental VRE populations while \(\text{vanC1 E. gallinarum}\) was dominant in residential wastewater. High-level VRE (\(\text{vanA E. faecium}\)) were only isolated from hospital sewer line samples, and all displayed intermediate resistance to ampicillin and ciprofloxacin but were sensitive to chloramphenicol and rifampin. They also had indistinguishable BOX-PCR genotypes. Twenty percent of the nosocomial isolates possessed the virulence-associated \(\text{esp}\) gene
variant that is unique to *E. faecium*. Two *esp*-positive *E. faecium* strains were isolated from surface waters, but were vancomycin-sensitive. Infections caused by low-level VRE can be difficult to treat as they sometimes demonstrate vancomycin susceptibility in vitro while being resistant in vivo. While the relative rareness of high-level VRE in sources tested other than hospital wastewater is encouraging, the high proportion of low-level VRE isolated from surface waters and the preponderance of high-level VRE in hospital wastewater are cause for concern and should be the subject of better surveillance.

**Introduction**

Enterococci can cause blood stream infections, endocarditis, and urinary tract infections (Gross *et al.* 1976; Megran 1992; Pfaller *et al.* 1998). Vancomycin resistant enterococci (VRE) have been isolated from approximately 30% of the patients in intensive care units in US hospitals (NNIS 2004; Rice *et al.* 2004). VRE have been detected in environmental waters, sewage, agricultural runoff, animal feces and feces of healthy human hosts in parts of Europe (Devriese *et al.* 1996; VanderAuwera *et al.* 1996; Aarestrup *et al.* 1998; Stobberin *et al.* 1999; Gambarotto *et al.* 2000; Dicuonzo *et al.* 2001; Guardabassi and Dalsgaard 2004). This widespread prevalence in Europe is mainly attributed to the practice of using the glycopeptide avoparcin in animal feeds for growth promotion (McDonald *et al.* 1997). In contrast, high-level VRE have seldom been reported in environmental waters or non-hospital related sources in the US (Coque *et al.* 1996; Harwood *et al.* 2001), although high-level VRE were isolated from marine waters in Washington and California (Roberts *et al.* 2009). Harwood et al isolated *vanA* VRE from hospital wastewater, whereas chicken feces and residential wastewater isolates exhibited the low level *vanC* genotype (Harwood *et al.* 2001).
Vancomycin resistance in enterococci is attributed to the possession of gene clusters designated vanA (E. faecium, E. faecalis), vanB (E. faecium, E. faecalis) and vanC (E. gallinarum, E. casseliflavus) (Cetinkaya et al. 2000). vanA and vanB mediated resistance can be plasmid-borne or chromosomally encoded as a transferable element (Arthur et al. 1993; Rice et al. 1998). vanA confers high-level resistance to vancomycin (MIC: 64 - >1000 µg ml\(^{-1}\)) and teicoplanin (MIC: 16 - 512 µg ml\(^{-1}\)) while vanB confers moderate to high-level resistance to vancomycin only (MIC: 4 - 1000 µg ml\(^{-1}\)). vanC is an intrinsic, chromosomally encoded, low-level type of resistance (MIC: 2 - 32 µg ml\(^{-1}\)), found in certain species that are generally non-pathogenic, but that occasionally cause disease (Cetinkaya et al. 2000). Other low-level VRE genotypes include vanD, vanE and vanG (Fines et al. 1999; Cetinkaya et al. 2000).

*E. faecalis* and *E. faecium* also possess virulence factors such as adhesins, cytolysins, gelatinase, and serine protease. The esp gene, which encodes the enterococcal surface protein, may facilitate colonization of the urinary tract (Shankar et al. 2001) and biofilm formation (Toledo-Arana et al. 2001; Heikens et al. 2007). esp was initially described in clinical *E. faecalis* isolates (Shankar et al. 1999). An esp variant was later discovered in *E. faecium* isolates from nosocomial infections (Eaton and Gasson 2001; Willems et al. 2001) and is now used as a marker of human fecal pollution in environmental waters (Scott et al. 2005). Several PCR methods have been developed for the detection of the *E. faecium* esp gene in microbial source tracking (MST) (McDonald et al. 2006; McQuaig et al. 2006; Brownell et al. 2007; Whitman et al. 2007; Ahmed et al. 2008a; Ahmed et al. 2008b) and clinical studies (Eaton and Gasson 2002; Leavis et al. 2003; Coque et al. 2005).
Recently a hospital-adapted sub-population of vancomycin resistant *E. faecium* (VREF) has been implicated in nosocomial outbreaks in five continents, including North America (Top *et al.* 2007; Valdezate *et al.* 2009). Multi-locus sequence typing (MLST) revealed a specific genetic lineage of VREF designated clonal complex-17 (CC-17), which is also characterized by intermediate to high resistance to ampicillin and ciprofloxacin (Leavis *et al.* 2006b; Deshpande *et al.* 2007; Top *et al.* 2007). Many of these isolates possess the *E. faecium* variant *esp* gene on a putative pathogenicity island (Willems *et al.* 2005; Leavis *et al.* 2006a; Leavis *et al.* 2006b; Top *et al.* 2008). Previous studies have documented the co-occurrence of the *esp* gene with the hyaluronidase (*hyl*) gene in VREF strains (Rice *et al.* 2003; Vankercikhoven *et al.* 2004; Klare *et al.* 2005). The *hyl* gene is a virulence-associated gene that potentially contributes to invasion of the nasopharynx (Rice *et al.* 2003).

The growing incidence of *vanA* and *vanB* VRE infections in hospitals in the US highlights the need for surveillance of the prevalence of VRE in surface waters, as well as associated matrices such as sediments and vegetation, to determine the overall threat to the community. Survival studies have demonstrated increased persistence of enterococci in sediments as compared to environmental waters, indicating that sediments may play a role in protecting the organisms from stressors such as elevated temperatures and ultraviolet radiation from the sun (Sherer *et al.* 1992; Howell *et al.* 1996; Anderson *et al.* 2005). Vegetation may play a similar role in providing protection and act as a reservoir for these organisms (Byappanahalli *et al.* 2003; Whitman *et al.* 2003), yet the prevalence of antibiotic-resistant bacteria in vegetation and sediment has been infrequently explored (Cordova-Kreylos and Scow 2007; Matyar *et al.* 2008).
Enterococci isolated from environmental water, sediment and vegetation samples as well as residential and hospital wastewater were tested for vancomycin resistance using culture-based methods followed by molecular typing. We hypothesized that *Enterococcus* spp. that are resistant to high levels of vancomycin (*vanA* and *vanB* genotypes) would be isolated from hospital wastewater whereas the majority of the enterococci isolated from environmental samples and residential wastewater will prove susceptible to vancomycin.

**Materials and methods**

*Sample collection and processing*

Water, sediment and vegetation samples were collected from Lake Carroll, Hillsborough River and Ben T Davis beach (Tampa Bay). The Lake Carroll site (28º 2.918’ N, 82º 29.828’ W) is on a small residential lake in West Tampa surrounded completely by suburban housing. The Hillsborough River site (28º 4.260’ N, 82º 22.671’ W) is at the University of South Florida's Riverfront Park, downstream of a substantial amount of protected, undeveloped land. The upper bay site, Ben T Davis Beach (27º 58.141’ N, 82º 34.522’ W), is west of the City of Tampa in Old Tampa Bay. Dominant vegetation species at the freshwater sites were *Alternanthera philoxeroides* (alligator weed), *Egaria densa* (Brazilian waterweed), *Hydrilla verticilata*, *Myriophyllum aquaticum* (parrot feather), and *Vallisneria Americana* (eel grass). The dominant vegetation species at the estuarine site was *Halodule wrightii* (shoal grass). Vegetation coverage at all of these sites ranged from moderate to heavy and sediments were composed of fine quartz sand. Samples were collected in sterile bottles, maintained at 4°C and processed within 4 hours of collection. Sediment and vegetation samples were diluted 1:10 with phosphate buffered dilution water (0.0425 g · L⁻¹ KH₂PO₄ and 0.4055 g · L⁻¹ MgCl₂; pH 7.2)
and particle-associated bacteria were dislodged using sonication (Anderson et al. 2005). Microorganisms in water (1ml, 10ml), sediment (2ml, 20ml) and vegetation (2ml, 20ml) samples were concentrated by membrane filtration through 0.45 µm pore size filters and the filters were incubated on mEI agar at 41ºC for 18-22 hours (U. S. Environmental Protection Agency 1997). Individual colonies with a blue halo (presumptive enterococci) were picked using sterile toothpicks and inoculated into Enterococcusel broth (EB) in 96-well microtitre plates. After incubation at 37ºC for approximately 22 hours, glycerol was added to each well and the EB plate was frozen at -80ºC until cultures were reanimated. Water (100 ml) and sediment (25 ml) samples were also filtered and the filters incubated on mEI agar with vancomycin (6 µg ml⁻¹) (to determine the percentage of VRE from the total enterococci). Water (3 liters) and sediment (25 ml) samples were also filtered and the filters incubated on mEI agar with vancomycin (32 µg ml⁻¹) for isolation of high-level VRE.

Wastewater samples were collected from the Falkenburg Road Advanced Wastewater Treatment Plant in Tampa, FL, from a hospital sewer line and from a septic pump truck (three samples each). Samples were handled as detailed above, but enterococci were isolated as follows. Hospital wastewater samples (1 ml and 100 µl) were filtered and incubated on mEI agar and mEI agar with vancomycin (6 µg ml⁻¹) in triplicate (to determine the percentage of VRE from the total enterococci). Residential and septic pump truck wastewater samples were filtered and filters were incubated on mEI agar (1 ml and 100 µl) and mEI agar with 6 µg ml⁻¹ vancomycin (10 ml and 25 ml) in triplicate.
Vancomycin susceptibility testing

The agar dilution screening method was used to screen for VRE (Swenson et al. 1994). Isolates grown in EB were streaked on trypticase soy agar (TSA) for further isolation and incubated overnight at 37°C. Individual colonies were inoculated in brain heart infusion (BHI) broth and grown overnight at 37°C. The turbidity of the overnight culture was adjusted to match the 0.5 McFarland standard and 10µl of the suspension was spot inoculated on BHI agar with vancomycin (6 µg ml⁻¹), which might potentially discourage the growth of a few low-level vanB and many low-level vanC VRE isolates. Isolates displaying growth were further tested by spot inoculation on BHI agar with 32 µg ml⁻¹ vancomycin. VRE isolates that grew at 6µg ml⁻¹ concentration of vancomycin but did not grow at 32 µg ml⁻¹ were reported as LL-VRE whereas the VRE isolates that grew at 32 µg ml⁻¹ of vancomycin were reported as HL-VRE. These results were reconfirmed by amplifying the vancomycin resistance genes of individual isolates. Vancomycin MICs for 50 randomly selected LL-VRE isolates were determined by both agar dilution and broth microdilution methods (4, 6 and 8 µg ml⁻¹ vancomycin).

DNA was extracted from the overnight broth cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) as per manufacturer’s instructions. Genes conferring vancomycin resistance were targeted in a multiplex PCR (Table 4) using extracted DNA from the individual isolates as template (Dutka-Malen et al. 1995). Positive controls used include Enterococcus faecalis A256 (vanA), Enterococcus faecium C68 (vanB), Enterococcus gallinarum ATCC 49573 (vanC1) and Enterococcus casseliflavus ATCC 700327 (vanC2/3). The vancomycin susceptible strain Enterococcus
faecalis ATCC 29212 was used as a negative control. The presence of products of the correct molecular size was confirmed using gel electrophoresis.

Isolates identified as HL-VRE by multiplex PCR and phenotype on vancomycin-amended media were further tested for susceptibility to ampicillin (12, 16 and 20 µg ml\(^{-1}\)), chloramphenicol (6, 8 and 10 µg ml\(^{-1}\)), ciprofloxacin (2, 4 and 6 µg ml\(^{-1}\)) and rifampin (1 and 2 µg ml\(^{-1}\)) as described in the Clinical and Laboratory Standards Institute guidelines using standard breakpoints (Clinical and Laboratory Standards Institute 2006). The choice of antibiotics was based on those identified as therapeutically important by the SENTRY Antimicrobial Surveillance Program (Deshpande et al. 2007). Isolates were tested in triplicate by both the agar dilution and broth microdilution methods.

Enterococcus faecalis ATCC 29212 was used as a negative control. Vancomycin MICs for HL-VRE isolates were determined by both agar dilution and broth microdilution methods (64, 128, 256 and 512 µg ml\(^{-1}\) vancomycin).

**Sequencing the 16S rRNA gene and vanA gene**

DNA extracted from individual isolates was amplified using the bacterial universal primers 8f and 1492r (Lane 1991) (Table 4). The PCR master mix was prepared using 13.5µl of Jumpstart ReadyMix Taq (Sigma, St. Louis, Missouri), 8.5µl sterile water, 1µl of each primer (10 µM) and 1µl of extracted DNA (5-15 ng µl\(^{-1}\)) adding up to a total volume of 25µl. PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 10 min. PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen, Valencia, CA), and were shipped to Macrogen Corp. (Rockville, MD). Each sample was sequenced in duplicate using the forward primer 8f and approximately 900 bp of clean sequences were
obtained. The vanA gene (~ 640bp) was amplified using previously published primers and PCR conditions (Dutka-Malen et al. 1995) and the PCR amplicons were sequenced in both forward and reverse directions using the vanA primer set. Sequences were assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI) and analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their identities.

Table 4. Primers used in this study.

<table>
<thead>
<tr>
<th>Amplified gene</th>
<th>Product size (bp)</th>
<th>Oligonucleotide sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>732</td>
<td>A1 (5’-GGGAAAAACGACAATTGC-3’) A2 (5’-GTACAATGCCGGCGGTGTA-3’)</td>
<td>Dutka-Malen et al, 1995</td>
</tr>
<tr>
<td>vanB</td>
<td>635</td>
<td>B1 (5’-ATGGGAAGCGGATAGTC-3’) B2 (5’-GATTTCCAGTCTCGACC-3’)</td>
<td>Dutka-Malen et al, 1995</td>
</tr>
<tr>
<td>vanC1</td>
<td>822</td>
<td>C1 (5’-GGTATCAAGGAACCCTC-3’) C2 (5’-CTTCCGACCATGCT-3’)</td>
<td>Dutka-Malen et al, 1995</td>
</tr>
<tr>
<td>vanC2/3</td>
<td>439</td>
<td>D1 (5’-CTCCTACGATTCTCTTTGG-3’) D2 (5’-CGAGCAAGACCTTTAAG-3’)</td>
<td>Dutka-Malen et al, 1995</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>1484</td>
<td>8f (5’-AGAGTTGTTGACTCAG-3’) 1492r (5’-GGTACCTGTACGACTT-3’)</td>
<td>Lane, 1991</td>
</tr>
<tr>
<td>esp</td>
<td>680</td>
<td>F (5’-TATGAAAGCACAATGCTC-3’) R (5’-ACGTGAAAATTCTCC-3’)</td>
<td>Scott et al, 2005</td>
</tr>
<tr>
<td>esp</td>
<td>510</td>
<td>14F (5’-AATGGATTCTTTAGCATCTG-3’) 12R (5’-AATTGAGTTCTTTAGCATCTG-3’)</td>
<td>Leavis et al, 2003</td>
</tr>
<tr>
<td>esp</td>
<td>956</td>
<td>esp11 (5’-TTGCTAGTTGAGCCGACC-3’) esp12 (5’-TTGCTAATGCTGCTTCACGACC-3’)</td>
<td>Leavis et al, 2003</td>
</tr>
<tr>
<td>hyl</td>
<td>661</td>
<td>hyl_Efm F (5’-GAGTAGAGGAATATCTTGGC-3’) hyl_Efm R (5’-AGGCTCCATTCTGT-3’)</td>
<td>Rice et al, 2003</td>
</tr>
<tr>
<td>BOXA2R</td>
<td>variable</td>
<td>5’- ACG TGG TTT GAA GAG ATT TTC G -3’</td>
<td>Malathum et al, 1998</td>
</tr>
</tbody>
</table>
**BOX-PCR genotyping of enterococci**

*Enterococcus* isolates were typed using the horizontal, fluorophore-enhanced, repetitive extragenic palindromic-PCR (HFERP) technique (Johnson et al. 2004). Working primer stock was prepared by mixing 0.09 µg of unlabeled BOX A2R primer (Malathum et al. 1998) (0.68 µg µl⁻¹) per µl and 0.03 µg of 6-FAM (fluorescein) labeled BOX A2R primer (0.74 µg µl⁻¹) per µl. The PCR master mix was prepared using 11.6µl of sterile water, 5x Gitschier buffer (Kogan et al. 1987), 2.5µl of 10% DMSO, 1.5 µl BOX A2R working primer, 0.4µl of 2% BSA, 1mM dNTP mixture, 1µl of Taq DNA polymerase and 1µl of extracted DNA, all adding up to a total volume of 25µl. The following PCR conditions were used: an initial denaturation at 95°C for 7 min, followed by 35 cycles of 90°C for 30 s, 40°C for 60 s, 65°C for 8 min. and a final extension at 65°C for 16 min. *Enterococcus faecium* C68 was used as the control strain in PCR and loaded onto individual gels to determine inter gel variability. A ladder plus non-migrating loading dye mixture was prepared by mixing 5 µl of Genescan-2500 ROX internal lane standard (Applied Biosystems, Foster City, CA) and 20 µl dye (150 mg Ficoll 400 per ml, and 25 mg blue dextran per ml). 12.5 µl of individual PCR products was mixed with 3.3 µl of the ROX-dye mixture, loaded in a 1.5% agarose gel and electrophoresced at 90V for 4 hours. Gel images were scanned using a Typhoon 8600 Variable Mode Imager (GE Healthcare).

*Screening for virulence factors*

Isolates were tested for the presence of the *esp* and *hyl* genes encoding the enterococcal surface protein of *E. faecium* and the hyaluronidase enzyme, respectively. The *esp* gene was amplified using one primer set employed by microbial source tracking (MST) studies and two sets of primers described in clinical studies (Table 4). The primer set used in
MST studies was described as a proposed marker of human fecal pollution (Scott et al. 2005). The primer set 14F and 12R was used to amplify the esp gene as described previously by Leavis et al (Leavis et al. 2003). Isolates negative for the presence of the esp gene were tested using another primer set esp11 and esp12 to ensure accuracy of results (Leavis et al. 2003).

The PCR master mix for amplification of the hyl gene (Table 4) was comprised of 12.5 µl of GoTaq Green, 8.5 µl of sterile water, 1 µl each of 10µM forward and reverse primers (Rice et al. 2003) and 2 µl of template DNA. PCR conditions were as follows: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 30 s and a final extension at 72°C for 5 min. Enterococcus faecium C68 was used as the positive control and Enterococcus faecalis ATCC 29212 was used as the negative control for detection of both virulence factors.

Statistical analysis

BOX-PCR gel images were imported into Bionumerics (Applied Maths, Belgium) and analyzed using the Pearson similarity coefficient in order to construct dendrograms using the unweighted pair group method with arithmetic mean (UPGMA) (optimization 1.0%, tolerance 0.5%). BOX-PCR patterns were also compared visually to confirm results. Differences in the frequency of observation of LL-VRE within each matrix between sites were calculated using the chi-square test (GraphPad InStat, La Jolla, USA).

Results

Vancomycin resistant enterococci isolated from the freshwater sites, Lake Carroll (LC) and Hillsborough River (HR), as well as those isolated from estuarine waters at Ben T
Davis (BTD) beach exhibited the VanC phenotype (henceforth referred to as low-level or LL-VRE) (Tables 5 and 6). The minimum inhibitory concentration (MIC) of vancomycin for fifty randomly selected LL-VRE isolates was 8 µg ml⁻¹. The majority of the VRE strains isolated from environmental matrices at the three sites tested positive for the vanC2/3 gene and were identified as *E. casseliflavus-flavescens* (the two species are indistinguishable by small subunit rRNA sequencing). One isolate from LC sediment and one isolate each from BTD water and sediment tested positive for the vanC1 gene. No VanA or VanB (high-level or HL-VRE) phenotypes were isolated from these sites. HL-VRE were not detected even when larger sample volumes were screened on mEI agar with 32 µg ml⁻¹ vancomycin, an inhibitory concentration for LL-VRE.

**Table 5.** VRE genotypes observed from environmental water and wastewater samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of isolates typed</th>
<th>Genotypes observed</th>
<th>% VRE (total <em>Enterococcus concentration)</em>&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Carroll</td>
<td>187&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vanA</td>
<td>vanB</td>
</tr>
<tr>
<td>Hillsborough River</td>
<td>192&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ben T Davis beach</td>
<td>188&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Residential wastewater</td>
<td>25&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Hospital wastewater</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25</td>
<td>29</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isolated on mEI (no vancomycin) and screened post-isolation  
<sup>b</sup> Pre-screened for vancomycin resistance on mEI + 6 µg ml⁻¹ vancomycin  
<sup>c</sup> 20 isolates from WWTP influent and 5 isolates from a septic pump truck  
<sup>d</sup> CFU ml⁻¹
The proportion of VRE (VRE as a percentage of total enterococci screened) observed in the water, sediment and vegetation samples (hereafter termed matrices) within each surface water site is listed in Table 6. In some cases, i.e. LC water and HR sediment, LL-VRE were by far the majority of total enterococci screened. Significant differences in the proportion of LL-VRE were observed in all site-by-site comparisons. Significant differences were also observed when each matrix was compared across the three sites. VRE proportions in estuarine waters were markedly and significantly lower than freshwater for all three matrices. LL-VRE proportions for each site were highest in either water or sediment samples, but never vegetation (Table 6). In fact, for the HR and BTD sites, the lowest VRE proportions were observed in vegetation samples.

Table 6. Low-level VRE as a percentage of total enterococci in each matrix (water, sediment, vegetation) at each site.

<table>
<thead>
<tr>
<th>Source</th>
<th>Water</th>
<th>Sediment</th>
<th>Vegetation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Carroll</td>
<td>97 (62)</td>
<td>35 (63)</td>
<td>68 (62)</td>
<td>66 (187)</td>
</tr>
<tr>
<td>Hillsborough River</td>
<td>53 (64)</td>
<td>73 (64)</td>
<td>30 (64)</td>
<td>52 (192)</td>
</tr>
<tr>
<td>Ben T Davis beach</td>
<td>25 (64)</td>
<td>47 (60)</td>
<td>3 (64)</td>
<td>24 (188)</td>
</tr>
</tbody>
</table>

Two isolates from a Hillsborough River vegetation sample carried the variant esp gene that is unique to E. faecium and is associated with human feces (Scott et al. 2005; Brownell et al. 2007). The BOXA2R genotypes of these two isolates were 99% similar.
Figure 12. Dendrogram demonstrating the similarity of BOX-PCR patterns of vanA VREF isolated from hospital wastewater, esp- positive isolates are underlined.

and DNA sequencing identified both as *E. faecium*. Both isolates were susceptible to vancomycin and tested negative for *vanA*, *vanB* and *vanC* by PCR.
Approximately 1.6% of the total enterococci (3 x 10^4 CFU ml\(^{-1}\); average of three separate sampling events) isolated from the residential wastewater demonstrated low-level vancomycin resistance and carried the \textit{vanC}1 genotype (Table 5). These LL-VRE were identified as \textit{E. gallinarum} by 16S rRNA gene sequencing. No HL-VRE were isolated from residential wastewater.

Approximately 35% (1.7 x 10^3 CFU ml\(^{-1}\)) of the enterococci (4.9 x 10^3 CFU ml\(^{-1}\); average of three separate sampling events) isolated from the hospital wastewater were vancomycin resistant, including both LL-VRE and HL-VRE (Table 5). Forty-six percent of the 54 VRE tested carried the \textit{vanA} gene and their BOXA2R patterns were 89% similar (Figure 12) while the rest of the isolates demonstrated the \textit{vanC}1 genotype. Since BOXA2R patterns of the control strain \textit{E. faecium} C68 were also 89% similar, the \textit{vanA} VRE BOX patterns were indistinguishable, and may represent clones.

Seven \textit{vanA} VRE isolates chosen at random were sequenced and identified to the species level as \textit{E. faecium} (GenBank Accession #s GQ489017 to GQ489023). The VREF strains were resistant to intermediate levels of ampicillin (MIC > 16 µg ml\(^{-1}\)) and ciprofloxacin (MIC > 4 µg ml\(^{-1}\)) but sensitive to chloramphenicol (MIC = 8 µg ml\(^{-1}\)) and rifampin (MIC < 2 µg ml\(^{-1}\)). The MIC for vancomycin was 512 µg ml\(^{-1}\). The \textit{esp} gene was detected in 20% (5/25) of the VREF isolates, and all three sets of \textit{esp} primers gave the same result. None of the isolates carried the \textit{hyl} gene. The \textit{vanA} gene sequences of \textit{esp} positive and \textit{esp} negative VREF isolates were 100% identical as determined by aligning the sequences on BLAST (GenBank Accession #s GQ489012 to GQ489016).
Discussion

Water, sediment, and vegetation samples were collected from two freshwater and one estuarine site in Florida for this study, which is the first study to evaluate VRE in vegetation samples and to compare VRE proportions in freshwater vs. estuarine waters and across different matrices. In fact, very few studies in the US have attempted to determine the vancomycin susceptibility of environmental enterococci (Harwood et al. 2001; Moore et al. 2008; Roberts et al. 2009). In our study, LL-VRE were readily isolated from modest volumes of environmental water samples (2ml to 100ml) without enrichment or use of vancomycin-amended media for the initial screening. Moore et al assessed *E. faecalis* and *E. faecium* from ocean waters and sewage in Southern California for vancomycin resistance (Moore et al. 2008) by screening on media amended with 16 μg ml\(^{-1}\) vancomycin. Under these conditions all isolates were vancomycin-susceptible; however, this concentration inhibits most LL-VRE. It is therefore not possible to compare the frequency of isolation of environmental enterococci from the California study with the current study.

Another study reported the detection of HL-VRE at public beaches in Washington and California (Roberts et al. 2009). The vast majority of LL-VRE enterococci were also excluded from this study, since bacteria were screened on mE agar supplemented with 18 μg ml\(^{-1}\) vancomycin. HL-VRE were infrequently detected over the eight year study, which is noteworthy because HL-VRE had not previously been detected outside hospital or wastewater settings in the US (Roberts et al. 2009). In contrast, we found no HL-VRE in any environmental water matrix, but a very high proportion of LL-VRE. Our samples were collected over a short time period (6 months), and in light of the findings of Roberts.
et al. and the prevalence of HL-VRE in hospital wastewater, greater efforts toward surveillance of VRE in these waters is appropriate.

The high prevalence of LL-VRE observed in the surface waters in this study is a cause for concern from the public health perspective. LL-VRE can cause serious infections such as endocarditis, bacteremia and meningitis, particularly in immunocompromised patients (Yoshimoto et al. 1999; Kurup et al. 2001; Reid et al. 2001; Dargere et al. 2002). A six-year survey in a hospital in Japan found LL-VRE associated with approximately 12% of all enterococcal bacteremia cases (Koganemaru and Hitomi 2008). Another study found no differences in the severity of illness and mortality rates between E. faecalis bacteremia and LL-VRE bacteremia (de Perio et al. 2006). Treatment of infections caused by intrinsically resistant LL-VRE can be difficult because they sometimes demonstrate vancomycin susceptibility in vitro while being resistant in vivo (Ratanasuwan et al. 1999; Reid et al. 2001). Recently a strain of E. casseliflavus/gallinarum possessing the vanA gene and one possessing both vanA and vanB genes were isolated from beaches in WA and CA (Roberts et al. 2009). Acquisition of high-level vancomycin resistance genes by LL-VRE is of particular concern because phenotypic identification from clinical samples can confound treatment strategy and infection control measures (Dutka-Malen et al. 1994; Yoshikazu 1996; Coombs et al. 1999; Roberts et al. 2009), and because they are so prevalent in the environment.

The proportion of LL-VRE (calculated as percentage of total enterococci) isolated from the environmental matrices was significantly higher than the proportion of LL-VRE isolated from the residential wastewater. Although very few vanC E. gallinarum were isolated from residential wastewater in Tampa eight years prior to this study, the
difference in screening methods does not permit a direct comparison with the proportion of LL-VRE isolated from residential wastewater in the current study (Harwood et al. 2001). The only documented isolation of HL-VRE from community wastewater in the US is the detection of 49 *E. faecium* isolates possessing the *vanA* gene and one possessing the *vanB* gene from a semiclosed agri-food system in Texas (Poole et al. 2005).

In this study, *vanA* VREF with indistinguishable BOX-PCR patterns were consistently isolated from the hospital wastewater during three separate sampling events (at least one month apart). In other studies multiple genotypes of *vanA* VREF were isolated from clinical samples or hospital wastewater using other typing methods such as pulsed-field gel electrophoresis (PFGE) (Thal et al. 1998; Ko et al. 2005; Kotzamanidis et al. 2009) and MLST (Ko et al. 2005; Caplin et al. 2008). Differences between genotyping methods could be responsible for the observance of indistinguishable VREF genotypes in our study and multiple VREF genotypes in other studies. BOX-PCR typing is not a very discriminatory typing method as evidenced by a difference in the PFGE patterns of a few nosocomial VREF isolated in our study (data not shown). VREF isolates demonstrated high-level vancomycin resistance and intermediate resistance to ampicillin and ciprofloxacin and the *esp* gene was detected in some of the isolates; characteristics used to define the hospital-adapted CC-17 cluster. Determining the relationship of the VREF strains isolated in this study to VREF isolates belonging to the CC-17 cluster using typing methods such as MLST or multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) can be of epidemiological significance (Willems et al. 2005; Leavis et al. 2006a; Top et al. 2008).
Twenty percent of the vanA VREF isolated in this study were esp positive but all isolates were negative for hyl. Pantosti et al reported the presence of the esp gene in 94% of clinical VREF isolates but none of them carried the hyl gene (Stampone et al. 2005). Some studies have reported the co-occurrence of the esp and hyl virulence genes in a small proportion of clinical VREF strains (Klare et al. 2005; Novais et al. 2005b) whereas others have found both genes in the majority of the isolates (Rice et al. 2003; Vankerckhoven et al. 2004). The association of these virulence factors in VREF seems to vary among populations.

VRE can potentially be disseminated into environmental waters by compromised sewer systems or improper treatment practices, which poses a health risk for the community (Harwood et al. 2001; Iversen et al. 2004; Novais et al. 2005a). Horizontal transfer of virulence determinants and antibiotic resistance genes from VRE to other bacteria in the environment has been documented in a number of studies (Schaberg and Zervos 1986; Huycke et al. 1998; Baquero 2004), and the recent finding of an HL-VRE E. casseliflavus/ gallinarum strain in WA with high frequencies of in vitro conjugal transfer accentuates this risk (Roberts et al. 2009). Surveillance studies should be initiated to monitor the presence of HL-VRE in environmental waters and associated matrices. Experiments to explore their ability to survive and proliferate in environmental matrices are also warranted based on these and other recent findings.

Acknowledgements

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*asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants 

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Occurrence of *Escherichia coli* and enterococci in Cladophora (Chlorophyta) in nearshore 

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RESEARCH SIGNIFICANCE

Members of the genus Enterococcus are commensals that inhabit the gastrointestinal tracts of humans and other mammals. The metabolic flexibility of enterococci facilitates their survival and proliferation in a wide range of environments such as soils, surface waters, sediments and vegetation associated with surface waters and food (Fujioka 1999; Eaton and Gasson 2001; Harwood et al. 2004). Accurate identification and classification of enterococci is important for both environmental and clinical purposes. It is difficult to identify some Enterococcus species due to their phenotypic similarity (Devriese et al. 1993). Therefore, molecular methods such as genotyping and genetic sequencing are increasingly employed for identification purposes (Malathum et al. 1998; Harwood et al. 2004).
BOX-PCR genotyping of enterococci

Genotyping methods have the capacity to process a large number of isolates (high throughput) and are cost-effective as compared to sequencing studies. Although BOX-PCR fingerprinting has been used to type enterococci in ecological studies (Brownell et al. 2007) and MST studies (Brownell et al. 2007; Hassan et al. 2007), till date, no study has demonstrated that the discrete clusters formed by enterococcal BOX-PCR patterns are phylogenetically related species/strains. The purpose of this study was to compare the ability of BOX-PCR typing to determine genetic relatedness of enterococci with that of the “gold standard” method, 16S rRNA gene sequencing. Enterococci isolated from two freshwater sites and one estuarine site were typed using BOX-PCR and their 16S rRNA genes were sequenced. It was hypothesized that the relationships projected by the genotypic BOX-PCR dendrograms will be similar to those obtained by phylogenetic analysis.

As hypothesized, BOX-PCR dendrograms were fairly congruent (77%) with the phylogenetic tree created by 16S rRNA sequencing. The majority of the strains within the different Enterococcus species could be clearly differentiated using BOX-PCR typing. In comparison, the resolution capacity of the 16S rRNA gene is limited when identifying closely related Enterococcus species such as E. faecium and E. mundtii. The BOXA2R profile of the genus Lactococcus clustered with the BOXA2R patterns of other Enterococcus species whereas it emerged as an outgroup in the phylogenetic tree. This indicates that closely related genera might not be as distinguishable using BOX-PCR typing. To the best of my knowledge, this is the first study to shed light on the relationships between enterococcal species and strains using both genotypic (BOX-PCR
typing) and phylogenetic (16S rRNA sequencing) data. Although BOX-PCR typing is an excellent tool for investigating strain diversity, genotypic studies relying solely on BOX-PCR typing should exercise caution while interpreting phylogenetic relationships projected by BOX-PCR dendrograms.

**Vancomycin resistance in enterococci**

Acquisition of antibiotic resistance genes and virulence determinants through conjugative plasmids and transposons make some *Enterococcus* species capable of causing disease (Tenover 2001). Enterococci can be resistant to a wide range of antimicrobial agents such as β-lactams, aminoglycosides and glycopeptides. Vancomycin is a glycopeptide antibiotic used to treat infections caused by gram-positive organisms. Vancomycin resistance in enterococci was first reported in 1986 in Europe and has since been isolated from clinical and environmental samples worldwide (Leclercq *et al.* 1988; Uttley *et al.* 1989). The plasmid-borne *vanA* and *vanB* genes confer moderate to high-level vancomycin resistance in enterococci while low-level resistance (*vanC1* and *vanC2/3* genes) is chromosomally encoded.

Vancomycin resistant enterococci (VRE) have been detected in environmental waters, sewage, agricultural runoff, animal feces and feces of healthy human hosts in parts of Europe (Aarestrup 1995; Devriese *et al.* 1996). In comparison, VRE are not commonly isolated from environmental waters or non-hospital related sources in the US (Coque *et al.* 1996; Harwood *et al.* 2001; Roberts *et al.* 2009). This study investigated the incidence of VRE in the water, sediment and vegetation samples from two freshwater and one estuarine site. Vancomycin susceptibility was determined using both culture-based (agar dilution method) and molecular (multiplex PCR targeting vancomycin resistance genes)
methods. VRE were also isolated from municipal and hospital wastewater samples. It was hypothesized that enterococci isolated from environmental matrices and municipal wastewater would be susceptible to vancomycin whereas vancomycin resistant enterococci would be isolated from hospital wastewater.

**Isolation of VRE from environmental sources**

Low-level VRE (LL-VRE) (< 32 µg ml\(^{-1}\)) isolated from environmental matrices demonstrated the \( vanC2/3 \) genotype and were identified as *Enterococcus casseliflavus-flavescens* by 16S rRNA gene sequencing. Although no high-level VRE were isolated from surface waters, the high proportion of LL-VRE in environmental matrices is a cause for concern from the public health perspective. LL-VRE have the potential to cause disease, particularly in immunocompromised and geriatric patients. Treatment of infections caused by LL-VRE can be problematic because they sometimes demonstrate vancomycin susceptibility in vitro while being resistant in vivo. *Enterococcus gallinarum* (\( vanC1 \) genotype) were isolated from municipal wastewater and were a much lower proportion (1.6%) than the proportion of LL-VRE isolated from environmental matrices (20% to 60%).

Multi-drug resistant enterococci with indistinguishable BOX-PCR genotypes were isolated from hospital sewer line samples. These isolates were identified as *Enterococcus faecium* possessing the \( vanA \) genotype and resistant to high levels of vancomycin (MIC = 512 µg/ml). They also demonstrated intermediate resistance to ampicillin (MIC > 16 µg/ml) and ciprofloxacin (MIC > 4 µg/ml). Twenty percent of these isolates carried the variant \( esp \) gene which may facilitate colonization of the urinary tract (Shankar et al. 2001) and biofilm formation (Toledo-Arana et al. 2001; Heikens et al. 2007). The
detection of the *vanA* genotype exclusively and the absence of the *vanB* genotype in hospital wastewater is an unusual finding.

This is one of the few studies in the US that has attempted to determine the vancomycin susceptibility of environmental enterococci. This is also the first study to evaluate VRE in vegetation samples and to compare VRE proportions in freshwater vs. estuarine waters and across different matrices. Dissemination of high-level VRE (HL-VRE) into the groundwater or recreational waters due to compromised sewer lines or improper treatment practices poses a health risk for the community. The acquisition of virulence factors and antibiotic resistance genes from HL-VRE by LL-VRE and other indigenous vancomycin susceptible enterococci accentuates this risk. There is a need for surveillance studies to monitor the presence of VRE and determine their ability to survive and persist in environmental matrices.

**References**


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ABOUT THE AUTHOR

Bina Nayak received a Bachelor’s degree in Microbiology from the Ramnarain Ruia College of Arts and Sciences in Mumbai, India in 1995. She enrolled in the University of South Florida, Tampa, FL in 2002 as a non-thesis Master’s student in the Department of Biology. Based on her excellent prowess, her committee recommended a change of program towards the Doctorate in Biology degree in Spring 2004.

During her Ph.D. program, Bina worked on two major projects involving research in landfill microbiology and environmental enterococci. She also worked as a Research Assistant on two projects with governmental funding agencies. She presented her research findings at several Southeastern branch (SEB) and General meetings of the American Society for Microbiology (ASM). She was awarded the Tharp Summer Research Fellowship from the USF, Department of Biology. She was employed part-time as a teaching assistant for the following courses: General Microbiology, Determinative Bacteriology and Microbial Physiology.