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MICROBIOLOGY OF WATER AND WASTEWATER: DISCOVERY OF A NEW GENUS NUMERICALLY DOMINANT IN MUNICIPAL WASTEWATER AND ANTIMICROBIAL RESISTANCES IN NUMERICALLY DOMINANT BACTERIA FROM OKLAHOMA LAKES

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Toby D. Allen
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MICROBIOLOGY OF WATER AND WASTEWATER: DISCOVERY OF A NEW GENUS NUMERICALLY DOMINANT IN MUNICIPAL WASTEWATER AND ANTIMICROBIAL RESISTANCES IN NUMERICALLY DOMINANT BACTERIA FROM OKLAHOMA LAKES

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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Dr. Ralph S. Tanner

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Dr. Kathleen E. Duncan

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Dr. David P. Nagle

_____
Dr. Mark A. Nanny

_____
Dr. Marvin Whiteley

"Science advances through tentative answers to a series of more and more subtle questions which reach deeper and deeper into the essence of natural phenomena" – Louis Pasteur

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

The environment remains largely unexplored. Although many studies address numerous issues, each study potentially opens doors for new viewpoints. The work in this dissertation constitutes a study in water and wastewater microbial ecology. The initial observations (detailed below) that lead to the research summarized by this dissertation were made, to varying degrees, in the Capstone in Microbiology Laboratory. This work represents a small portion of what we can learn from studying the aquatic environment. The three chapters contained in this dissertation were written in the style of the American Society for Microbiology.

The first chapter of this work summarizes the discovery of an ecologically important wastewater microorganism, *Cloacibacterium*. Although municipal wastewater has been studied for many years, this organism has somehow remained obscured from the light of discovery. *Cloacibacterium* is a member of the *Flavobacteriaceae*, a family of bacteria that constitutes part of a larger, phylogenetically cohesive group sometimes referred to as the *Flavobacterium-Cytophaga* group. *Cloacibacterium* was discovered primarily because of an important ecological and evolutionary trait, high numbers. This strain was first isolated in the Capstone Laboratory during an investigation of the impact of treated wastewater on the microbial ecology of the Canadian River. This microorganism was recognized as a novel strain in part, because of an initial BIOLOG analysis. This organism exhibited unusually low

similarities to hits from BIOLOG's current Gram-negative database, which prompted the phenotypic and phylogenetic characterization study summarized in Chapter One. BIOLOG analysis also proved important for screening for other isolates with a similar metabolic fingerprint, thus leading to the characterization of other strains of *Cloacibacterium*. Although members of the *Flavobacterium-Cytophaga* group have been long-established as members of the microbiota of wastewater treatment plants (Güde, 1980; Benedict and Carlson, 1971), only recently have researchers began to realize how numerically dominant this group is in municipal wastewater (Liu *et al*., 2005; Wagner *et al*., 1994; Van Ommen Kloeke and Geesey, 1999). Also only recently, members of the *Flavobacterium-Cytophaga* group have been implemented as important in the removal of phosphate from municipal wastewater (Van Ommen Kloeke and Geesey, 1999), an important process to the treatment of wastewater. A short outline for an experiment designed to determine whether *Cloacibacterium* is involved in this process appears at the end of the second chapter.

The second chapter continues work with *Cloacibacterium*, and addresses the question of its origins in municipal wastewater. This genus was detected, and its numbers present in untreated wastewater was estimated. In addition, other possible environments were screened for the presence of *Cloacibacterium* sp. Although none were found in the current study, other environments are waiting to be explored. The current literature suggests at least three other habitats where *Cloacibacterium* sp. may be

present, according to clone library sequencing studies (O'Sullivan *et al.*, 2002; Munson *et al.*, 2004; Geissler *et al.*, unpublished). As stated above, Chapter Two concludes the studies with *Cloacibacterium* by bringing forth an untested hypothesis that addresses the question of the ecological role of *Cloacibacterium* in wastewater treatment plants. The *Flavobacterium-Cytophaga* group contributes a significant proportion of the total phosphatase activity in wastewater, indicating their role in the removal of phosphate (Van Ommen Kloeke and Geesey, 1999). This hypothesis would test whether or not *Cloacibacterium* is part of the phosphate-removing microbiota in wastewater. This untested hypothesis would be the logical next step in the study of *Cloacibacterium*. The research outlined in Chapter Two was conducted according to a protocol approved by the Institutional Review Board of the University of Oklahoma, Norman Campus.

The third chapter constitutes a comparative study of wastewater-contaminated vs. noncontaminated surface waters. The initial observation that lead to this research was made from an investigation conducted in the Capstone Laboratory. A community of bluegill (*Lepomis macrochirus*) was observed living in a plume of treated municipal wastewater from the wastewater treatment plant in Norman, OK. Having taken part in ecology studies involving the impact of treated wastewater on the microbiota of the Canadian River, this observation sparked an interest regarding the change in bluegill microbiota from contaminated vs. uncontaminated fresh waters. The experiments detailed in Chapter Three were designed to survey these

differences. However, the results pointed to a possibility of environmental reservoirs of multiple antimicrobial resistant organisms living in conditions where no apparent antibiotic input exists. In addition to the high-levels of antimicrobial-resistance in numerically-dominant organisms, an unprecedented level of resistance to ciprofloxacin (in both clinical and environmental microorganisms) was discovered. The idea of environmental reservoirs of antimicrobial resistance is not new, but is supported by the results of this study. Chapter Three concludes with a short summary of further research which would shed more light on some of these findings, including whether *Microbacterium* sp. are intrinsically resistant to ciprofloxacin, and what mechanisms are responsible for its high level of ciprofloxacin resistance.

In conducting this work, my viewpoint on environmental microbiology has changed. To most concisely state the summary of this work, novel microbiology happens. Serendipity is the driving force of many discoveries, but knowing when something novel is at hand is what makes discoveries take shape.

CHAPTER ONE

Cloacibacterium normanensis gen. nov., sp. nov., A Gram-Negative Pigmented Bacterium Isolated from Municipal Wastewater

ABSTRACT

Three bacterial isolates from municipal wastewater were investigated for their phenotypic, biochemical, and molecular characters. All strains were isolated from municipal wastewater, and the type strain, NRS1^T, was isolated as a numerically dominant heterotrophic bacterium. The isolates NRS1^T, NRS30, and NRS32 were Gram-negative, catalase and oxidase positive, nonmotile, non spore-forming, yellow to orange pigmented rods. Pigments were of the carotenoid-type, and no flexirubin-type pigments were detected. All strains degraded gelatin, starch, esculin, casein, and weakly degraded DNA. None degraded cellulose, chitin, agar, urea, pectin, alginate, uric acid, xanthine, or hypoxanthine. Indole was produced by all strains. All strains were methyl red and Voges-Proskauer negative, and nitrate was not reduced. Pyruvate was observed as an end-product from growth on unbuffered glucose. The predominant respiratory quinone was menaquinone MK-6. The DNA base composition was 31 mole % G+C. 16S rRNA gene sequence analysis indicated that these three strains, as well as two uncharacterized strains, MRS7 and MRS14, were members of the *Chryseobacterium-Bergeyella-Riemerella* branch of the family *Flavobacteriaceae*. This bacterium could not be ascribed to any known genus by cellular fatty acid analysis or BIOLOG analysis. All strains were phylogenetically unaffiliated with any described genus from the 16S ribosomal gene sequence analysis. It is therefore proposed that the unknown bacterium be classified in a new genus

Cloacibacterium gen. nov., as *Cloacibacterium normanensis* gen. nov. sp. nov. The type species of the genus *Cloacibacterium* is *Cloacibacterium normanensis* strain NRS1^T (=ATCC BAA-825^T) (=DSM 15886^T) (=CCUG 46293^T) (=CIP 108613^T). The GenBank accession number for the 16S gene sequence of strain NRS1^T is AJ575430. The habitat for *Cloacibacterium* is municipal wastewater, where it was discovered as a numerically dominant species.

INTRODUCTION

The Gram-negative, yellow-orange pigmented (usually), non-spore forming bacteria commonly referred to as the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group is a long-described group (Bergey *et al.*, 1923), and has since undergone much reclassification and subsequent emended descriptions (Bernardet *et al*., 1996; Vandamme *et al.*, 1994; Holmes *et al.*, 1986a; 1986b). The earlier taxa were classified according to characteristics that included gliding motility and pigment production (which turned out to be common characters of heterogenous groups), and has subsequently acquired many heterogenous and misclassified taxa (Bernardet *et al.*, 1996). The strain of *Flavobacterium aquatile*, the type strain of the genus, is not considered representative of the genus *Flavobacterium* (Holmes and Owen, 1979; Bernardet *et al.*, 1996). *Flavobacterium aquatile*, originally described by Bergey *et al*. (1923) is represented by a single strain that possesses characteristics such as gliding motility and swarming that are more similar to members of the *Cytophaga* (Holmes and Owen, 1979; Bernardet *et al.*, 1996). This led to the proposal that *Flavobacterium breve* be instated as the type strain (Holmes and Owen, 1979), which was subsequently denied (Wayne, 1982). The current taxonomy of the CFB group is more correct as more genera are described and misclassified taxa are correctly classified using 16S gene sequence analyses and DNA-DNA hybridizations. This is due to the work of many researchers including Gherna and Woese (1992), Bernardet *et*

al. (2002), Paster *et al.* (1994), Nakagawa and Yamasato (1993; 1996, Vandamme *et al*. (1996), and Hirsch *et al*. (1998), among others.

The family *Flavobacteriaceae* make up a large group of diverse bacteria that are found in many habitats, including: freshwater and marine waters and sediment, rice field soil, microbial mats, hypersaline lakewater, activated sludge, human sputum, dental plaque, cerebrospinal fluid, trachea, human wound, diseased dogs and cats, diseased fish, and diseased birds (Bernardet and Nakagawa, 2003). Some isolates have been found from unusual habitats, including psychrophillic members from Antartic sea ice, lakes, and sediment (McCammon *et al.*, 1998; Bowman and Nichols, 2002; Humphry *et al*., 2001). There is also evidence of uncultured flavobacteria as intracellular endosymbionts of insects (Bernardet and Nakagawa, 2003). Members of the flavobacteria can be readily isolated from chlorinated water supplies (du Moulin, 1979; Ridgeway and Olson, 1982), and may possess some ability for chlorine resistance (du Moulin, 1979; Ridgeway and Olson, 1982). Members of the *Bacteroides* have been shown to be predominant isolates of the human colon microbiota, accounting for about 30% of culturable isolates from stool (Moore and Holdeman, 1974; Holdeman *et al.*, 1976). Similarly, cytophagas are common in activated sludge and other parts of wastewater treatment plants (Güde, 1980; Benedict and Carlson, 1971), where they may play a role in the removal of phosphate from wastewater (Van Ommen Kloeke and Geesey, 1999).

Members of the CFB group are known for their ability to degrade

complex organic matter (e.g. agar, chitin, cellolose, pectin, xylan, and DNA). The fish pathogens produce various proteolytic enzymes (Bernardet and Nakagawa, 2003). The ability to degrade crystalline cellulose by members in the CFB group has a taxonomic significance, and is limited to members of the genus *Cytophaga* (Nakagawa and Yamasato, 1996). Some strains have been shown to degrade halogenated aromatic hydrocarbons (Carvalho *et al.*, 2002). Strains of *Flavobacterium* have been shown to degrade pesticides (Kaufman and Kearney, 1965), as well as crude oil and defined hydrocarbon mixtures (Atlas and Bertha, 1972). Cloning studies from environmental samples have detected members of the CFB group from river biofilms (O'Sullivan *et al.*, 2002), testifying to their survival in oligotrophic environments.

Current genera of the family *Flavobacteriaceae* include: *Aequorivita*, *Arenibacter*, *Bergeyella*, *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Croceibacter*, *Elizabethkingia*, *Empedobacter*, *Flavobacterium*, "*Fucobacter*", *Gelidibacter*, *Kaistella*, *Mesonia*, *Muricauda*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salgentibacter*, *Tenecibacterium*, *Ulvibacter*, *Vitellibacter*, *Weeksella*, and *Zobellia* (Bernardet and Nakagawa, 2003; Kim *et al*., 2005; 2004). Of these genera, *Aequorivita, Arenibacter, Cellulophaga, Croceibacter*, "*Fucobacter*", *Gelidibacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, and *Zobellia* are considered to be marine organisms (Bernardet and

Nakagawa, 2003).

All members of the family *Flavobacteriaceae* are Gram-negative, non-spore forming, and are either nonmotile or exhibit motility by gliding. All members except *Polaribacter irgensii* are non-flagellated (Bernardet *et al.*, 2002). Colonies are pigmented by carotenoids and/or flexirubins, or are nonpigmented. Menaquinone 6 is either the only respiratory quinone, or the major respiratory quinone, and their DNA base composition is 27-44 mole % G+C (Bernardet *et al*., 2002). The characteristics listed above are important for classifying new members of the *Flavobacteriaceae* (Bernardet *et al.*, 2002).

The discovery of flexirubin-type pigments (Reichenbach and Kleinig, 1974; Weeks, 1981) has led to a simple assay for flexirubins to screen for members of the CFB group. Flexirubin-type pigments have been subsequently defined as chemotaxonomic markers for this group. The flexirubin-type pigments are primarily associated with the freshwater, soil, or clinical isolates whereas the carotenoid-type pigments are primarily associated with marine species (Bernardet and Nakagawa, 2003).

The CFB group has distinctive elements of their cellular fatty acid composition. Branched-chain fatty acids are dominant, particularly 15:0 *iso*, making up from 15 to 45% (and in some cases higher) of the total (Reichenbach, 1999). A large proportion of the branched-chain fatty acids are the *iso*-type. Other predominant fatty acids include straight chain 16:1, 16:0, and 15:0. Also, there is collectively a large amount (15-55% total) of 2-

hydroxy and 3-hydroxy fatty acids (Reichenbach, 1999). The 2-hydroxy branched-chain fatty acids are rarely found in other bacteria and are considered chemotaxonomic markers for this group (Fautz *et al.*, 1981).

Another interesting phenotype of the CFB group are distinctive changes in cell morphologies under different culture conditions. For example, a strain of *Flexibacter* exhibited long, threadlike cells that were up to 400 µm under nutrient-rich conditions, but exhibited much shorter rods under nutrient-limiting conditions, when grown at a lower temperature, or when in stationary phase (Simon and White, 1971). Similar pleomorphic cell morphologies have been described for other members of the CFB group (Reichenbach, 1999), with cell populations consisting of short and very long rods, indicating that this phenotype may also be used as a taxonomic screening method for the CFB.

This report includes the phenotypic, biochemical, and molecular characterization of three novel strains (NRS1^T, NRS30, and NRS32), as well as the phylogeny of two additional uncharacterized strains (MRS7 and MRS14). Phylogenetically, all five strains fall into the *Riemerella-Bergeyella-Chryseobacterium* branch of the family *Flavobacteriaceae*.

METHODS AND MATERIALS

Strains and isolation. Strain NRS1^T was isolated as a most numerous culturable heterotroph from a dilution series in half-strength tryptic soy broth (TSB) (Difco Laboratories) inoculated with untreated wastewater from the wastewater treatment plant in Norman, OK (Cleveland Co.). Strains NRS30, NRS32, NRS10 and NRS19 were isolated from the wastewater treatment plant in Norman, OK by direct plating on nutrient agar (NA) (Difco Laboratories). Colonies that exhibited a yellow-orange pigment were then screened by phenotype microarray (BIOLOG, Hayward, CA), and compared to the metabolic profile of strain NRS1^T. Strains NRS1^T, NRS30 and NRS32 were chosen for characterization. Strains NRS10 and NRS19 were partially characterized and the results of the characterization appear in the Appendix. Two uncharacterized strains, MRS7 and MRS14, were isolated from the wastewater treatment plant in Moore, OK (Cleveland Co.), and screened by the methods listed above. Tryptic soy broth (TSB) or tryptic soy agar (TSA) (Difco Laboratories) was used for routine culture unless otherwise stated. All incubations were at 30ºC unless stated otherwise.

Phenotypic characterization. Gram stain, catalase and oxidase tests, indole production, methyl red, Voges-Proskauer, starch hydrolysis, esculin hydrolysis, cellulose degradation, litmus milk reaction, and gelatin hydrolysis (plate method) were performed as previously described (Smibert and Krieg, 1991). Hydrolysis of casein, DNA, and urea, and reduction of nitrate were

determined by inoculation on the appropriate media (Difco Laboratories). Citrate utilization was determined by using Simmons' citrate agar (Difco Labotatories). Relationship to oxygen was tested by using fluid thioglycollate medium (Difco Laboratories). Hydrolysis of xanthine, hypoxanthine and uric acid were performed as previously described (Bowman *et al.*, 1996). Pectin hydrolysis was determined by the method of Hildebrand (1971) with the following modifications: mineral solution (20 ml l^{-1}), vitamin solution (10 ml l^{-1}), and trace metals (5 ml l^{-1}) (Tanner, 2002). Hydrolysis of alginate and chitin were performed by methods previously described (West and Colwell 1984). Resistance to antimicrobials was determined by the method of Bauer *et al.* (1966) using BBL Sensi-Discs (Becton Dickinson and Company, Sparks, MD). BIOLOG analysis was performed according to the manufacturer's instructions (Hayward, CA). Fermentation of glucose was examined in an unbuffered medium (l^{-1}) (20 ml minerals, 10 ml vitamins, 1 ml trace metals, 5 g dextrose, 0.5 g yeast extract; pH 7.1-7.3), and in a buffered medium (same as above, except 30 g l^{-1} N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) and an initial pH of 7.6). Fermentation products of glucose were determined by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (4.6 $\times$ 250 mm, 5 μm particle size) and a mobile phase of 0.005 N H_2SO_4 (1 ml $min.^{-1}$) (Bio-Rad, Richmond, CA). Glucose consumption was assayed by the oxidation of *o*-dianisidine in the presence of glucose oxidase and peroxidase at 425 nm (Raabo and Terkildsen, 1960) using a Spectronic 20 D spectrophotometer

(Milton Roy Co., Rochester, NY). pH range and optimum was determined by incubation in 1.5% tryptone broth supplemented with one of the following (30 g l^{-1}): HOMOPIPES (homopiperazine-N,N'-bis-2-[ethanesulfonic acid]) (pH 4 and 5), MES (2-[N-morpholino]ethanesulfonic acid) (pH 6), TES (pH 7 and 8), or CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) (pH 9). Growth was monitored spectrophotometrically at 600 nm.

API-Zym. The strains were characterized biochemically by Dr. Enevold Falsen (Culture Collection, University of Göteborg) (CCUG) by using the API-Zym test system according to the manufacturer's instructions (API Bio-Mérieux, France).

FAME analysis. Fatty acid methyl ester (FAME) analysis was performed by Microcheck, Inc, (Northfield, VT). Analysis was performed according to methods previously described (Miller, 1982; Sassar, 1990).

Pigments. Pigmented cells were subjected to the KOH test for flexirubin-type pigments as previously described (Hirsch and Reichenbach, 1981) for preliminary analysis. Cells were grown on TSA and the pigments were extracted with acetone. The cell debris was removed by centrifugation and the crude extract was analyzed by a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA).

Microscopy. Cells were observed using an Olympus CH-2 phase-contrast microscope (Olympus Corp., Lake Success, NY). Cells were measured by an ocular micrometer, calibrated with a stage micrometer (1.0 mm in 0.01 mm). A phase-contrast micrograph (FIGURE 1) was taken using an Olympus BX61

epifluorescent microscope equipped with a Spot RT Slider digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Phylogenetic analysis. Mole % G + C, menaquinone analysis, 16S rDNA analysis, and the phylogenetic tree appearing in FIGURE 4, were performed by Dr. Paul Lawson. The mol % G + C content of DNA was determined by HPLC according to Mesbah *et al*. (1989) except the methanol content of the mobile phase was decreased to 8% and the temperature was increased to 37^{o}C. Isoprenoid quinones were extracted as described by Collins *et al*. (1977) and analyzed by HPLC as described by Groth *et al*. (1997). The 16S rRNA genes of the isolates were amplified by PCR using universal primers pA (positions 8 to 28, *Escherichia coli* numbering) and pH (positions 1542 to 1522). The amplified products were purified by using a QIAquick PCR purification kit and then sequenced using primers directed toward conserved positions of the rRNA gene, a dRhodamine terminator cycle sequencing kit, and an automatic DNA sequencer. The closest known relatives of the new isolates were determined by performing database searches using the program FASTA (Pearson and Lipman, 1985). 16S gene sequences from described bacteria were retrieved from GenBank and aligned with the newly determined sequence using the program SEQtools (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al*., 1997), and a phylogenetic tree (FIGURE 4) was constructed according to the neighbour-joining method (Saitou and Nei, 1987) with the programs SEQtools and TreeView (Page, 1996). The

stability of the groupings was estimated by bootstrap analysis (1000 replicates) using the same programs.

Pairwise analysis of 16S rDNA sequences was performed using the BLAST 2 sequences tool of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) (Tatusova and Madden, 1999). The phylogenetic tree in FIGURE 5 was generated by the neighbor-joining method (Saitou and Nei, 1987) using CLUSTAL X, v.1.83 (Higgins and Sharp, 1988). The sequences were aligned manually using the same software. Bootstrap analysis was performed using 1000 replicates.

RESULTS AND DISCUSSION

Cells were rod-shaped (FIGURE 1) and varied greatly in length according to the type of medium used. The most striking difference occurred between broth and plate cultures. In broth, most cells were observed to be very long and threadlike (up to 27 µm), with random bends. Cultures from agar plates were much shorter rods (5 to 9 µm). Similar pleomorphic cell morphologies have been described for other members of the *Flavobacterium-Cytophaga* group (Simon and White, 1971; Hirsch and Reichenbach, 1981; Kondo *et al.*, 2001). For example, a similar cell shape change occurred with *Flexibacter* strain FS-1 which exhibited long, threadlike cells that were up to 400 µm in length under nutrient-rich conditions, but exhibited much shorter rods under nutrient-limiting conditions, when grown at a lower temperature, or when in stationary phase (Simon and White, 1971). After 48 hours, colonies grown on TSA were round, convex, with an entire margin, and very waxy. For most strains, colonies were measured to be 1.5 mm, colonies of strain MRS7 were somewhat smaller (1.0) mm. All strains produced a yellow to orange pigment (FIGURE 2).

Pigments. The KOH test for flexirubin-type pigments was negative for all strains. The KOH test was developed by researchers during the investigation of the pigments of strains of *Flexibacter* and *Cytophaga* (Reichenbach and Kleinig, 1974). The KOH test for flexirubin-type pigments was an important development because it allowed for easy differentiation

FIGURE 1: Phase-contrast micrograph of *Cloacibacterium* sp. (1,000 × magnification).

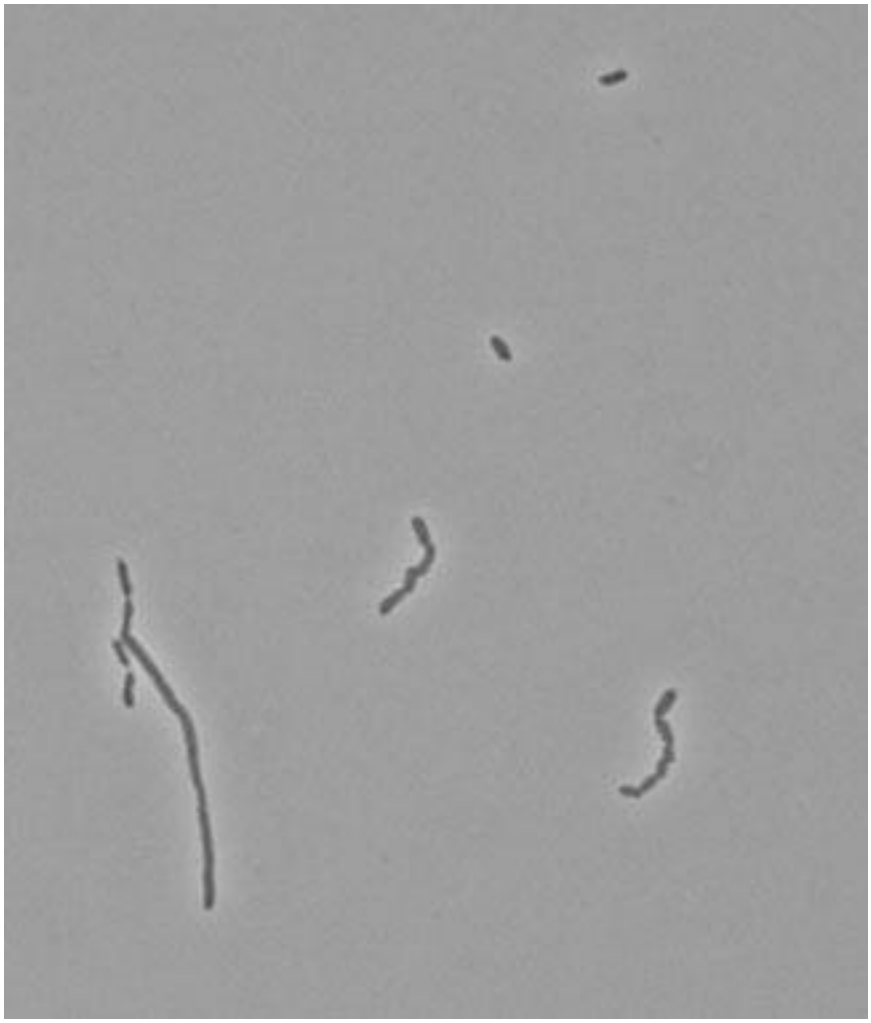


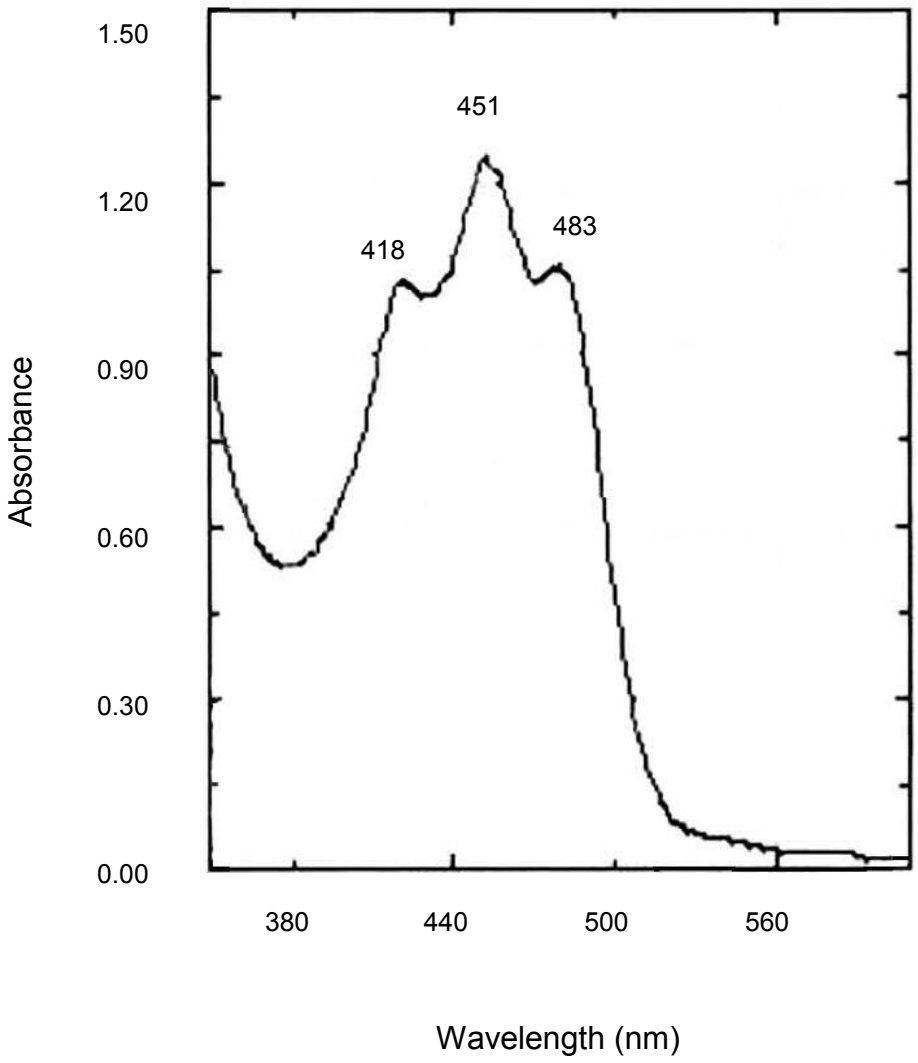
FIGURE 2: Yellow-orange pigmented colonies of *Cloacibacterium* strain NRS1^{T} growing on TSA.

between these two genera. The absorbance spectra of crude pigment extract from all strains exhibited a triple-peak signature characteristic of carotenoid pigments (Schmidt *et al*., 1994) (FIGURE 3), and exhibited absorbance maxima in the visible region at 418 nm, 451 nm, and 483 nm. This result is in contrast with the majority of aquatic (and terrestrial) members of the family *Flavobacteriaceae*, which exhibit primarily flexirubin-type pigments. Carotenoid-type pigments are more closely associated with marine members of the *Flavobacteriaceae* (Bernardet and Nakagawa, 2003).

All strains stained Gram-negative, and were catalase and oxidase positive. None exhibited any sign of gliding motility as observed with a wet mount. All strains hydrolyzed starch, esculin, gelatin, casein, and weakly hydrolyzed DNA. Indole was produced by all strains. None hydrolyzed the following: citrate, urea, chitin, pectin, alginate, uric acid, xanthine or hypoxanthine. All strains were methyl red and Voges-Proskauer negative, and nitrate was not reduced. None degraded cellulose or agar. In unbuffered media, a major end-product of glucose fermentation under aerobic conditions was pyruvate (29-34% carbon recovery) (data not shown). The final pH of all samples in which pyruvate was present was around 4.7. Pyruvate was not observed in a heavily buffered medium (30 g l^{-1}) (final pH was 7.2-7.4). Weak growth occurred under anoxic conditions (N_2 headspace), but pyruvate was not observed. All strains grew under microaerophilic to facultatively anaerobic conditions in fluid thioglycollate medium.

BIOLOG analysis. BIOLOG analysis did not produce an identification for any

FIGURE 3: Scanning absorbance spectrum of pigment extract from *Cloacibacterium* species.



of the strains based on the current Gram-negative MicroLog database. All strains utilized the following: α-cyclodextrin, dextrin, glycogen, cellobiose, gentabiose, α-D-glucose, maltose, turanose, L-alanylglycine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, inosine, uridine, glucose-1-phosphate, and glucose-6-phosphate. Differences among strains are summarized in TABLE 1. None utilized the following: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, I-erythritol, L-fucose, *m*-inositol, lactulose, D-mannitol, L-rhamnose, D-sorbitol, xylitol, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-asparagine, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, D,L-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol. Overall, two notable patterns of discrimination emerged from the differences in substrate utilization patterns from BIOLOG analysis. Strains NRS1^{T} and MRS7 exhibited unique patterns compared to the three other strains as a whole (TABLE 1). The explanation for this observation remains unknown, however in the case of MRS7 this may be explained by taxonomic divergence (discussed below).

TABLE 1: Differences among *Cloacibacterium* strains by BIOLOG analysis.

Substrate	NRS1^T	NRS30	NRS32	MRS7	MRS14
Tween 40	–	+	+	+	+
Tween 80	–	+	+	+	+
L-arabinose	–	–	–	+	–
D-fructose	–	–	+	+	+
D-galactose	–	+	–	+	+
α-D-lactose	–	+	–	+	–
D-mannose	+	–	–	+	–
D-melbiose	–	–	–	+	+
β-methyl-D-glucoside	–	–	–	+	–
D-psicose	–	–	–	+	–
D-raffinose	–	–	–	–	+
Sucrose	–	–	+	–	+
D-trehalose	–	+	+	+	+
Methyl pyruvate	–	–	–	+	+
Monomethylsuccinate	–	+	+	+	+
Acetic acid	–	–	–	+	–
α-ketobutyrate	+	–	–	+	+
α-ketovalerate	–	–	+	–	–
L-alanine	–	–	+	–	–
L-aspartic acid	–	+	+	–	–
L-ornithine	–	+	+	+	+
L-serine	–	+	+	–	+
L-threonine	–	+	+	+	+
Urocanic acid	–	+	+	–	+
Thymidine	–	+	+	+	+
Glycerol	+	+	+	+	–
D,L-α-glycerol phosphate	+	+	+	+	–

API-Zym. Using the API-Zym kit, positive reactions were obtained for alkaline phosphatase, esterase C4 (weak reaction), esterase lipase C8, leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase and phosphoamidase. Cystine arylamidase, α-glucosidase and β-glucosidase was found to be variable among strains examined (TABLE 2). Activity for lipase C14, typsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acteyl-β-glucosaminidase, α-mannosidae and α-fucosidase was not detected.

FAME analysis. Analysis of fatty acid methyl esters revealed branched-chain fatty acids were predominant in the NRS strains (TABLE 3). The most predominant fatty acids were 15:0 *iso* (40-46%), 15:1 *iso* (undetermined unsaturation position) (7-10%),13:0 *iso* (8-9%), 17:0 *iso* 3-OH (5-9%), and to a lesser extent 15:0 *anteiso* (5-6%) and 15:0 *iso* 3-OH (4%). There were several similarities to close phylogenetic relatives of the NRS strains. Most noteworthy is the predominance of 15:0 *iso*, which is also the case in strains of *Riemerella* and *Bergeyella* (Vandamme *et al*., 1994; Vancanneyt *et al*., 1999), and has been reported as the dominant fatty acid of the *Cytophagales* (Reichenbach, 1999). Another fatty acid common to the NRS strains as well as in *Bergeyella* and *Riemerella* is the 17:0 *iso* 3-OH. The NRS strains also have several fatty acids in common with *Riemerella*; including 13:0 *iso*, 15:0 *anteiso*, and 15:0 *iso* 3-OH (Vancanneyt *et al*., 1999). Another observation was the occurrence of 2-OH and 3-OH fatty acids, which sum to 13-16% in the NRS strains (TABLE 3). This is a characteristic of the *Cytophagales*,

TABLE 2: Differences among *Cloacibacterium* strains and related genera by API-Zym analysis.

Activity	NRS1[T]	NRS30	NRS32	*Bz*[‡]	*Rc*[†]	*Ra*[*]
Cystine arylamidase	–	–	+(w)	+	+(w)	–
α-glucosidase	+	+(w)	–	–	+	+
β-glucosidase	+	–	–	–	–	–
Alkaline phosphatase	+	+	+	+	+	+
Esterase C4	+(w)	+(w)	+(w)	+(w)	+(w)	–
Esterase lipase C8	+	+(w)	+	+	+(w)	+
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+(w)	+
α-chymotrypsin	+	+	+(w)	–	+	+
Acid phosphatase	+	+	+	+	+	+
Phosphoamidase	+	+	+	+	+	+
Lipase C14	–	–	–	–	–	–
Trypsin	–	–	–	–	–	–
α-galactosidase	–	–	–	–	–	–
β-galactosidase	–	–	–	–	–	–
β-glucuronidase	–	–	–	–	–	–
N-acteyl-β-glucosaminidase	–	–	–	–	–	–
α-mannosidae	–	–	–	–	–	–
α-fucosidase	–	–	–	–	–	–

(w) denotes a weak positive reaction.
[‡]*Bergeyella zoohelcum* (ATCC 43767[T]).
[†]*Riemerella columbina* (DSM 16469[T]).
[*]*Riemerella anatipestifer* (ATCC 11845[T]).

TABLE 3: Cellular fatty acid composition of *Cloacibacterium* strains.

	NRS1^T	NRS30	NRS32
$C_{12:0\ 3\text{-}OH}$	2.1	1.0	2.1
$C_{13:0\ iso}$	7.9	9.2	9.2
$C_{13:0\ anteiso}$	1.1		1.0
$C_{14:0\ iso}$	3.3	2.8	3.4
$C_{15:0}$	1.1		
$C_{15:0\ iso}$	40.8	43.0	45.8
$C_{15:0\ anteiso}$	5.0	5.7	6.2
$C_{15:0\ iso\ 3\text{-}OH}$	3.5	4.4	3.9
$C_{15:1\ iso\ F}$*	7.8	7.0	10.2
$C_{15:1\ anteiso\ A}$*	5.4	4.0	
$C_{16:0}$		1.3	
$C_{16:0\ iso}$	1.7		1.0
$C_{16:0\ 3\text{-}OH}$	1.0		
$C_{16:0\ iso\ 3\text{-}OH}$	2.5	1.9	1.8
$C_{16:1\ (\omega 5c)}$	1.0		
$C_{17:0\ iso\ 3\text{-}OH}$	5.3	8.6	4.8

*Unknown unsaturation position.

which possess 2-OH and 3-OH fatty acids summing to 15-55% (Reichenbach, 1999). Some noteworthy differences include the absence of 15:0 *iso* 2-OH and 17:1 *iso* from the NRS strains, both of which have been reported as predominant in *Bergeyella* (Vandamme *et al*., 1994). Another difference the NRS isolates possess is the predominance of a 15:1 *iso* (undetermined unsaturation position), which has not been noted as predominant in the Cytophagales (Reichenbach, 1999).

Antimicrobial resistance. All strains were resistant to one or more aminoglycosides (including one intermediate resistance). Only one strain (MRS7) was resistant to ampicillin. Two of the strains (NRS32 and MRS14) were resistant to nalidixic acid. Antibiotic resistances are shown in TABLE 4.

Temperature and pH range. All strains grew from 18°C to 36°C, none grew at 4°C, nor at 40°C or above. The optimum for all strains was around 30°C. All strains grew at pH 7 and 8, some strains also grew at pH 6 and 9 (TABLE 5). The optimum for all strains was around 7.

Based on phenotypic characteristics, BIOLOG analysis, and FAME analysis, the unidentified isolates from municipal wastewater did not correspond to any recognized Gram-negative bacterium. From the comparative 16S rRNA gene sequence analysis, it is evident that the unknown organism represents a previously unknown taxon. Phylogenetically, the novel bacterium is a member of the *Chryseobacterium-Bergeyella-Riemerella* branch of the family *Flavobacteriaceae*. Together, strains NRS1^{T}, NRS30, NRS32, MRS7 and MRS14 form an association with a cluster of

TABLE 4: Antimicrobial resistances* of *Cloacibacterium* strains.

Strain	Resistance
NRS1^T	erythromycin, kanamycin, streptomycin
NRS30	erythromycin, kanamycin
NRS32	erythromycin, nalidixic acid intermediate resistance: kanamycin
MRS7	ceflaclor, ampicillin, kanamycin, intermediate resistance: ceftriaxone, streptomycin
MRS14	nalidixic acid intermediate resistance: kanamycin

*Antibiotics tested using the method of Bauer *et al.* (1966): ampicillin (10 µg), carbenicillin (100 µg), cefaclor (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), sulfathiazole (0.25 mg), tetracycline (30 µg), and trimethoprim (5 µg), each.

TABLE 5: pH range for growth of *Cloacibacterium* strains[*].

pH	NRS1[T]	NRS30	NRS32
4	–	–	–
5	–	–	–
6	+	–	+
7	+	+	+
8	+	+	+
9	–	+	–

[*]Growth was measured in half strength TSB incubated at 30ºC using absorbance values at 600 nm.

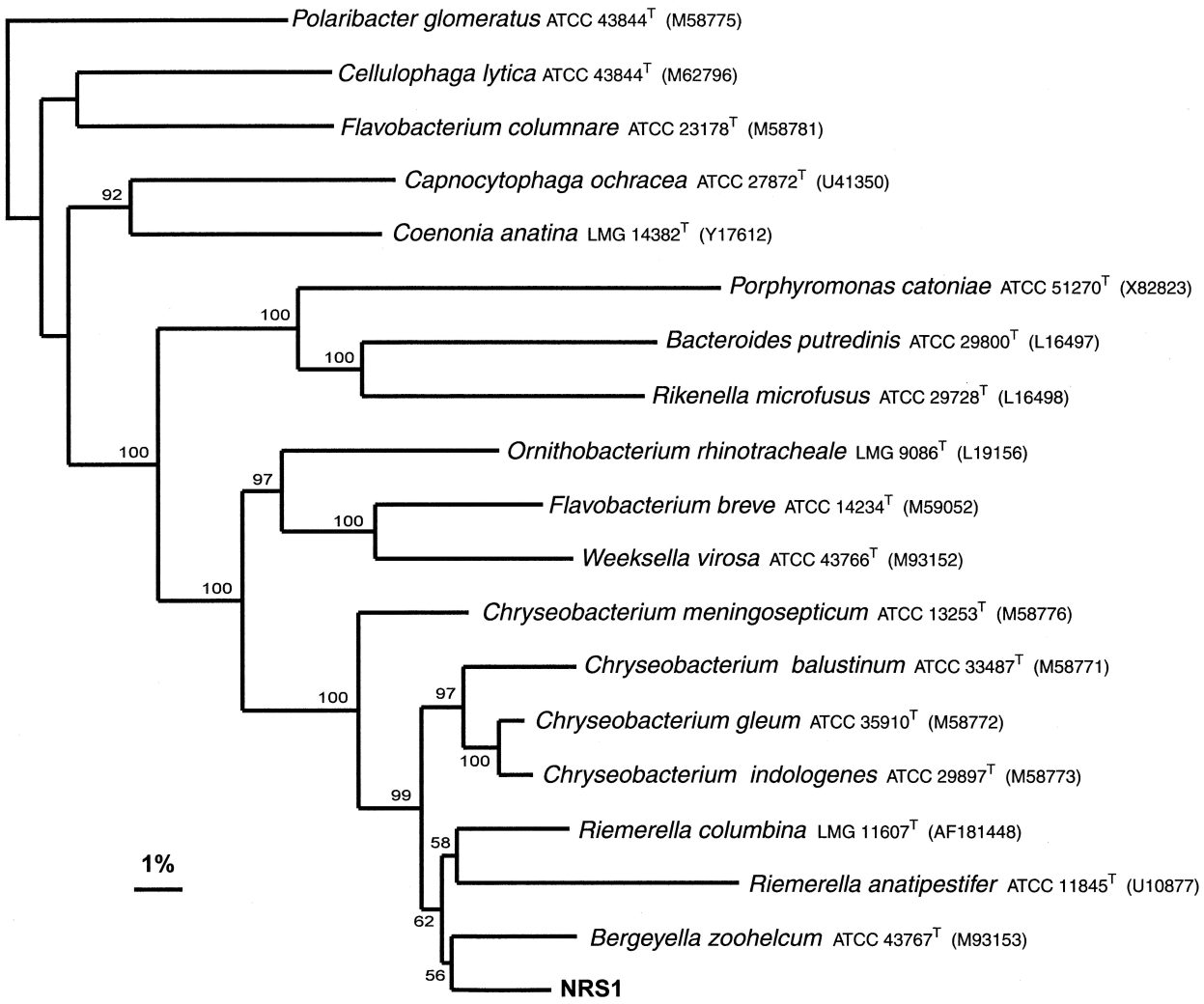
organisms, which includes *Riemerella anatipestifer*, *Riemerella columbina*, and *Bergeyella zoohelcum* (FIGURE 4). The unknown isolate also shared 16S rRNA gene sequence homology with some uncultured bacteria, including an uncultured uranium mining waste clone of 99.2% (Geissler *et al.*, unpublished) (GenBank accession number AJ519403), with a human oral clone (Munson *et al.*, 2004) (GenBank accession number AY278614) of 98.7%, and with a river biofilm clone (O'Sullivan *et al.*, 2002) (GenBank accession number AY038761) of 97.4% with bootstrap values of 100% (data not shown). Among described bacteria, the unidentified isolate displayed highest sequence homology to *Riemerella anatipestifer* (88.8%), *Riemerella columbina* (94.6%), and *Bergeyella zoohelcum* (94.4%). Strains NRS1^T, NRS30, NRS32, MRS7, and MRS14 share close sequence similarities (TABLE 6), and are likely to be of the same genus (FIGURE 5). However, according to the phylogenetic tree in FIGURE 5, strain MRS7 may represent a second species of *Cloacibacterium*. This observation agrees with the BIOLOG data (TABLE 1). This strain exhibited a 98.5% 16S gene sequence similarity to strain NRS1^T (TABLE 6). DNA:DNA hybridization would be a useful tool in discriminating between the two species.

The only known habitat for these strains is municipal wastewater. Conditions within the wastewater plants surveyed may allow for the survival and multiplication of these organisms. The presence of strain NRS1^T at the indicated high number in wastewater indicates that this organism is ecologically important in its habitat. This organism may play a role in the

The following captions are for the phylogenetic trees on pp. 29-30.

FIGURE 4 (p. 29): Phylogenetic tree of *Cloacibacterium normanensis* strain NRS1^{T} and related Gram-negative bacteria constructed using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replicates, are given at branching points. The bar corresponds to a 1% difference in sequences.

FIGURE 5 (p. 30): Phylogenetic tree of *Cloacibacterium* strains and closely related bacteria constructed using the neighbor-joining method. Bootstrap values, expressed as whole numbers (1000 replicates) are given at branching points. The bar corresponds to a 1% difference in sequences.


Polaribacter glomeratus ATCC 43844^T (M58775)
Cellulophaga lytica ATCC 43844^T (M62796)
Flavobacterium columnare ATCC 23178^T (M58781)
Capnocytophaga ochracea ATCC 27872^T (U41350)
Coenonia anatina LMG 14382^T (Y17612)
Porphyromonas catoniae ATCC 51270^T (X82823)
Bacteroides putredinis ATCC 29800^T (L16497)
Rikenella microfusus ATCC 29728^T (L16498)
Ornithobacterium rhinotracheale LMG 9086^T (L19156)
Flavobacterium breve ATCC 14234^T (M59052)
Weeksella virosa ATCC 43766^T (M93152)
Chryseobacterium meningosepticum ATCC 13253^T (M58776)
Chryseobacterium balustinum ATCC 33487^T (M58771)
Chryseobacterium gleum ATCC 35910^T (M58772)
Chryseobacterium indologenes ATCC 29897^T (M58773)
Riemerella columbina LMG 11607^T (AF181448)
Riemerella anatipestifer ATCC 11845^T (U10877)
Bergeyella zoohelcum ATCC 43767^T (M93153)
NRS1
92
100
100
100
97
100
100
100
97
100
99
58
62
56
1%

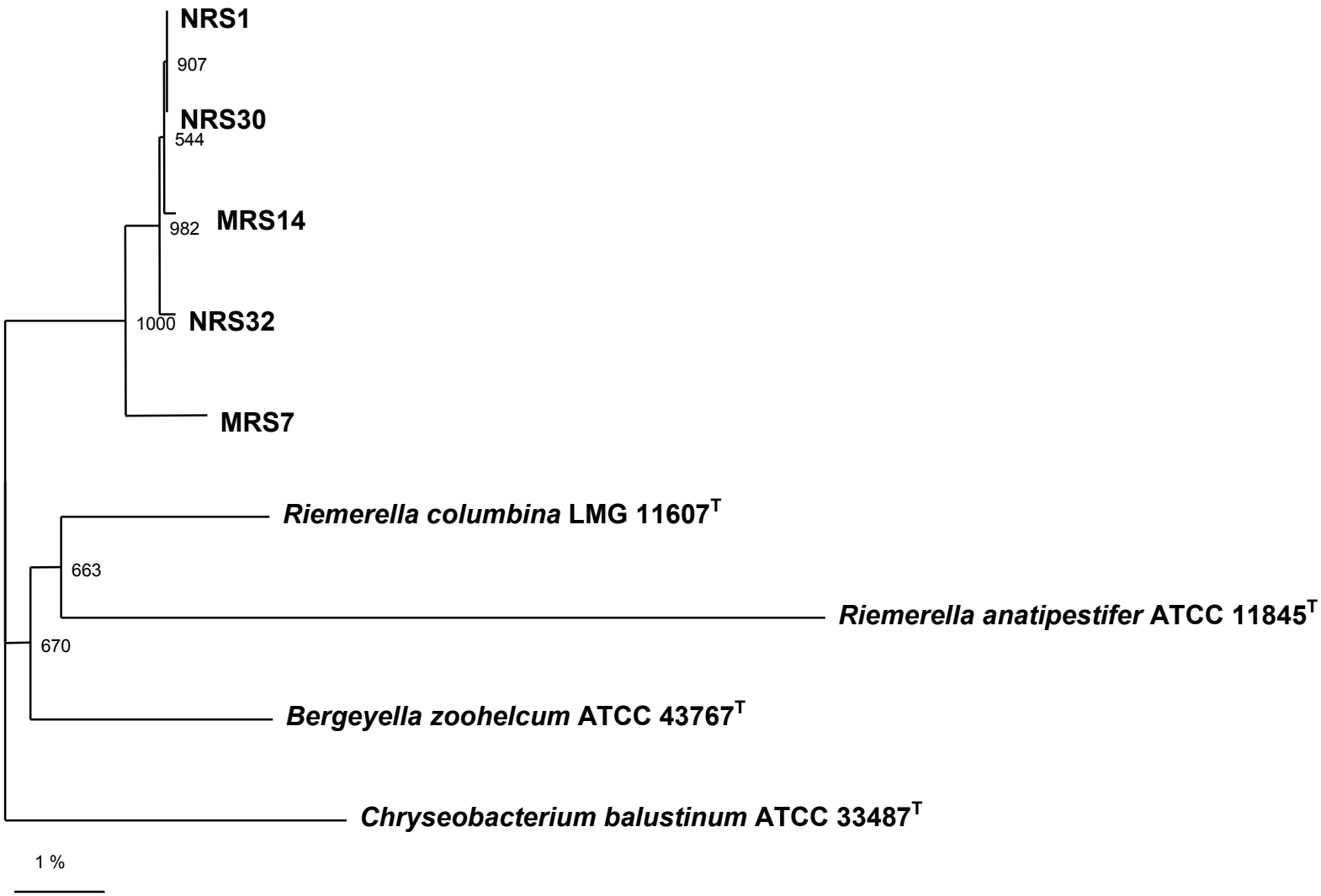

NRS1
907
NRS30
544
982
MRS14
1000
NRS32
MRS7
Riemerella columbina LMG 11607T
663
Riemerella anatipestifer ATCC 11845T
670
Bergeyella zoohelcum ATCC 43767T
Chryseobacterium balustinum ATCC 33487T
1 %

TABLE 6: Percent similarities of the 16S gene sequences of *Cloacibacterium* strains.

	NRS1^T	**MRS7**	**MRS14**	**NRS30**	**NRS32**
NRS1^T	——	98.45	99.79	100	99.59
MRS7	98.45	——	98.37	98.45	98.31
MRS14	99.79	98.37	——	99.79	99.51
NRS30	100	98.45	99.79	——	99.59
NRS32	99.59	98.31	99.51	99.59	——

removal of phosphate in wastewater. A summary of the experiments that could be used to test this hypothesis appears at the end of Chapter Two. Another possible ecological role of this bacterium is the breakdown of complex macromolecules (i.e. polysaccharides and proteins) within untreated wastewater. In contrast to strains NRS1^T, NRS30, and NRS32, strains of *Riemerella* have been found only from birds and pigs (Segers *et al.*, 1993; Vancanneyt *et al.*, 1999). Likewise, strains of *Bergeyella* have been found mainly from animal bite wounds in humans, throughout the oral and nasal cavities of dogs, and from clinical specimens (Vandamme *et al.*, 1994; Hugo *et al.*, 2004). In contrast to the NRS strains, both *Riemerella* and *Bergeyella* sp. are not considered free-living (Vandamme *et al.*, 2004; Hugo *et al.*, 2004), and have been found primarily in the respiratory tracts of their respective hosts.

Based on tree topology considerations and sequence divergence values of 5% or greater with the aforementioned taxa, the unidentified bacterium was only distantly related to these taxa and merits classification at a similar taxonomic rank (i.e., genus). Therefore based on phenotypic and phylogenetic findings, the unknown isolate from untreated wastewater merits classification in a new genus, for which the name *Cloacibacterium normanensis* gen. nov., sp. nov. is proposed. Strain NRS1^T is the type strain, and has been deposited in the American Type Culture Collection (ATCC BAA-825^T), the German Collection of Microorganisms (DSM 15886^T), The Culture Collection of the University of Göteborg (CCUG 46293^T), and the

Collection de l'Institute Pasteur (CIP 108613^T). *Cloacibacterium* strains NRS30, NRS32, MRS7, and MRS14 have been deposited in the Culture Collection of the University of Göteborg, and have been assigned the accession numbers CCUG 48043, CCUG 48044, CCUG 50908, and CCUG 50907 respectively. TABLE 7 lists the characteristics useful for distinguishing *Cloacibacterium* from its closest phylogenetic relatives.

Description of *Cloacibacterium* gen. nov. (Clo.a´ci.bac. ter.i.um. L. fem. n. *cloaca*, sewer, canal; N. L. neut. n. *bacterium*, from Gr. *bakterion*, a small rod; N. L. neut. n. *Cloacibacterium*, bacterium of the sewer). Cells were Gram-negative, non-motile, non-spore forming, and were pleomorphic rod-shaped. Strains grew under aerobic to facultatively anaerobic conditions. Growth was observed between 18°C to 36°C. No growth was observed at 4°C or at 40°C and above. The optimum pH for all strains was around 7. Catalase and oxidase tests were positive. Starch, esculin, gelatin, and casein were hydrolyzed, and DNA was weakly hydrolyzed. Indole was produced by all strains. Nitrate was not reduced. The KOH test for flexirubin-type pigments was negative. The pigments produced were carotenoid-type pigments. Fatty acids consisted mainly of branched-chain fatty acids with 13:0 *iso*, 15:0 *iso*, 15:1 *iso*, and 17:0 *iso*-3-OH predominating. The predominant respiratory quinone was MK-6. The G + C content of DNA was 31 mol %. The type species is *Cloacibacterium normanensis*.

TABLE 7: Differentiating characteristics between *Cloacibacterium* and phylogenetically related genera.

	Cloacibacterium	*Bergeyella*[*]	*Reimerella*[†]	*Chryseobacterium*[*]
Pigment	+ (carotenoid)	–	–[¶]	+ (flexirubin)[‡]
DNase	+	–	ND	(+)
Gelatinase	+	+	(+)	+
Urease	–	+	v	v
Indole	+	+	v	v
Esculin hydrolysis	+	–	v	(+)
Starch hydrolysis	+	–	v	ND
Acid from glucose	+	–	v	(+)
Growth at:				
37°C	+	+	+	+
42°C	–	–[#]	(+)	v
Growth on:				
MacConkey's agar	–	–	–	(+)
Mol % G + C	31	35-37	29-37	33-38
Habitat	free living	parasitic or saprophytic	parasitic	free living or parasitic

[*] Data were obtained from Vandamme *et al.* (1994).
[†] Data were obtained from Segers *et al.* (1993), and from Vancanneyt *et al.* (1999).
+Positive for all strains studied; (+), positive for most strains studied; –, negative for all strains studied.
v Varies within, or between strains and species; ND, not determined.
[‡] Some strains of *Chryseobacterium meningosepticum* are nonpigmented (Vandamme *et al*., 1994).
[#] Present in one strain of *Bergeyella zoohelcum* (Vandamme *et al.*, 1994).
[¶] *Riemerella columbina* produces a grey-white or beige pigment (Vancanneyt *et al.*, 1999).

Description of *Cloacibacterium normanensis* sp. nov. *Cloacibacterium normanensis* (nor.man.en´sis. N. L. masc. adj. *normanensis*, pertaining to the city of Norman, Oklahoma, U.S.A., from where the organism was first isolated). Cells were Gram-negative, non-motile, non-spore forming, and were pleomorphic rod-shaped. Colonies grown on TSA were round, convex, with an entire margin, and very waxy, and were about 1.5 mm in diameter. Strains grew under aerobic to facultatively anaerobic conditions. Growth was observed between 18°C to 36°C. No growth occurred at 4°C or at 40°C or above. Optimum temperature for growth was 30°C. All strains grew at pH 7 and 8, some strains also grew at pH 6 and 9. The optimum pH for all strains was 7. Catalase and oxidase tests were positive. Indole was produced by all strains. Starch, esculin, gelatin and casein was hydrolyzed, DNA was weakly hydrolyzed. Urea, chitin, pectin, alginate, uric acid, xanthine, or hypoxanthine was not hydrolyzed. The methyl red and Voges-Proskauer tests were negative. Nitrate was not reduced. Cellulose and agar were not degraded. A major end-product of glucose fermentation under unbuffered aerobic conditions was pyruvate. Pyruvate was not observed under heavily buffered conditions. Using the API-Zym kit, positive reactions were obtained for alkaline phosphatase, esterase C4 (weak reaction), esterase lipase C8, leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase and phosphoamidase. Cystine arylamidase, α-glucosidase and β-glucosidase were variable between strains. Activity for lipase C14, typsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acteyl-β-

glucosaminidase, α-mannosidae and α-fucosidase were not detected. The KOH test for flexirubin-type pigments was negative. The pigments produced were of the carotenoid-type. Fatty acids consisted mainly of branched-chain fatty acids with 13:0 *iso* (8-9%), 15:0 *iso* (41-46%), 15:1 *iso* (7-10%), and 17:0 *iso*-3-OH (5-9%) predominating. The predominant respiratory quinone was MK-6. The G + C content of DNA was 31 mol %. Isolated from municipal wastewater, which is the only known habitat for this organism. The type strain is NRS1^T which was deposited in the American Type Culture Collection (ATCC BAA-825^T), the German Collection of Microorganisns (DSM 15886^T), Culture Collection, University of Göteborg (CCUG 46293^T), and the Culture Collection de l'Institute Pasteur (CIP 108603^T). The GenBank accession number for the 16S gene sequence of strain NRS1^T is AJ575430.

CHAPTER TWO

Enumeration of *Cloacibacterium* species using a Most Probable Number-Polymerase Chain Reaction Method

ABSTRACT

A pair of oligonucleotide primers (Cloac-001f and Cloac-001r) was designed specific to the newly described genus *Cloacibacterium*. The primer pair was employed to detect and enumerate populations of *Cloacibacterium* sp. *in situ* using whole-cells in an MPN-PCR study. The primer pair did not amplify purified DNA from *Riemerella anatipestifer* (ATCC 11845^T) or *Bergeyella zoohelcum* (ATCC 43767^T). Washed whole cells were used in the PCR reaction after a freeze-thaw cycle and an extended initial denaturation temperature to achieve cell lysis. Contaminating cells from *Pseudomonas fluorescens* (ATCC 13525) did not affect the sensitivity of the PCR reaction. MPN-PCR detected up to 74.2% of *Cloacibacterium* sp. present as compared to spread-plate colony counting. *Cloacibacterium* sp. was detected in wastewater influent from the wastewater treatment plant in Norman (Cleveland Co., OK) at 1.38×10^5 cells ml^{-1}, but was not detected in wastewater effluent. Similarly, *Cloacibacterium* sp. was detected in untreated wastewater from the wastewater treatment plant in Moore (Cleveland Co., OK) at 1.38×10^4 cells ml^{-1}. Representative positive samples from Moore and Norman wastewater were sequenced producing a 557 bp fragment that exhibited 99.1% similarity to the theoretical target sequence of *Cloacibacterium*. *Cloacibacterium* sp. was not detected in untreated wastewater influent from the wastewater treatment plant in El Reno (Canadian Co., OK), nor was *Cloacibacterium* sp. detected in purified DNA

isolated from human stool specimen (n=10). *Cloacibacterium* sp. was also not detected from purified DNA isolated from hydrocarbon-contaminated soil. This study showed no evidence to support that *Cloacibacterium* is part of the normal microbiota of the human lower GI tract. The only habitat described thus far for *Cloacibacterium* has been municipal wastewater, which is supported by this study. The primers used for this study are good candidates for further environmental detection and enumeration of *Cloacibacterium* species.

INTRODUCTION

Several strains of *Cloacibacterium* species were recently isolated from untreated municipal wastewater from treatment plants in Norman and Moore, OK. *Cloacibacterium normanensis* strain NRS1^T was isolated as a most numerous culturable heterotrophic bacterium from the treatment plant in Norman. The presence of this organism in untreated wastewater at the indicated high number suggests a possible human GI tract origin for this genus, even though *Cloacibacterium* does not possess the usual characteristics of the GI microbiota (namely oxidase negative, optimum temperature of 36ºC). However, this hypothesis would explain why *Cloacibacterium* was observed at the indicated high number in untreated wastewater because a constant source of input would be identified. The presence of *Cloacibacterium* in untreated wastewater at the indicated high number also suggests *Cloacibacterium* is an important organism in the microbial ecology of wastewater. If the source of *Cloacibacterium* in wastewater treatment plants is not from human input, then *Cloacibacterium* must be a normal inhabitant of wastewater where environmental conditions are well-suited for this organism.

The microbiota of the human colon is a complex ecosystem. Out of the estimated 10^{11} to 10^{14} bacteria per g, (Finegold *et al.*, 1983; Moore and Holdeman, 1974) more than 400 species are estimated to be present (Holdeman *et al*., 1976; Moore and Holdeman, 1974). Out of these, only 30-

40 species make up about 99% of the bacteria present (Drasar, 2003). Many colonic bacteria have fastidious growth requirements, and most are strict anaerobes (Lay *et al.*, 2005; Finegold *et al.*, 1983), but previous studies have estimated that at least 60% of the bacterial species present have been recovered in pure culture (Moore and Holdeman, 1974; Holdeman *et al.*, 1976). More recently, 16S gene amplification and sequencing of strict anaerobes has revealed that over 90% belong to established species, which still leaves a very large number of undescribed colonic bacteria (Namsolleck *et al*., 2004).

The closest phylogenetic relatives of *Cloacibacterium* (genera of *Bergeyella* and *Riemerella*) were isolated from the respiratory systems of domestic and wild birds (Vancanneyt *et al.*, 1999; Segers *et al.*, 1993) or from the respiratory tract of canines and dog-bite wounds (Vandamme *et al.*, 1994). Most strains of *Bergeyella* and *Riemerella* have been isolated from host organisms, and are not considered to be free-living environmental organisms (Vandamme *et al*., 2004; Hugo *et al*., 2004). However other, more distant relatives of *Cloacibacterium* such as cytophagas (*Cytophaga*-like bacteria) (CLB) have been found in predominant numbers from sewage treatment plants (Güde, 1980). These organisms are common in freshwater and soil, and seem to be adapted for the degradation of large and difficult to degrade macromolecules (e.g. chitin and cellulose) (Reichenbach, 1999). The study by Güde (1980) revealed that CLB were present in much higher numbers from the treated effluent than from untreated influent, indicating that

environmental factors are more favorable for CLB from the effluent rather than the influent. Due to their degradation profiles, CLB are enriched more after the initial treatment processes, when recalcitrant substrates are readily available (Reichenbach, 1999). In contrast, *Cloacibacterium* sp. are numerically dominant from untreated influent. Due to the differential parameters employed by Güde (1980) (KOH positive, gliding motility, and the degradation of cellulose, chitin, and pectin), the possibility of confusing CLB with *Cloacibacterium* sp. is very small.

Since no methods exist for the enrichment and selection for *Cloacibacterium* sp., this genus can be difficult to isolate if it is present in low numbers. The presence of many yellow-orange pigmented genera makes enumerating *Cloacibacterium* sp. from environmental samples an even more difficult task.

The polymerase chain reaction (PCR) is a rapid, specific and sensitive tool (Saiki *et al.*, 1988). PCR has been used as a powerful diagnostic method for many applications including quantification of amplifiable target DNA. One such method couples the PCR with the most probable number technique (MPN) (Cochran, 1950). MPN-PCR is a powerful, robust, quantitative approach that has successfully enumerated specific targeted groups of bacteria (Picard *et al.*, 1992; Leung *et al.*, 1997; Fredslund *et al*., 2001; Degrange and Bardin, 1995; Savill *et al.*, 2001; Degrange *et al.*, 1998; Michotey *et al.*, 2000). MPN-PCR can be very specific, detecting down to the genus or even the species and subspecies levels (Picard *et al.*, 1992),

depending on the target sequence and primers used.

Several studies have focused on 16S rDNA probe based methods for detection of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group from environmental samples (Kirchman *et al.*, 2003; O'Sullivan *et al.*, 2002; Weller *et al.*, 2000; Manz *et al.*, 1996). However, most of these studies focus on detection of the group as a whole rather than focusing on particular genera.

In the present study the goal was to design a pair of oligonucleotide primers specific for the newly-described genus *Cloacibacterium*, to detect and enumerate members of this genus in its natural environment. The intent was to answer two questions posed from the initial observation: 1) What numbers of *Cloacibacterium* sp. are present in untreated wastewater? And 2) Is *Cloacibacterium* an inhabitant of the normal human lower GI microbiota? The polymerase chain reaction was employed, coupled to the most probable number technique (MPN-PCR) to probe both wastewater and human stool samples for the presence of this organism.

METHODS AND MATERIALS

Environmental sampling and strains used. Samples of untreated wastewater influent and treated effluent were aseptically taken from wastewater treatment plants in Norman (Cleveland Co., OK), Moore (Cleveland Co., OK), and El Reno (Canadian Co., Oklahoma). Stool samples were self-collected from ten individuals who had not undergone recent antibiotic therapy, in accordance with a protocol approved by the Institutional Review Board of University of Oklahoma, Norman Campus. Strains of *Bergeyella zoohelcum* (ATCC 43767^T) *Riemerella anatipestifer* (ATCC 11845^T), and *Pseudomonas fluorescens* (ATCC 13525) were obtained from the American Type Culture Collection (Manassas, VA). *Cloacibacterium* strains NRS1^T, MRS7, MRS14, NRS30, and NRS32 were used as control strains.

DNA extraction from pure cultures. DNA was extracted from pure culture strains using bead-mill homogenation in the presence of SDS for cell lysis (Miller *et al*., 1999), and isolated by a phenol, chloroform-isoamyl alcohol method previously described (Rios-Hernandez *et al.*, 2003) (TABLE 8).

DNA extraction from stool samples. DNA was extracted from fresh stool samples using the QIAamp DNA Stool Mini Kit (Qiagen cat. no. 51504) (Qiagen, Valencia, CA). Approximately 200 mg of each stool sample was used to extract DNA according to the manufacturer's instructions for bacterial pathogen detection. DNA from stool was quantified and assayed for purity by

TABLE 8: Outline of DNA isolation procedure (Rios-Hernandez *et al*., 2003).

1) Load suspended cells in a 2 mL tube containing 1.0 g zirconia/silica beads (0.1 mm) (BioSpec Products, Inc, Bartlesville, OK).
2) Add, 300 μL TE buffer (10 mM tris-HCl*, 1 mM EDTA†, pH 8.0).
3) Add 300 μL tris lysis buffer (500 mM tris, 10 mM NaCl, 10% SDS‡, pH 8.0).
4) Add 600 μL chloroform: isoamyl alcohol (24:1).
5) Agitate mixture with a mini-beadbeater (BioSpec Products, Inc. Bartlesville, OK) for 60 s. at 4200 rpm.
6) Centrifuge mixture for 5 min. at 5,000 × g.
7) Add 500 μL phenol to the supernatant, and centrifuge for 5 min. at 5,000 × g.
8) Repeat phenol extraction as needed.
9) Add 5 μL RNase (10,000 U/μL) (Stratagene), and incubate at 37° C for 1 hr.
10) Add 500 μL phenol, and centrifuge at 5,000 × g for 5 min.
11) Add 500 μL chloroform: isoamyl alcohol (24:1) to supernatant and centrifuge at 5,000 × g for 5 min.
12) Precipitate DNA by adding 2 vol. cold 100% ethanol, and 0.1 vol. 3 M sodium acetate (pH 7). Hold at −20° C overnight.
13) Centrifuge precipitated DNA solution at 18,000 × g for 20 min., drain supernatant.
14) Wash pellet with 500 μL 70% cold ethanol, centrifuge at 18,000 × g for 5 min.
15) Wash pellet with 500 μL 100% cold ethanol, centrifuge at 18,000 × g for 5 min.
16) Remove supernatant and allow samples to air-dry for 5-15 min., resuspend DNA in 30 μL nuclease-free water, and store at −20° C.

*(hydroxymethyl)aminomethane hydrochloride.
†ethylenediaminetetraacetic acid.
‡sodium dodecyl sulfate.

A_{260} and by $A_{260/280}$ respectively using a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The final template concentration used in PCR reactions ranged from 1,020 ng to 0.407 ng.

Other DNA used in this study. DNA previously purified by the method of Bornemann *et al.* (1996) from crude oil-contaminated soil from the Tallgrass Prairie Preserve, near Pawhuska, OK (Mehta, 2004) was also used to determine the range of habitats for *Cloacibacterium* species. The soil DNA was previously quantified to be 5 ng μl^{-1}, and was a gift from the laboratory of Dr. Kathleen Duncan.

Oligonucleotide primers. *Cloacibacterium*-specific primers were designed using the 16S gene sequence from strain NRS1^T. Short sequences that were specific to *Cloacibacterium* sp. were chosen and verified for their specificity using the BLASTn tool of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997). The primers were then verified for their general suitability using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rosen and Skaletsky, 2000). The primers used to detect *Cloacibacterium* sp. were: Cloac-001f (5'-TATTGTTTCTTCGGAAATGA) and Cloac-001r (5'-ATGGCAGTTCTATCGTTAAGC) and were custom-synthesized by Invitrogen (Carlsbad, CA). General characteristics of the primer pair are listed in TABLE 9. This primer pair targeted a 557 bp region of the 16S gene of *Cloacibacterium* species (FIGURE 6). Universal 16S eubacterial primers were used to demonstrate the presence of amplifyable DNA from stool DNA

TABLE 9: Properties of *Cloacibacterium*-specific primers used.

Primer	Length (bp)	Position*	T_m(ºC)	%GC
Cloac-001f	20	33	53.42	30.00
Cloac-001r	21	589	56.22	42.86

*From the 1,475 bp sequence of the 16S ribosomal gene of *Cloacibacterium normanensis* strain NRS1^T.

FIGURE 6: The 16S gene sequence of *Cloacibacterium normanensis* strain NRS1^{T} and sites of primer annealing.

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1 agcgggaggc ctaacacatg caagccgagc gg**tattgttt cttcggaaat ga**gagagcgg
>>>>>>>>>>>>>>>>>>

61 cgtacgggtg cggaacacgt gtgcaacctg cctttatctg ggggatagcc tttcgaaagg
121 aagattaata ctccataaca tattgattgg catcaattaa tattgaaagc tccggcggat

181 agagatgggc acgcgcaaga ttagctagtt ggtgaggtaa cggctcacca aggcgatgat

241 ctttaggggg cctgagaggg tgatccccca cactggtact gagacacgga ccagactcct

301 acgggaggca gcagtgagga atattggtca atgggtgcaa gcctgaacca gccatcccgc

361 gtgaaggacg actgccctat gggttgtaaa cttcttttgt atagggataa acctaccctc

421 gtgagggtag ctgaaggtac tatacgaata agcaccggct aactccgtgc cagcagccgc

481 ggtaatacgg agggtgcaag cgttatccgg atttattggg tttaaagggt ccgtaggcgg

541 acttataagt cagtggtgaa agcctgtc**gc ttaacgatag aactgccat**t gatactgtaa
<<<<<<<<<<<<<<<<<<<<

601 gtcttgagta tatttgaggt agctggaata agtagtgtag cggtgaaatg catagatatt

661 acttagaaca ccaattgcga aggcaggtta ccaagatata actgacgctg agggacgaaa

721 gcgtggggag cgaacaggat tagataccct ggtagtccac gccgtaaacg atgctaactc

781 gtttttgggc tttagggttc agagaccaag cgaaagtgat aagttagcca cctggggagt

841 acgctcgcaa gagtgaaact caaaggaatt gacgggggcc cgcacaagcg gtggattatg

901 tggtttaatt cgatgatacg cgaggaacct taccaagact taaatgggaa ttgacagttt

961 tagaaataga actttcttcg gacaattttc aaggtgctgc atggttgtcg tcagctcgtg

1021 ccgtgaggtg ttaggttaag tcctgcaacg agcgcaaccc ctgtcactag ttgccatcat

1081 tcagttgggg actctagtga gactgcctac gcaagtagag aggaaggtgg ggatgacgtc

1141 aaatcatcac ggcccttacg tcttgggcca cacacgtaat acaatggccg gtacagaggg

1201 cagctacaca gcgatgtgat gcaaatctcg aaagccggtc tcagttcgga ttggagtctg

1261 caactcgact ctatgaagct ggaatcgcta gtaatcgcgc atcagccatg gcgcggtgaa

1321 tacgttcccg ggccttgtac acaccgcccg tcaagccatg gaagctgggg gtacctgaag

1381 tcggtgaccg taaaaggagc tgcctagggt aaaactagta actagggcta agtcgtaaca

1441 aggtagccgt accggaaggt gcggctggat cacct

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preparations as well as from *Bergeyella zoohelcum* (ATCC 43767^T) and *Riemerella anatipestifer* (ATCC 11845^T), which were used as negative control strains. The universal primer pair used was: 357f (5'-CTCCTACGGGAG-GCAGCAG) and 907r (5'-CCGTCAATTCCTTTGAGTTT) (Johnson, 1994).

PCR conditions. The polymerase chain reaction was performed using a Mastercycler Gradient 5331 thermocycler (Eppendorf, Hamburg, Germany). PCR conditions for purified DNA were as follows: Initial DNA denaturation at 94º C for four minutes, then 30 cycles of DNA denaturation for 30 s. at 94º C, primer annealing for 30 s. at 53º C, DNA extention for 30 s. at 72º C, and a final extention of 72º C for four minutes. PCR conditions using whole cells as a template were the same as above except the initial denaturation was extended to ten minutes (after a freeze/thaw cycle) to achieve lysis. DNA was amplified from *Cloacibacterium* sp. by PCR in a total volume of 25 μL. Five microliters of template DNA was added in a mixture of 1 × PCR amplification buffer (10 × buffer contains 500 mM KCl and 100 mM Tris-HCl [pH 9.0]), 500 mM betaine, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 100 μM of each primer, and 0.625 U of *Taq* DNA polymerase (Fisher Scientific, Fair Lawn, NJ). Environmental samples that exhibited a positive PCR amplification for *Cloacibacterium* were then reamplified for sequencing using a total volume of 50 μL under the conditions listed above except the template DNA remained at five μL. The PCR product was then purified using a QIAquick PCR Purification Kit (Qiagen no. 28104) (Qiagen, Valencia, CA). Sequencing was performed via the dideoxy method of Sanger

et al. (1977) at the Oklahoma Medical Research Foundation DNA Sequencing Facility (OMRF, Oklahoma City, OK).

Agarose gel electrophoresis. Purified genomic DNA or PCR-amplified DNA was visualized by gel electrophoresis in TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA [pH 8.0] with 0.8% agarose (wt./vol.) The gels were stained with ethidium bromide (0.05 mg l^{-1}) and photographed under a UVP 312 nm UV transilluminator (UVP, Inc., Upland, CA) equipped with a Kodak EDAS 290LE digital camara (Eastman Kodak Co., Rochester, NY).

MPN-PCR enumeration. Untreated sewage samples were collected from wastewater treatment plants in Norman (Cleveland Co., OK), Moore (Cleveland Co., OK), and El Reno (Canadian Co., OK). Pure cultures of whole cells and wastewater samples were serially diluted tenfold in triplicate using either half-strength tryptic soy broth (TSB) (Difco Laboratories), or a pyrophosphate buffer (pH 7.1) respectively, then harvested by centrifugation at 18,000 × g, washed with 500 µl 1% saline, resuspended in 30µl nuclease-free water, and frozen at -20ºC until PCR was performed. Cultures of *Cloacibacterium normanensis* strain NRS1^{T} were used to compare enumerations by MPN-PCR to spread-plate colony counting using tryptic soy agar (TSA) (Difco Laboratories) to determine the sensitivity of the MPN-PCR. All incubations were at 30ºC. Total heterotroph counts from wastewater samples were estimated by culture-dependent MPN using half-strength TSB (Difco Laboratories), or by spread-plate colony counting using TSA.

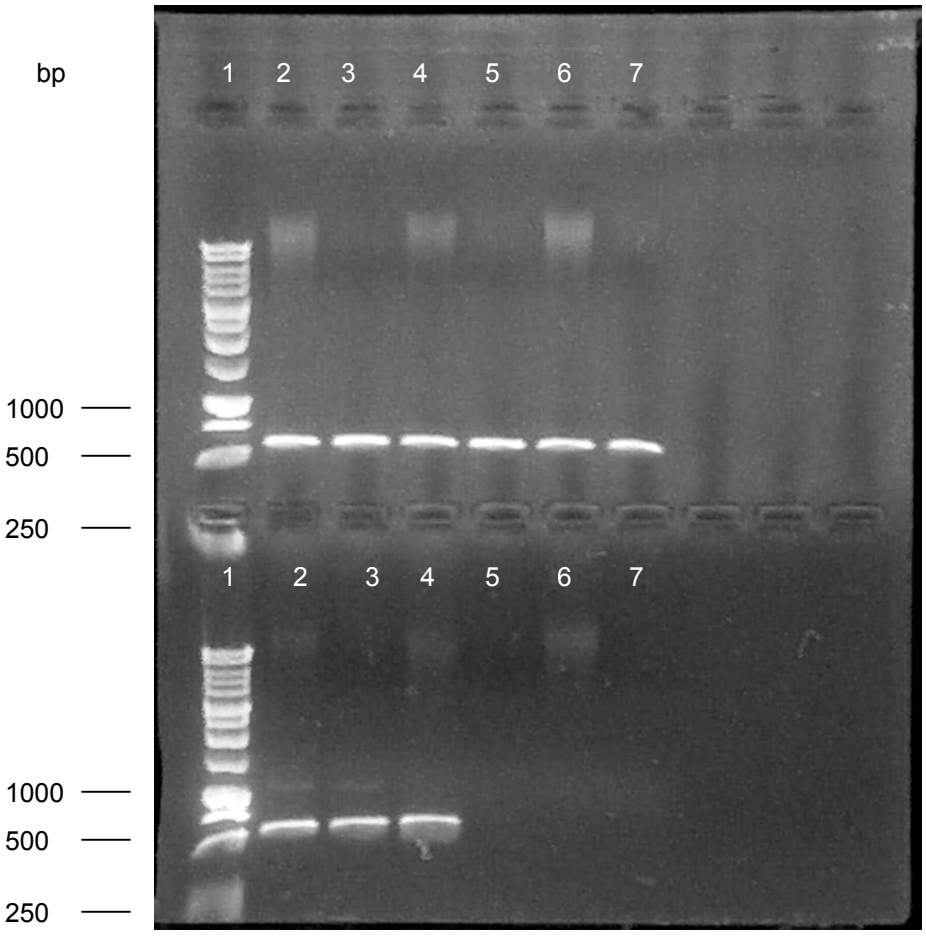
RESULTS AND DISCUSSION

A simple, straightforward method for detecting and enumerating *Cloacibacterium* species *in situ* was developed. The primer pair (Cloac-001f/r) was specific for *Cloacibacterium* and competition due to nonspecific annealing was not observed. The primer pair was used to estimate the number of *Cloacibacterium* species from environmental samples.

PCR optimization and primer specificity. The primer pair Cloac-001f/r was tested against genomic DNA isolated from all known strains of *Cloacibacterium* (NRS1^T, MRS7, MRS14, NRS30, and NRS32). After PCR, DNA from all strains produced a single band that migrated just above the 500 bp band of the marker, indicating that the product matched the expected 557 bp product (FIGURE 7). No noticeable difference was detected when the number of PCR amplification cycles was increased from 30 to 40 (data not shown).

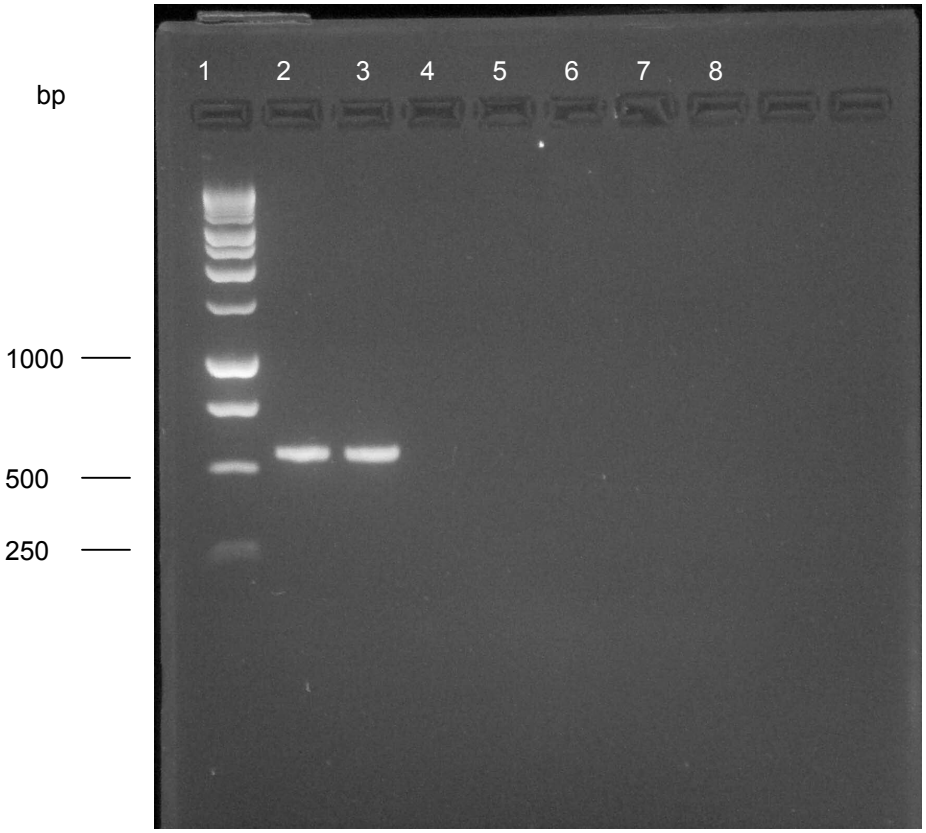
The primer pair Cloac-001f/r was specific to *Cloacibacterium* strains. These primers were tested against two close phylogenetic neighbors of *Cloacibacterium*; *Riemerella anatipestifer* (ATCC 11845^T) and *Bergeyella zoohelcum* (ATCC 43767^T) under low stringency conditions (T_a= 48ºC), and increased the stringency of the annealing temperature to 64.1ºC. As seen in FIGURE 8, the primer pair Cloac-001f/r amplified genomic DNA only from *Cloacibacterium* species under low stringency conditions, with no nonspecific product observed. PCR product from genomic DNA isolated from

FIGURE 7: Specificity of primers Cloac-001f/r to genomic DNA isolated from different strains of *Cloacibacterium*.



Top lanes: 1) 1 Kb ladder (Promega), 2) MRS7 (1:10), 3) MRS7 (1:100), 4) MRS14 (1:10), 5) MRS14 (1:100), 6) NRS30 (1:10), 7) NRS30 (1:100).
Bottom lanes: 1) 1Kb ladder (Promega), 2) NRS32 (1:10), 3) NRS32 (1:100), 4) NRS1 (1:10), 5) *Bergeyella zoohelcum* (ATCC 43767^T) (1:10), 6) *Riemerella anatipestifer* (ATCC 11845^T) (1:10), 7) no template control.

FIGURE 8: Specificity of primers Cloac-001f/r to genomic DNA isolated from *Cloacibacterium* species under low-stringency conditions.



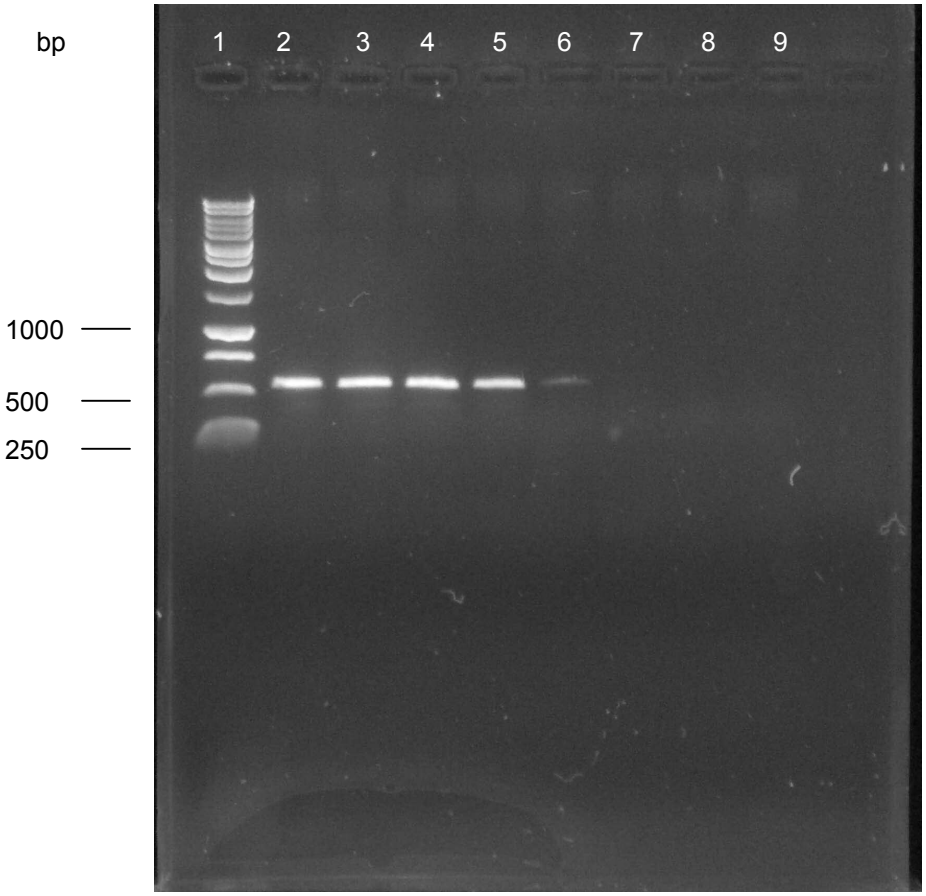
Lanes: 1) 1 Kb ladder (Promega), 2) NRS1^{T} (1:5), 3) NRS1^{T} (1:10), 4) *Bergeyella zoohelcum* (ATCC 43767^{T}) (1:5), 5) *Bergeyella zoohelcum* (ATCC 43767^{T}) (1:10), 6) *Riemerella anatipestifer* (ATCC 11845^{T}) (1:5), 7) *Riemerella anatipestifer* (ATCC 11845^{T}) (1:10), 8) no template control.

Cloacibacterium sp. was visualized with annealing temperatures up to 58.7ºC (FIGURE 9), indicating the robustness of the primer pair.

MPN-PCR of wastewater samples. Substances that interfere with PCR (e.g. humic acids) may be co-purified with DNA (Steffan *et al.*, 1988; Zhou *et al.*,1996) In order to better quantify *Cloacibacterium* species in wastewater, a whole cell PCR method was employed. This method would eliminate the error introduced from manipulation of DNA, given that efficient cell lysis could be achieved. A previous study showed that whole cells were successfully used as template for quantitative PCR in bacteria from soil (Pillai *et al.*, 1991). However, their procedure utilized a differential sedimentation process, whereas this study used dilution to eliminate or lessen the concentration of interfering substances such as humic acids, chelators, or organic contaminants that are present in untreated sewage (Tsai *et al.*, 1993). Washed whole cells of *Cloacibacterium normanensis* strain NRS1^T were used to determine the sensitivity of the PCR reaction. This method amplified a single DNA fragment corresponding to the target size, with good sensitivity (FIGURE 10).

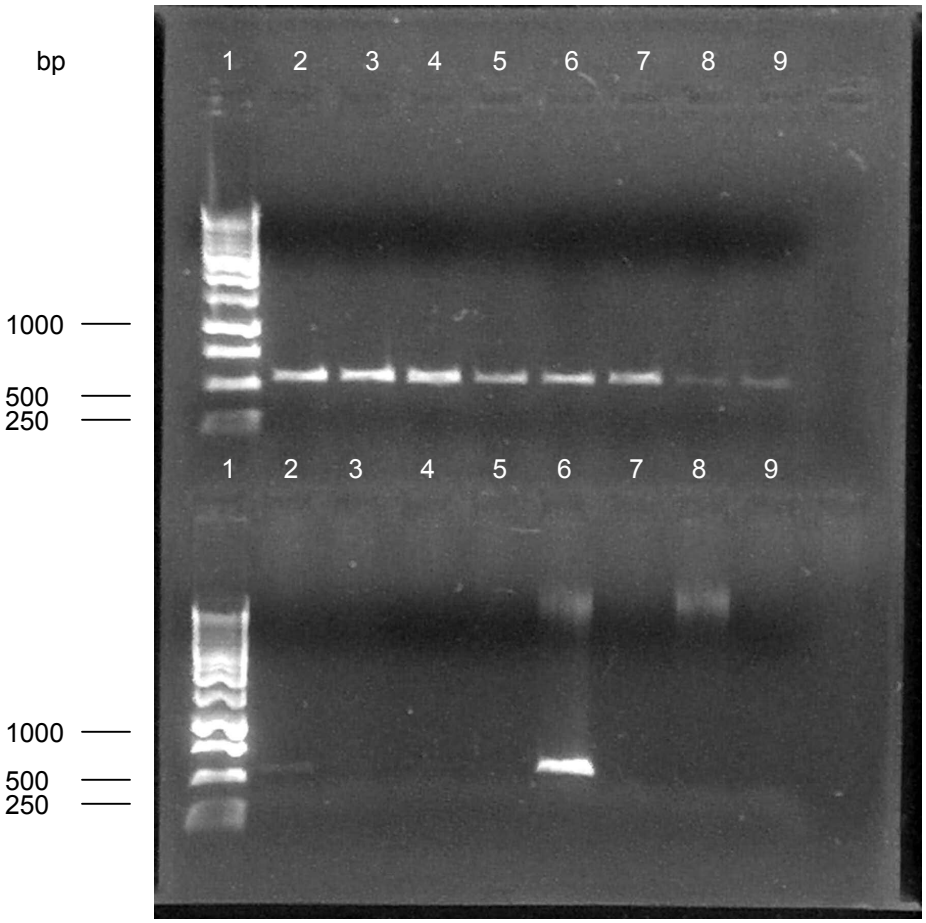
MPN-PCR enumeration using *C. normanensis* strain NRS1^T was compared to spread-plate colony counting. MPN-PCR estimated the concentration to be 1.44×10^7 ml^{-1} whereas spread-plating detected a mean of 4.03×10^7 CFU ml^{-1} (1.94×10^7 to 6.12×10^7 CFU ml^{-1}). Up to 74.2 % of *Cloacibacterium* was detected in this assay. *Cloacibacterium normanensis* strain NRS1^T was mixed with approximately 4×10^6 CFU ml^{-1} of

FIGURE 9: PCR product from DNA isolated from *Cloacibacterium* sp. under an increasing gradient of annealing temperatures.



Lanes: 1) 1 Kb ladder (Promega), 2) T_a = 48.3 ºC, 3) T_a = 50.7 ºC, 4) T_a = 53.4 ºC, 5) T_a = 56.1 ºC, 6) T_a = 58.7 ºC, 7) T_a = 61.1 ºC, 8) T_a = 62.9 ºC, 9) T_a = 64.1 ºC.

FIGURE 10: PCR product from whole cells of *Cloacibacterium* sp.



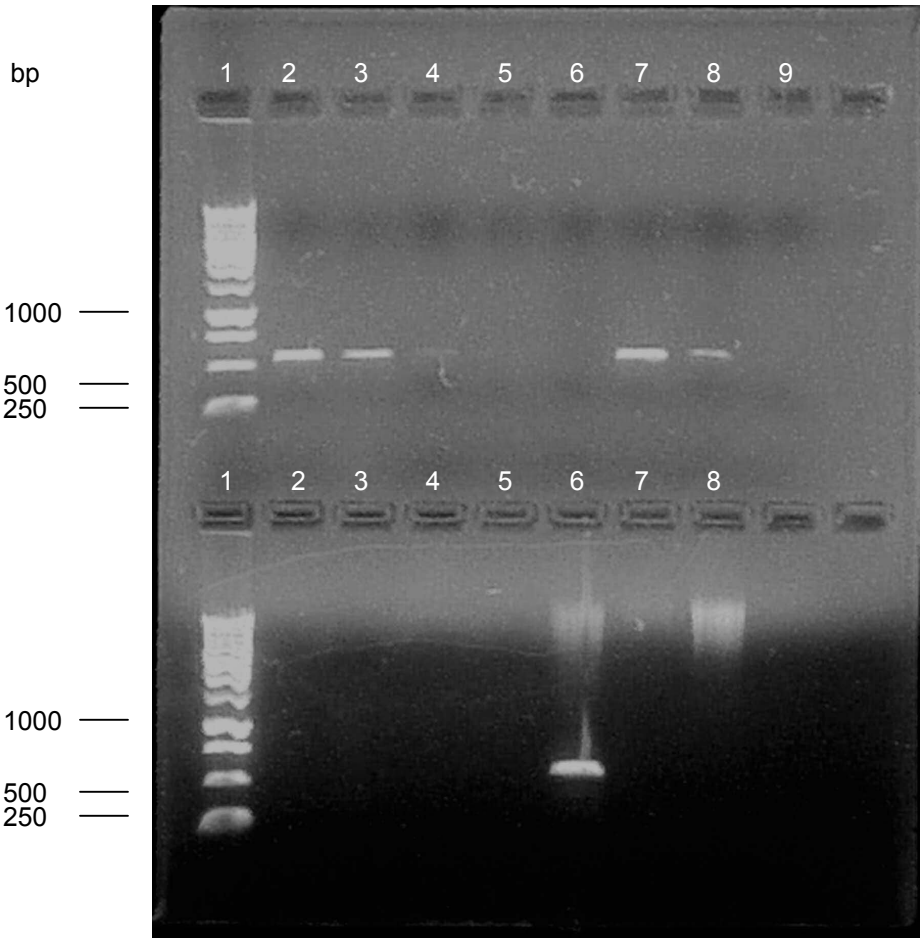
Top lanes: 1) 1 Kb ladder (Promega), 2) NRS1^T (1:10), 3) NRS1^T (1:10), 4) NRS1^T (1:10), 5) NRS1^T (1:100), 6) NRS1^T (1:100), 7) NRS1^T (1:100), 8) NRS1^T (1:1,000), 9) NRS1^T (1:1,000).
Bottom lanes: 1) 1 Kb ladder (Promega), 2) NRS1^T (1:1,000), 3) NRS1^T (1:10,000), 4) NRS1^T (1:10,000), 5) NRS1^T (1:10,000), 6) NRS1^T DNA (1:10), 7) *Bergeyella zoohelcum* (ATCC 43767^T) (1:10), 8) *Riemerella anatipestifer* (ATCC 11845^T) (1:10), 9) no template control.

Pseudomonas fluorescens ATCC 13525 to determine whether foreign DNA would affect PCR enumerations. As seen in FIGURE 11, foreign DNA did not effect the sensitivity of the MPN-PCR.

Washed cells from the wastewater treatment plants from Norman, Moore, and El Reno were used as template to determine whether *Cloacibacterium* spp. were present. *Cloacibacterium*-specific signal was found in samples from the Norman and Moore plants (FIGURES 12 and 13), but not from the El Reno plant. After MPN-PCR was run on the Moore and Norman samples, a significant number of *Cloacibacterium* was estimated to be present (FIGURE 14). *Cloacibacterium* spp. from the Norman sample was estimated to be 1.38×10^5 ml^{-1}, and from the Moore sample to be 1.38×10^4 ml^{-1}. The background heterotroph population for the Norman plant (estimated by culture-dependent MPN) was 9.33×10^7 ml^{-1}, for the Moore plant (spread-plate colony counting) was 8.69×10^5 ml^{-1}, and for the El Reno plant (spread-plate colony counting) was 3.98×10^6 ml^{-1} (FIGURE 14). The theoretical detection limit of *Cloacibacterium* from our method was estimated to be less than 200 ml^{-1} according to Cochran's tables (1950).

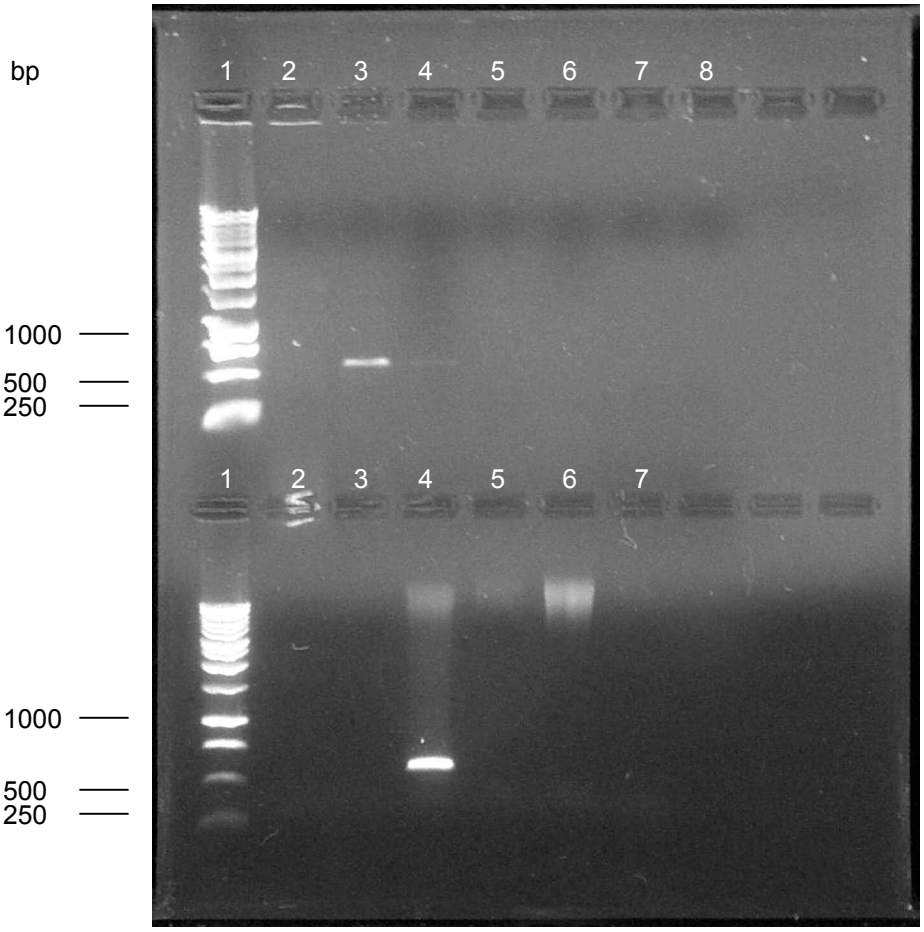
PCR was run a second time using representative *Cloacibacterium*-positive PCR reactions from both Norman and Moore samples as template DNA. This method of enhancing the amount of PCR product produced a significantly greater amount of product (FIGURE 15). This PCR product was cleaned and sequenced. The resulting DNA fragments were both 557 bp sequences matching the expected target length of 557 bp. The amplified

FIGURE 11: PCR product from whole cells of *Cloacibacterium* sp. with and without foreign contaminating cells.



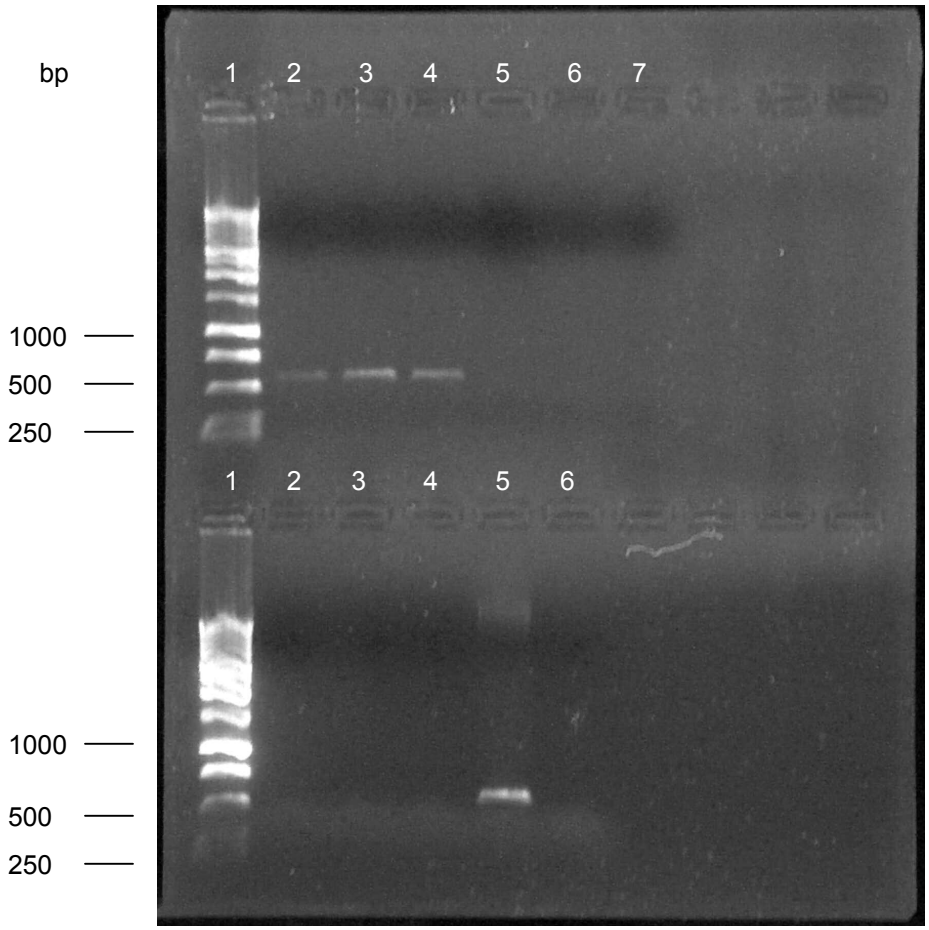
Top lanes: 1) 1 Kb ladder (Promega), 2) NRS1^T (1:10), 3) NRS1^T (1:100), 4) NRS1^T (1:1,000), 5) NRS1^T (1:10,000), 6) NRS1^T (1:100,000), 7) mix (1:10), 8) mix (1:100), 9) mix (1:1,000).
Bottom lanes: 1) 1 Kb ladder (Promega), 2) mix (1:10,000), 3) mix (1:100,000), 4) *Pseudomonas fluorescens* (ATCC 13525) (1:10), 5) *Pseudomonas fluorescens* (ATCC 13525) (1:100), 6) NRS1^T DNA (1:10), 7) no template control, 8) *Riemerella anatipestifer* (ATCC 11845^T) (1:10).

FIGURE 12: Detection of *Cloacibacterium* sp. from Norman wastewater samples.



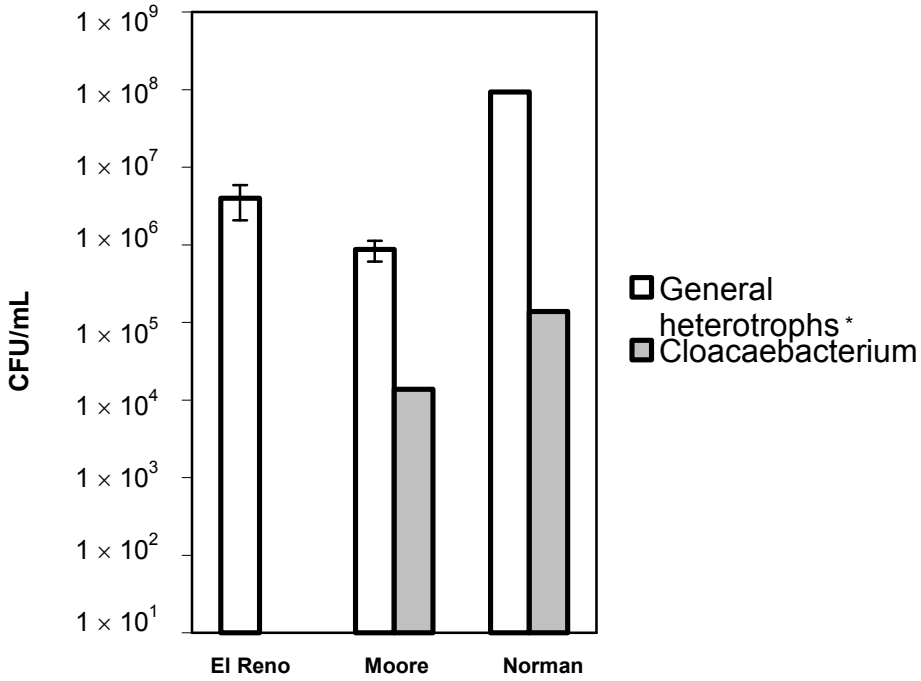
Top lanes: 1) 1 Kb ladder (Promega), 2) untreated wastewater (uw), 3) uw (1:10), 4) uw (1:100), 5) uw (1:1,000), 6) uw (1:10,000), 7) treated wastewater (tw), 8) tw (1:10).
Bottom lanes: 1) 1 Kb ladder (Promega), 2) tw (1:100), 3) tw (1:1,000), 4) NRS1^{T} DNA (1:10), 5) *Bergeyella zoohelcum* (ATCC 43767^{T}) (1:10), 6) *Riemerella anatipestifer* (ATCC 11845^{T}) (1:10), 7) no template control.

FIGURE 13: Detection of *Cloacibacterium* sp. from Moore wastewater samples.



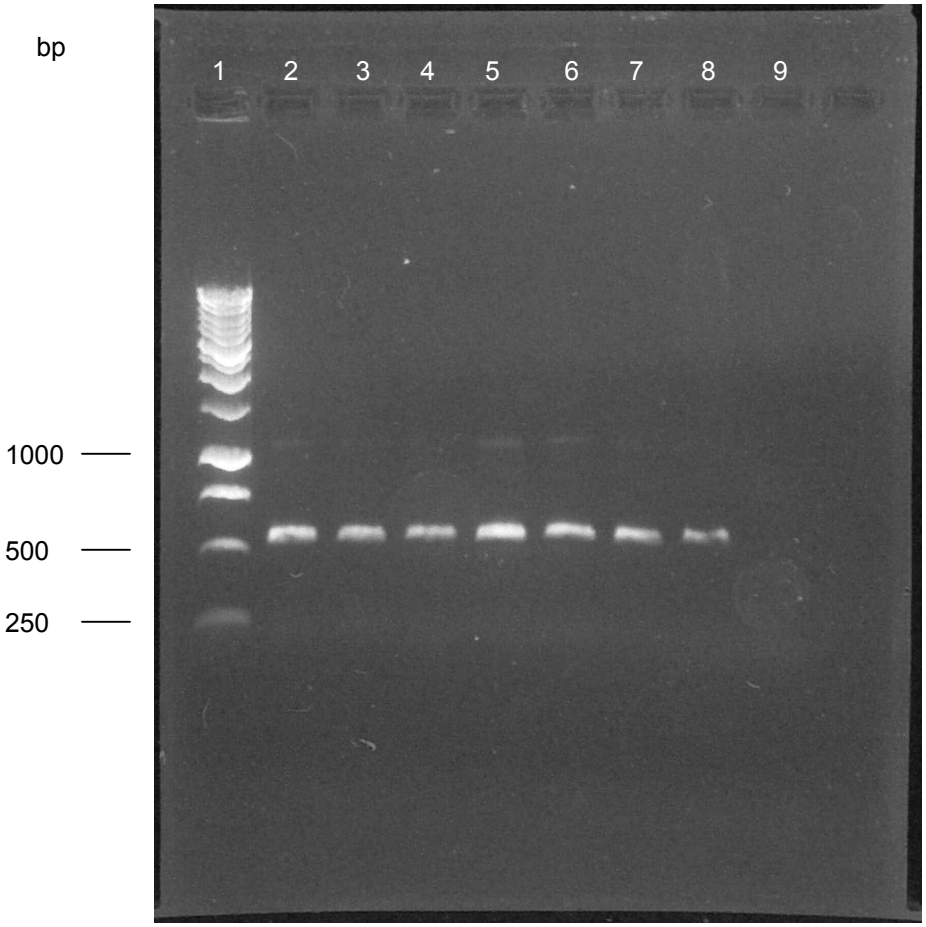
Top lanes: 1) 1 Kb ladder (Promega), 2) untreated wastewater (uw), 3) uw, 4) uw, 5) uw (1:10), 6) uw (1:10), 7) uw (1:10).
Bottom lanes: 1) 1 Kb ladder (Promega), 2) uw (1:100), 3) uw (1:100), 4) uw (1:100), 5) NRS30 (1:10), 6) no template control.

FIGURE 14: Enumeration of general heterotrophic bacteria and *Cloacibacterium* sp. from the wastewater plants surveyed.



*General heterotrophs from El Reno and Moore were enumerated by spread-plate colony counting. General heterotrophs from Norman and *Cloacibacterium* sp. from all sources were estimated by culture-dependent MPN and MPN-PCR respectively.

FIGURE 15: Amplification of *Cloacibacterium*-positive PCR reactions from Norman and Moore wastewater for sequence determination.



Lanes: 1) 1 Kb ladder (Promega), 2) Norman wastewater (NW), 3) NW (1:5), 4) NW (1:10), 5) Moore wastewater (MW), 6) MW (1:5), 7) MW (1:10), 8) NRS30 (1:10), 9) no template control.

DNA fragments from Norman and Moore samples exhibited a sequence homology to one another of 100%, and exhibited sequence homologies to all *Cloacibacterium* strains from 96.77% to 99.82%.

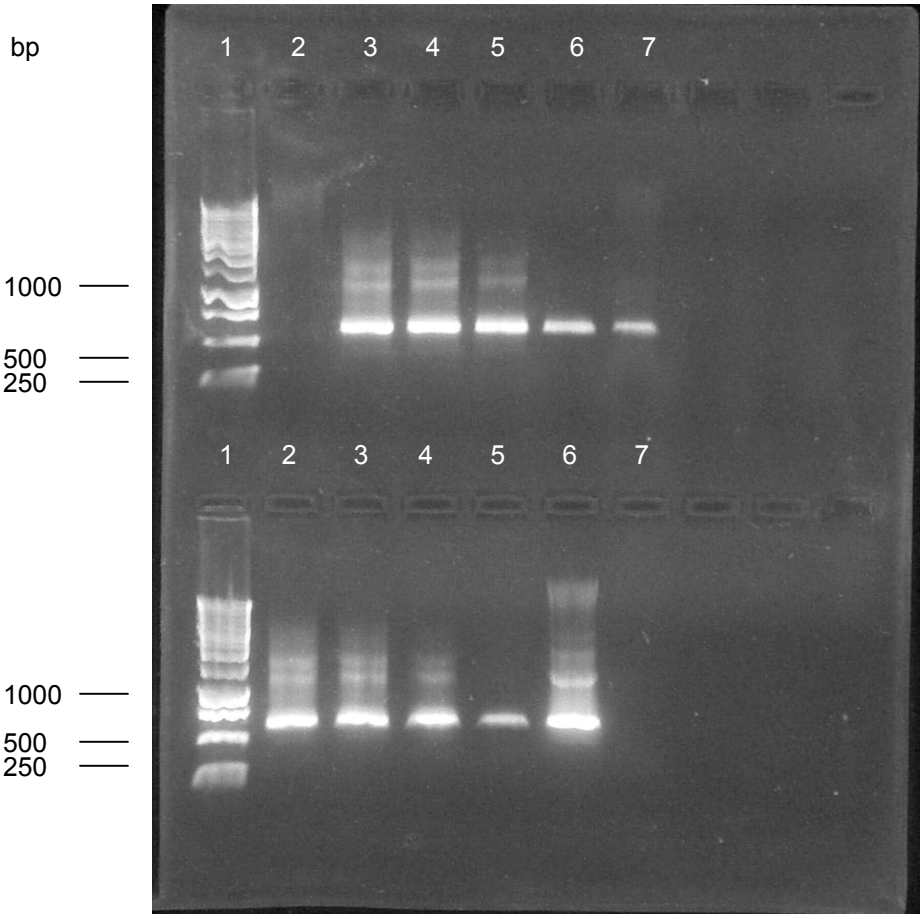
PCR of stool samples. Because *Cloacibacterium normanensis* was first observed to be present in relatively high numbers in municipal wastewater, it would be desirable to determine whether or not *Cloacibacterium* originated from fecal input into the plant.

Due to the high levels of suspended solids, humic acids, and inhibitory substances in stool samples, the whole cell method could not be used. DNA was therefore purified prior to PCR amplification. The yield of total DNA isolated using the QIAmp DNA stool mini kit yield was good, however the purity was less than what was expected (i.e. ~1.8-2.0) (TABLE 10). In order to estimate the yield of eubacterial DNA purified from this method, universal eubacterial primers were employed for PCR on genomic DNA isolated from representative stool samples. The resulting gel indicated the presence of amplifiable eubacterial DNA even with a dilution factor of 10^4 (FIGURE 16). Signal for *Cloacibacterium* was not detected from any of the ten human stool samples (TABLE 10) (an example is given in FIGURE 17). Representative stool DNA preparations were then "seeded" with purified *Cloacibacterium* DNA and PCR was performed to determine whether the high levels of genomic DNA isolated from stool would inhibit the PCR reaction. As seen in FIGURE 18, signal from *Cloacibacterium* sp. was detected and bands remained at a constant intensity with most concentrations of stool DNA

TABLE 10: Properties of DNA isolated from stool specimen.

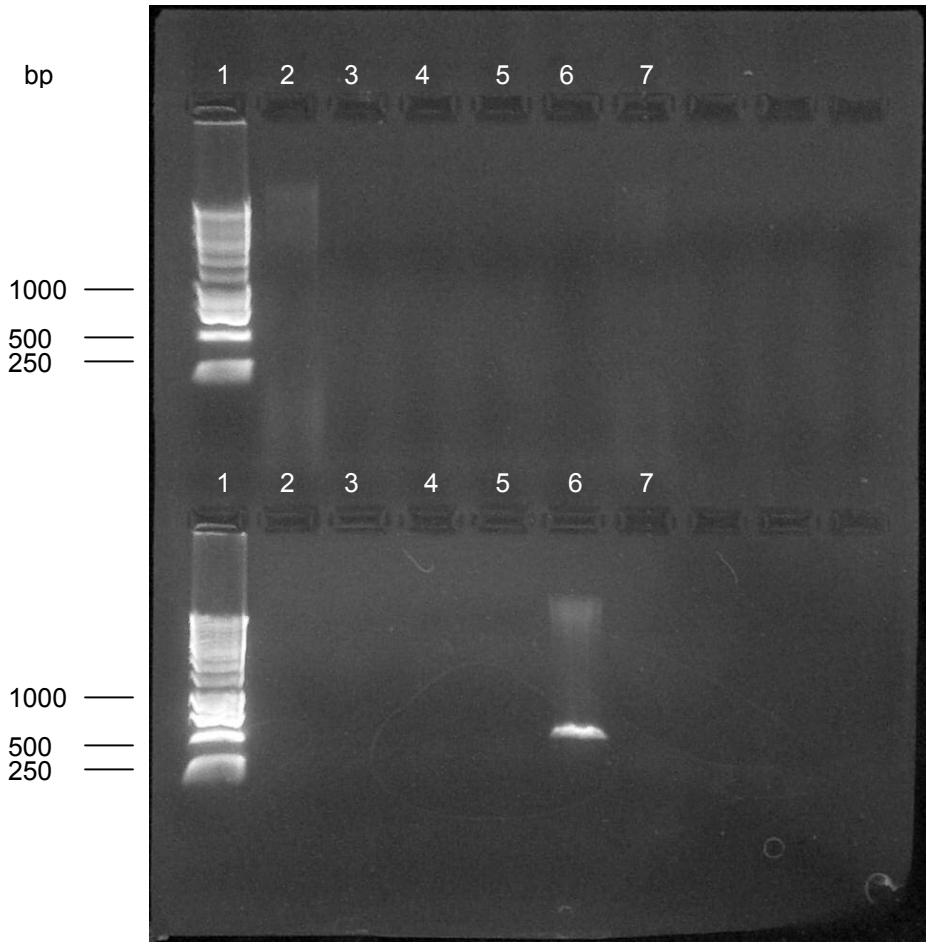
Specimen	Conc. (μg ml^{-1})	DNA purity ($A_{260/280}$)	*Cloacibacterium* sp. detected
A	86.88	1.34	–
B	193.25	1.34	–
C	81.50	1.37	–
D	96.75	1.57	–
E	193.50	1.42	–
F	162.00	1.39	–
G	184.50	1.26	–
H	155.00	1.49	–
I	125.33	1.62	–
J	204.00	1.27	–

FIGURE 16: Amplifiable bacterial DNA from stool specimen using universal eubacterial primers (357f and 907r).



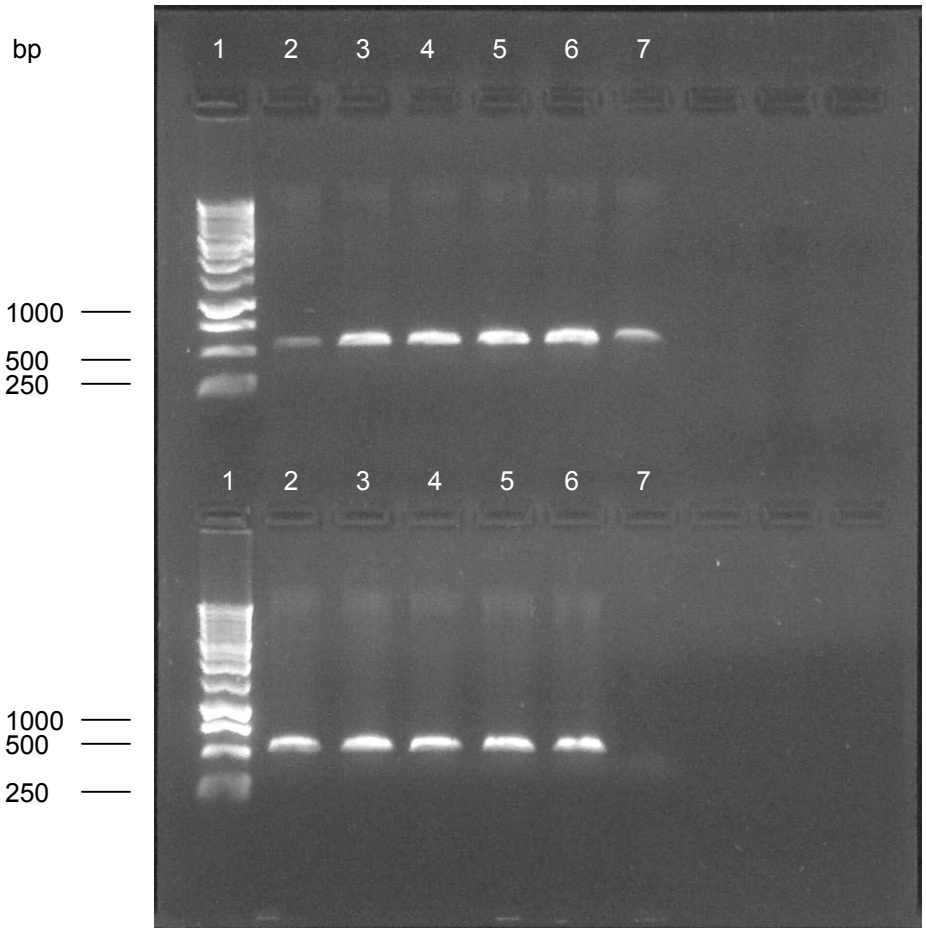
Top lanes: 1) 1 Kb ladder (Promega), 2) I, 3) I (1:10), 4) I(1:100), 5) I (1:1,000), 6) I (1:10,000), 7) J.
Bottom lanes: 1) 1 Kb ladder (Promega), 2) J (1:10), 3) J (1:100), 4) J 1:1,000), 5) J (1:10,000), 6) NRS 30 (1:10), 7) no template control.

FIGURE 17: PCR product was not detected from stool samples I or J using *Cloacibacterium*-specific primers Cloac-001f/r.



Top lanes: 1) 1 Kb ladder (Promega), 2) I, 3) I (1:10), 4) I(1:100), 5) I (1:1,000), 6) I (1:10,000), 7) J.
Bottom lanes: 1) 1 Kb ladder (Promega), 2) J (1:10), 3) J (1:100), 4) J 1:1,000), 5) J (1:10,000), 6) MRS7 (1:10), 7) no template control.

FIGURE 18: Amplifiable *Cloacibacterium* DNA in the presence of DNA isolated from stool specimen (I and J).



Top lanes: 1) 1 Kb ladder (Promega), 2) I+MRS7 3) I (1:10)+MRS7 4) I (1:100)+MRS7, 5) I (1:1,000)+MRS7, 6) I (1:10,000)+MRS7, 7) J+MRS7.
Bottom lanes: 1) 1 Kb ladder (Promega), 2) J (1:10)+MRS7, 3) J (1:100)+MRS7, 4) J (1:1,000)+MRS7, 5) J (1:10,000)+MRS7, 6) MRS7 (1:20), 7) no template control.

(except the undiluted sample).

These results provided evidence that the source of *Cloacibacterium* in the wastewater treatment plants surveyed was not likely from human input. However, due to the detection limit as well as the limited number of individual stool specimen processed, the possibility of *Cloacibacterium* sp. present as part of human GI microbiota in low numbers cannot be excluded.

In summary, many members of the family *Flavobacteriaceae* play the role of degraders of complex macromolecules in a diverse array of environments. While *Cloacibacterium* species are numerically dominant in municipal wastewater, the results of this study showed that there was no evidence to support that *Cloacibacterium* sp. is part of the normal microbiota of the human lower GI tract. The habitat for *Cloacibacterium* sp. was determined to be wastewater influent, where they were detected in moderate numbers. Most likely this genus can be found in other environments as well. For example, uncultured environmental clones from river biofilm samples (O'Sullivan *et al*., 2002), and uranium mining waste (Geissler *et al*., unpublished), as well as a clone from human carious dental lesion (Munson *et al*., 2004) exhibited high 16S rDNA sequence homology to *Cloacibacterium normanensis* strain NRS1^{T}, indicating other possible habitats for this genus. The numbers of *Cloacibacterium* sp. that were being reintroduced back into the environment were below the detection limit for our survey (< 200 cells ml^{-1}). Although a source of input was not elucidated, the environmental conditions in untreated wastewater may be favorable for the multiplication of

Cloacibacterium populations. The primers used in this study are good candidates for further environmental detection and enumeration of *Cloacibacterium* species.

Possible ecological role of *Cloacibacterium* sp. in sewage plants and future investigations. Members of the family *Flavobacteriaceae* are common in activated sludge and other parts of wastewater treatment plants (Güde, 1980; Benedict and Carlson, 1971), where they account for a relatively large proportion of the bacterial community. For example, the study by Güde (1980), found that CLB constituted 7 to 10% of the total heterotrophic count from activated sludge (with a seasonal decrease during summer months). In another study involving the community structure of a sulfate-reducing, microaerophilic, wastewater biofilm, members of the *Flavobacteriaceae* were shown to constitute 23% of the bacterial community, based on clone library sequencing of 16S DNA (Okabe *et al*., 2003). The conditions of the biofilm in this study are believed to be representative of the conditions in sewer lines.

The *Flavobacteriaceae* are not considered part of the colonic microbiota (Bernardet and Nakagawa, 2003), however, their numerical dominance within wastewater treatment plants signifies an important ecological role in this habitat. Some light has recently been shed on their possible ecological role in wastewater. Using an oligonucleotide probe specific for the *Cytophaga-Flavobacterium* group (CF319a) (Manz *et al.*, 1992), researchers have found that this group constituted a significant portion

(11-24%) of the microbial community of activated sludge from wastewater plants that employ enhanced biological phosphate removal (Liu *et al*., 2005; Wagner *et al*., 1994; Van Ommen Kloeke and Geesey, 1999). It is noteworthy that the *Cytophaga-Flavobacterium* group-specific probe used in these studies, CF319a, exhibited a 100% sequence homology to all strains of *Cloacibacterium*, indicating that *Cloacibacterium* sp. may have contributed to the findings listed above. A phylogenetic relative of *Cloacibacterium* (*Chryseobacterium defluvii*) was isolated from a phosphate-removing mixed culture obtained from activated sludge (Kämpfer *et al*., 2003), adding more evidence for the argument that this group may be important in the removal of phosphate from wastewater.

More importantly, members of the *Cytophaga-Flavobacterium* group have been shown to directly and significantly contribute to phosphate removal from activated sludge (Van Ommen Kloeke and Geesey, 1999). In this study, a combination of fluorescent staining for phosphatase activity and fluorescent *in situ* hybridization (FISH) were employed. Using probe CF319a, members of the *Cytophaga-Flavobacterium* group constituted 9.6% of the total community, 17-20% of phosphatase-active cells, and 35-45% of the total phosphatase activity measured (Van Ommen Kloeke and Geesey, 1999). Again, this study employed the *Cytophaga-Flavobacterium*-specific probe, CF319a, which includes the possibility of *Cloacibacterium* contributing to these results.

Based on these studies, *Cloacibacterium* sp. may play a role in the

removal of phosphate from wastewater. This organism was found in high numbers from untreated wastewater, and exhibited activities for several phosphatases (acid phosphatase, alkaline phosphatase and phosphoamidase) (TABLE 2), which are important for the release of organic phosphates. The phosphates released by the action of these enzymes are taken up by phosphate-accumulating organisms (PAO) and are stored inside the cell as inorganic polyphosphate (metachromatic or volutin) granules.

This hypothesis could be approached by asking two questions: First, what proportion of the total phosphatase activity in wastewater treatment plants is being produced by *Cloacibacterium* sp.? And second, what proportion of the phosphatase activity produced from members of the *Flavobacterium-Cytophaga* group is contributed by *Cloacibacterium* sp.?

This hypothesis could easily be tested by taking an approach similar to that of Van Ommen Kloeke and Geesey (1999). To determine the proportion of the total phosphatase activity produced by *Cloacibacterium* sp., first samples of untreated wastewater and activated sludge would be collected and screened for the presence of *Cloacibacterium* sp. using primers Cloac-001f/r and methods described above. Samples positive for the presence of *Cloacibacterium* sp. would be stained for local phosphatase activity by using the fluorogenic substrate ELF-P, then the samples would be costained by FISH using the *Cloacibacterium*-specific forward primer (Cloac-001f conjugated to a fluorescent dye) as a hybridization probe. Images would be produced by epifluorescence microscopy, and assayed using image analysis

software. The proportion of total phosphatase activity by *Cloacibacterium* sp. would then be calculated. This research would also estimate the density of *Cloacibacterium* sp. from activated sludge.

To determine the proportion of phosphatase activity produced from members of the *Flavobacterium-Cytophaga* group by *Cloacibacterium* sp., samples would be stained as outlined above using probe ELF-P (for local phosphatase activity) and Cloac-001, then stained using the *Flavobacterium-Cytophaga*-specific probe, CF319a conjugated to a second fluorescent dye. Images would be analyzed as outlined above, and the quantitative contribution of phosphatase activity by *Cloacibacterium* would then be compared to that of the *Flavobacterium-Cytophaga* group. This research would determine whether *Cloacibacterium* sp. are important for the release of phosphate, a precursor for the removal of phosphate by PAO (Blackall *et al.*, 2002). While *Cloacibacterium* sp. may be important in the release of phosphate in wastewater, it is also not known whether *Cloacibacterium* sp. accumulate the released phosphate. To test this, samples would first be stained with probe Cloac-001 as described above, then costained with 4',6-diamidino-2-phenylindole (DAPI), which stains polyphosphate granules (yellow emission) differentially than from cells (blue emission) (Allan and Miller, 1980). The results of this proposed research would potentially reveal the ecological role of *Cloacibacterium* sp. in wastewater and activated sludge samples.

CHAPTER THREE

The Microbiota of Oklahoma Lakes and Evidence for Natural Reservoirs of Antimicrobial Resistance

ABSTRACT

The acquisition of transferable multiple antimicrobial resistances has posed a major problem to microbiologists since the mid-twentieth century. The widespread use of antibiotics in humans and livestock has resulted in a dramatic increase in multiple antibiotic resistant (MAR) bacteria in virtually every facet of the environment. Previous studies have shown that the levels of antimicrobial resistances correspond to the level of wastewater contamination in the environment. A study comparing treated wastewater effluent to rural lakes was performed to determine the differences in antimicrobial resistances, dominant bacterial species, and enumeration of three populations: general heterotrophic bacteria, presumptive coliforms, and presumptive *E. coli.* Two of the lakes surveyed (Mountain Lake and American Horse Lake) are known to have little human impact and no apparent source of fecal contamination from human (sewage) or livestock sources. The dominant heterotrophic bacteria of the Norman wastewater outfall plume were strains of *Aeromonas.* The dominant heterotrophic bacteria from Mountain Lake were strains of *Aeromonas* and *Enterobacter*. The dominant heterotrophic bacteria from American Horse Lake were strains of *Pectobacterium*, *Citrobacter*, and a strain of *Microbacterium* that exhibited an unusually high resistance to ciprofloxacin. The dominant heterotrophic bacteria from Lake Thunderbird were strains of *Aeromonas*, *Plesiomonas*, and *Chryseobacterium.* Collectively, isolates from lake origin exhibited more

MAR than from the outfall plume, and isolates from the two lakes with the least human impact (Mountain Lake and American Horse Lake) exhibited more MAR than from the outfall plume. Isolates from Mountain Lake exhibited the most MAR strains, but two isolates from American Horse Lake exhibited unusual antibiotic resistances, *Citrobacter* strain AHW1G2 was resistant to eight of the antibiotics tested, and *Microbacterium* strain AHW1G3 was resistant to the synthetic antimicrobials ciprofloxacin and nalidixic acid. The high number of antimicrobial resistant strains from aquatic environments that are known to have limited human or livestock impact provide possible evidence that natural reservoirs of antimicrobial drug resistance exist in the environment.

INTRODUCTION

The acquisition of transferable resistance by bacteria to antimicrobial drugs has been well-documented and has unequivocally posed a major problem to bacteriologists since the mid-twentieth century (Watanabe, 1963). It has been well established that the widespread use of antibiotics in humans and livestock has resulted in multiple antibiotic resistances in bacteria (Aarestrup and Wegener, 1999; Feary *et al.*, 1972; Isenberg and Berkman, 1971; Novick, 1981). Studies have shown that withdrawing the use of antibiotics resulted in a decrease of antibiotic resistances in livestock (Langlois *et al.*, 1983; 1988). Ciprofloxacin-resistant bacteria were shown to increase after fluoroquinolone treatment of chicken flocks (Humphrey *et al*., 2005), which decreased again after treatment. Resistance to antimicrobial drugs in environmental strains has been correlated with the level of drug exposure (Anderson *et al.*, 1973; Linton *et al.*, 1974). Many bacteria isolated from wastewater treatment plants harbor multiple antibiotic resistances (MAR) (Gallert *et al.*, 2005; Walter and Vennes, 1985), and are readily transferable (Sturdevant and Feary, 1969; Mach and Grimes, 1982; Králikova *et al.*, 1984; McPherson and Gealt, 1986). For example, one study showed the percentage of ampicillin-resistant coliforms increased from the influent to the effluent (Iwane *et al.*, 2001). It is clear that human-impacted effluents contribute to the level of antimicrobial resistances in aquatic environments (Goñi-Urriza *et al.*, 2000; Esiobu *et al.*, 2002; Iwane *et al.*, 2001; Gallert *et al.*,

2005). However, MAR bacteria have also been isolated from waters with less apparent human impact (Bell *et al.*, 1983; Kelch and Lee, 1978; Ash *et al.*, 2002). Additionally, multiple-drug-resistant bacteria have been found in treated drinking water supples (Armstrong *et al.*, 1981; Calomoris *et al.*, 1984) and the treatment process may be selective for the multiplication of MAR bacteria (Armstrong *et al.*, 1982).

Recently some debate has occurred on whether bacteria carrying antibiotic resistances exist in natural reservoirs isolated from human-impacted environments, or exist only under instances of direct or indirect contamination from human or livestock waste. Previous research has suggested that a natural reservoir for antibiotic resistance genes does exist (Gilliver *et al.*, 1999; Sherley *et al.*, 2000). These two examples show that multiple antibiotic resistances occur in bacteria isolated from wild mammals. In contrast, another study suggested no such reservoir exists (Österblad *et al.*, 2001). In the study by Österblad *et al.* (2001), enterobacteria isolated from wild deer, moose, and vole exhibited very low levels of antimicrobial resistance, suggesting that the use of antibiotics is the primary cause of antibiotic resistances. In addition, it was noted that a higher population (and more antibiotic use) exists in the area where Gilliver *et al*. (1999) conducted their study than where Österblad *et al.* (2001) conducted their study. In addition to human population density, there are other considerations to be made when evaluating the contrasting results of these studies. Wide variations of antimicrobial resistance patterns based on bacterial species, geographic

location, and host species have been demonstrated (Sherley *et al.*, 2000). Differences in bacterial species composition have also been reported as a source of sample variation in antibiotic resistances in aquatic bacteria (Niemi *et al.*, 1983; Esiobu *et al.*, 2002).

The threat that MAR bacteria pose to public health is well understood. The transfer of antimicrobial resistance genes to pathogenic species has been documented (Blake *et al.*, 2003; Bell *et al.*, 1980). For example, environmental strains of fecal coliform bacteria have been shown to transfer single or multiple antimicrobial resistance to environmental *Salmonella* sp. (Bell *et al.*, 1980). Blake *et al.* (2003) demonstrated that antibiotic resistances were transferred from commensal bacteria to *E. coli* O157, as well as from *Salmonella* sp. back to commensal bacteria. Another concern is the acquisition of multiple drug resistances by opportunistic bacteria (von Graevenitz, 1977; Levy, 2002).

The use of coliform bacteria and *E. coli* as indicators of fecal contamination of waters has been a long-standing standard practice (Geldreich, 1966). *E. coli* is considered a specific indicator of fecal contamination (U.S. Environmental Protection Agency, 1986). However, *E. coli* has been questioned as an appropriate indicator because of its low survivability in the environment outside the colon (Jin *et al.*, 2004). Krumperman (1983) demonstrated that *E. coli* isolated from human origin or domestic animals in cases where antibiotics were routinely given had higher multiple antibiotic resistances than in cases where antibiotics were absent.

The extent to which *E. coli* is MAR may be used to help determine the source of contamination (Krumperman, 1983). However, this approach has shown variation between samples (Parveen *et al.*, 1997). Antibiotic resistance patterns have been used to discriminate between human and nonhuman sources in *E. coli* (Carroll *et al.*, 2005), fecal streptococci, and fecal coliform bacteria (Wiggins *et al.*, 1999; Harwood *et al.*, 2000). These studies and others (Kelch and Lee, 1978) provide evidence that bacteria with similar antimicrobial resistances originated from the same sources. Positive correlations of antibiotic resistance among different genera from common samples have been demonstrated, indicating a similar selective pressure among groups (Kelch and Lee, 1978).

The current study was performed to determine what differences exist in the microbiota of one sample site, contaminated with treated sewage, compared with rural lakes in Oklahoma. The lakes ranged from pristine to moderately impacted by human activity. Potential sources of impairment or maximum daily load violations on these lakes were within safety limits set and documented by the U. S. Environmental Protection Agency (1999), or the Oklahoma Department of Environmental Quality (2004). This study compared three populations of bacteria from each site (general heterotrophic bacteria, presumptive coliforms, and presumptive *E. coli*) including enumeration, antimicrobial susceptibility, and dominant heterotrophic species from each sample site.

METHODS AND MATERIALS

Surface water samples were aseptically taken from outfall of the wastewater treatment plant in Norman (Cleveland Co., OK), and these samples were compared to those of the following rural lakes: Mountain Lake (Carter Co., OK), American Horse Lake (Blaine Co., OK), and Lake Thunderbird (Cleveland Co., OK). The wastewater treatment plant in Norman currently has a maximum flow capacity of 30 million gallons per day, and has an average flow rate of 12 million gallons per day and is designed to treat wastewater for a population of over 100,000 (www.ci.norman.ok.us/). The lake areas surveyed range from virtually pristine to moderately utilized by human occupants. The number of inhabitants per square kilometer for Blaine and Carter counties is 33 and 143, respectively (www.factfinder.census.gov). However, the Lake Thunderbird area has the highest amount of human impact. The number of inhabitants per square kilometer for Cleveland Co. is 1,005, and the lake area contains homes, campsites, horse stables and equestrian trails, swimming beach, boat docks, and a marina. All lakes sampled are sources of drinking water for wildlife and domestic animals, and are classified as public and private water supplies (Oklahoma Department of Environmental Quality, 2004). Collection of water samples occurred between mid August to early September, and the water temperature varied between 24°C to 27°C.

General heterotrophic bacteria were enumerated by a most probable

number (MPN) assay from each sample site using half-strength tryptic soy broth (TSB) (Difco Laboratories). Most numerous culturable heterotrophic species were then isolated from each using tryptic soy agar (TSA) (Difco Laboratories). Presumptive coliform bacteria and *E. coli* were enumerated by pour-plate colony counting using CHROMagar ECC (Paris, France). Presumptive coliform bacteria and *E. coli* were sub-cultured onto EMB agar (Difco Laboratories) for verification, then isolated using TSA. Presumptive strains of *E. coli* were further confirmed using the methyl red and Voges-Proskauer tests. All strains were assayed for antibiotic resistance using the method of Bauer *et al.* (1966). Antibiotics tested using BBL Sensi-Discs (Becton-Dickinson and Company, Sparks, MD) were: ampicillin (10 µg), carbenicillin (100 µg), cefaclor (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), sulfathiazole (0.25 mg), tetracycline (30 µg), and trimethoprim (5 µg). Intermediate antimicrobial resistances shown in TABLES 12-15 were not scored as resistant, and appear only for comparison. Strains of *Aeromonas* are generally regarded as resistant to ampicillin (Rogol *et al.*, 1979; Havelaar *et al.*, 1987). Despite the report of ampicillin-sensitive strains of *Aeromonas* (Rahim *et al.*, 1984), a previous report showed that 99% of environmental isolates of *Aeromonas* are resistant to ampicillin (Goñi-Urriza *et al.*, 2000). Therefore, ampicillin resistance for strains identified as *Aeromonas* were not scored for this report. Most heterotrophic bacterial

isolates and presumptive coliforms were subjected to BIOLOG analysis for identification according to manufacturer's instructions (Hayward, CA). Isolates from the same sample and isolation conditions that exhibited similar cell and colony morphologies were not subjected to BIOLOG analysis. In these cases, representative strains were then chosen for analysis. All incubations were at 30°C.

RESULTS AND DISCUSSION

A total of 59 isolates were collectively recovered from surface water samples: 14 from Norman outfall, 12 from Mountain Lake, 15 from American Horse Lake, and 18 from Lake Thunderbird.

As expected, enumerations of general heterotrophic bacteria, presumptive coliforms, and presumptive *E. coli* were all higher from the Norman wastewater outfall plume than from the lakes surveyed (TABLE 11). Presumptive *E. coli* was essentially absent from the lakes surveyed, but was present in low numbers from Lake Thunderbird (TABLE 11). The essential absence of *E. coli* from the lakes surveyed demonstrates the apparent lack of fecal contamination at these sites. All lakes surveyed were in compliance with the EPA approved Oklahoma Water Quality Standards set by the Oklahoma Department of Environmental Quality (available at http://www.epa.gov/waterscience/standards/wqslibrary/ok/ok.html) (2003). The limits set by the Oklahoma DEQ for total coliform bacteria present in public and private water supplies are a mean of 50 ml^{-1}, and no more than 5% of samples taken can be over 200 ml^{-1} for a 30 day period. The limits set for *E. coli* present in primary body contact recreation waters are a mean of 1.26 ml^{-1}, and no sample can be over 4.06 ml^{-1}.

Most of the isolates recovered from the Norman wastewater outfall plume were presumptive *E. coli.* Additionally, two strains of *Aeromonas* were recovered as most numerous culturable heterotrophic bacteria (TABLE 12).

TABLE 11: Bacterial enumerations from surface water samples.

Site	Heterotrophs ml^{-1}*	Coliforms ml^{-1}†	*E. coli* ml^{-1}†
Norman wastewater outfall	8.0×10^5	4.8×10^2	3.1×10^1
Mountain Lake	1.4×10^4	2.7×10^1	$<2.3 \times 10^{-1}$
American Horse Lake	6.8×10^3	1.5×10^1	$<1.2 \times 10^{-1}$
Lake Thunderbird	1.4×10^4	2.1×10^1	2.4×10^{-1}

*Determined by a three-point MPN assay.
†Determined by pour-plate colony counting using CHROMagar ECC.

TABLE 12: Antimicrobial resistances of Norman wastewater outfall isolates.

Strain	ID/type	Antimicrobial resistance
Most numerous culturable heterotrophic bacteria		
OW1	*Aeromonas*[*]	carbenicillin, cefaclor, nalidixic acid intermediate resistance: erythromycin
OW2	"*Aeromonas*"[‡]	cefaclor, tetracycline, nalidixic acid, oxytetracycline intermediate resistance: erythromycin
Presumptive coliforms and *E. coli*		
OE6	*E. coli*[†]	Intermediate resistance: erythromycin
OE7	*E. coli*[†]	erythromycin, nalixic acid intermediate resistance: streptomycin
OE9	*E. coli*[†]	erythromycin, nalidixic acid
OE10	*E. coli*[†]	ampicillin, carbenicillin, doxycycline, erythromycin, oxytetracycline, nalidixic acid
OE11	*E. coli*[†]	ampicillin, carbenicillin, doxycycline, erythromycin, oxytetracycline, nalidixic acid, sulfathiazole, trimethoprim, tetracycline, streptomycin
OE12	*E. coli*[†]	erythromycin intermediate resistance: streptomycin
OE13	*E. coli*[†]	erythromycin intermediate resistance: ampicillin
OE16	*E. coli*[†]	erythromycin intermediate resistance: ampicillin
OE17	*E. coli*[†]	erythromycin
OE18	*E. coli*[†]	erythromycin intermediate resistance: streptomycin
OE19	*E. coli*[†]	erythromycin
OE20	*E. coli*[†]	erythromycin

[*]Determined by BIOLOG analysis.
[†]Determined by CHROMagar ECC phenotype, and verified by EMB phenotype, and MR/VP assay.
[‡]Presumptive identification based on BIOLOG analysis.

Although *Aeromonas* spp. are not considered part of the normal human GI microbiota, this result is not surprising as strains of *Aeromonas* are common in treated wastewater and waters contaminated with wastewater (Holmes, 1996; Araujo *et al*., 1989; Monfort and Baleux, 1991; Poffé and Op de Beeck, 1991). Strains of *Aeromonas* have been reported to be from 10^3 to 10^5 CFU ml^{-1} in treated sewage (Holmes, 1996), and have been correlated with coliforms in contaminated waters (Araujo *et al.*, 1989).

The dominant heterotrophic bacteria isolated from the lakes surveyed include strains of: *Aeromonas*, *Chryseobacterium*, *Citrobacter*, *Enterobacter*, *Microbacterium*, *Pectobacterium*, and *Plesiomonas* (TABLES 13-15). The ubiquituous presence of *Aeromonas* from the lakes surveyed was expected since *Aeromonas* spp. are considered part of the normal microbiota of aquatic environments (Hazen *et al.*, 1978; Holmes, 1996). However, since some of the *Aeromonas* strains in this study were MAR as well as numerically dominant in the lakes surveyed, a potential health risk does exist. Strains of *Aeromonas* have been associated with gastrointestinal infections, wound infections and septicemia in humans (Daily *et al.*, 1981; Gosling, 1996; Cahill, 1990). An interesting observation is that *Aeromonas* strain MLW2G2 had identical multiple antibiotic resistances to several of the coliforms isolated from the same site (TABLE 13). All strains identified as *Aeromonas* in this study were resistant to ampicillin, but this resistance does not appear in the tables nor was ampicillin scored as part of the multiple antibiotic profiles for these strains due to their intrinsic resistance to ampicillin (Goñi-Urriza *et al.*,

TABLE 13: Antimicrobial resistances of Mountain Lake isolates.

Strain	ID/type	Antimicrobial resistance
Most numerous culturable heterotrophic bacteria		
MLW1G1	No ID*	ampicillin, cefaclor, carbenicillin, kanamycin, streptomycin, tetracycline intermediate resistance: erythromycin, ceftriaxone, gentamycin, oxytetracycline
MLW1G2	No ID*	ampicillin, cefaclor, carbenicillin, streptomycin, tetracycline intermediate resistance: erythromycin, chloramphenicol, ceftriaxone, gentamycin, kanamycin, oxytetracycline
MLW2G1	*Enterobacter**	ampicillin, cefaclor, carbenicillin intermediate resistance: erythromycin
MLW2G2	*Aeromonas**	cefaclor, erythromycin intermediate resistance: carbenicillin
Presumptive coliforms and *E. coli*		
MLC1	ND†	ampicillin, cefaclor, erythromycin intermediate resistance: carbenicillin
MLC3	ND†	ampicillin, cefaclor, erythromycin intermediate resistance: carbenicillin, tetracycline
MLC5	*Enterobacter**	ampicillin, cefaclor, erythromycin intermediate resistance: streptomycin
MLC6	*Enterobacter**	ampicillin, cefaclor, erythromycin
MLC7	ND†	ampicillin, cefaclor, erythromycin intermediate resistance: carbenicillin
MLC8	No ID*	ampicillin, cefaclor, erythromycin intermediate resistance: carbenicillin
MLC9	No ID*	ampicillin, cefaclor, erythromycin intermediate resistance: carbenicillin
MLC11	ND†	ampicillin, cefaclor, erythromycin

*Determined by BIOLOG analysis.
†Not determined.

TABLE 14: Antimicrobial resistances of American Horse Lake isolates.

Strain	ID/type	Antimicrobial resistance
<u>Most numerous culturable heterotrophic bacteria</u>		
AHW1G1	*Pectobacterium*[*]	no resistance
AHW1G2	*Pectobacterium*[*]	intermediate resistance: erythromycin
AHW1G3	*Microbacterium*[#]	ciprofloxacin, nalidixic acid
AHW2G2	*Citrobacter*[*]	ampicillin, cefaclor, chloramphenicol, gentamycin, carbenicillin, kanamycin, oxytetracycline, tetracycline intermediate resistance: erythromycin, ceftriaxone, streptomycin
AHW2G3[‡]	ND[†]	ampicillin, cefaclor, carbenicillin intermediate resistance: erythromycin

[*]Determined by BIOLOG analysis.
[†]Not determined.
[‡]Trimethoprim not tested.
[#]Determined by partial 16S sequence analysis.

TABLE 14: Antimicrobial resistances of American Horse Lake isolates (cont.).

Strain	ID/type	Antimicrobial resistance
Presumptive coliforms and *E. coli*		
AHC16	No ID*	ampicillin, cefaclor, erythromycin, carbenicillin
AHC17	No ID*	ampicillin, cefaclor, erythromycin, carbenicillin
AHC18	ND†	ampicillin, cefaclor, erythromycin, carbenicillin intermediate resistance: doxycycline
AHC19	ND†	carbenicillin Intermediate resistance: ampicillin, erythromycin
AHC20	ND†	ampicillin, carbenicillin, erythromycin, cefaclor intermediate resistance: streptomycin
AHC21	No ID*	ampicillin, carbenicillin, erythromycin
AHC22	*Aeromonas**	cefaclor intermediate resistance: erythromycin
AHC23	ND†	ampicillin, erythromycin, cefaclor intermediate resistance: carbenicillin
AHC24	ND†	ampicillin, carbenicillin, erythromycin
AHC25	ND†	ampicillin, erythromycin intermediate resistance: cefaclor

*Determined by BIOLOG analysis.
†Not determined.

TABLE 15: Antimicrobial resistances of Lake Thunderbird isolates.

Strain	ID/type	Antimicrobial resistance
<u>Most numerous culturable heterotrophic bacteria</u>		
TLW1G1	*Plesiomonas*[*]	ampicillin, carbenicillin
TLW1G2[#]	*Aeromonas*[*]	cefaclor, carbenicillin Intermediate resistance: erythromycin
TLW1G3[#]	ND[†]	no resistance
TLW2G1[#]	*Chryseobacterium*[*]	ampicillin, cefaclor, carbenicillin, kanamycin Intermediate resistance: erythromycin
TLW2G2[#]	*Aeromonas*[*]	carbenicillin
TLW2G3[#]	ND[†]	ampicillin, cefaclor, carbenicillin Intermediate resistance: erythromycin

[*]Determined by BIOLOG analysis.
[†]Not determined.
[#]Trimethoprim not tested.

TABLE 15: Antimicrobial resistances of Lake Thunderbird isolates (cont.).

Strain	ID/type	Antimicrobial resistance
<u>Presumptive coliforms and *E. coli*</u>		
TC1	No ID[*]	ampicillin, cefaclor, erythromycin
TC2	ND[†]	ampicillin, cefaclor, erythromycin
TC3	ND[†]	ampicillin, cefaclor, erythromycin
TC4	ND[†]	erythromycin
TC5	ND[†]	ampicillin, cefaclor Intermediate resistance: erythromycin
TC6	ND[†]	ampicillin, cefaclor Intermediate resistance: erythromycin
TC7[#]	ND[†]	ampicillin, cefaclor, erythromycin
TC8	ND[†]	ampicillin, cefaclor, erythromycin
TC9	ND[†]	ampicillin, cefaclor Intermediate resistance: erythromycin
TC10	ND[†]	ampicillin, erythromycin, carbenicillin
TE1[#]	*E. coli*[‡]	ampicillin, carbenicillin Intermediate resistance: erythromycin
TE2[#]	*E. coli*[‡]	ampicillin Intermediate resistance: erythromycin

[*]Determined by BIOLOG analysis.
[†]Not determined.
[‡]Determined by CHROMagar ECC phenotype, and verified by EMB phenotype, and MR/VP assay.
[#]Trimethoprim not tested.

2000). This result agrees with previous findings that different Gram-negative genera from the same source can exhibit similar antimicrobial resistance patterns (Kelch and Lee, 1978). This result may be indicative of a similar antibmicrobial pressure exhibited on the bacteria in the sample and possible horizontal gene transfer of R-factors (Adams *et al.*, 1998), although this was not tested in the current study.

Collectively, of the lake isolates, a striking pattern of multiple antimicrobial resistance emerged. Of the 45 isolates collected from the lake water samples, 82.2% were resistant to two or more antimicrobials, 62.2% were resistant to three or more, and 17.8% were resistant to four or more (TABLE 16).

Interestingly, isolates from the Norman wastewater outfall plume exhibited a lower overall multiple antimicrobial resistance pattern than from the lakes surveyed (TABLE 16). This result could be due to the species composition of the strains from the outfall plume were mostly *E. coli* (Sherley *et al.*, 2000; Niemi *et al.*, 1983; Esiobu *et al.*, 2002). This observation does not agree with previous observations that levels of resistance correspond to the level of drug exposure (Anderson *et al.*, 1973; Linton *et al.*, 1974). In the current study, frequencies of multiple antibiotic resistance was observed from lakes in sparsely-populated areas with no apparent antibiotic input that in many cases exceeded the frequency of multiple antibiotic resistance from treated wastewater, an environment that receives antibiotic input. However, the findings of this study agree with the findings of Gilliver *et al.*, (1999), that

TABLE 16: Multiple antimicrobial resistances of water isolates.

Site	Total isolates	MAR isolates resistant to at least* two	three	four
Norman wastewater outfall	14	6 (42.9%)	4 (28.6%)	3 (21.4%)
Mountain Lake	12	12 (100%)	11 (91.7%)	2 (16.7%)
American Horse Lake	15	11 (73.3%)	9 (60%)	5 (33.3%)
Lake Thunderbird	18	14 (77.8%)	9 (44.4%)	1 (5.6%)
Lake totals	45	37 (82.2%)	29 (62.2%)	8 (17.8%)

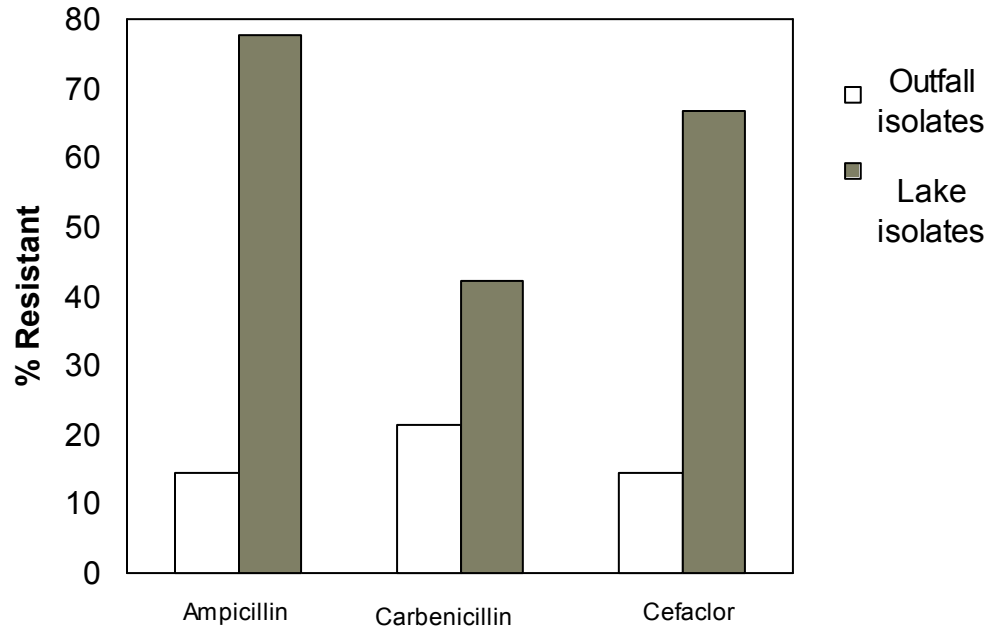
*Intermediate resistances were not considered.

multiple antibiotic resistant bacteria can be isolated from environments with little or no apparent antibiotic input.

In general, most of the resistances observed were to β-lactams (FIGURE 19), where a higher percentage of lake isolates were resistant to ampicillin and carbenicillin than wastewater outfall isolates. The same observation was made for the cephalosporin cefaclor (FIGURE 19). Resistance to erythromycin was high from both wastewater outfall and the lakes surveyed (FIGURE 20). To a lesser extent, a higher percentage of isolates with resistances to tetracyclines was found from wastewater outfall (FIGURE 21). Also to a lesser extent, percentages of isolates with resistances to nalidixic acid, trimethoprim, and sulfathiazole were higher from wastewater outfall (FIGURE 22), however resistance to ciprofloxacin was found from one of the lakes surveyed whereas none was found from wastewater outfall.

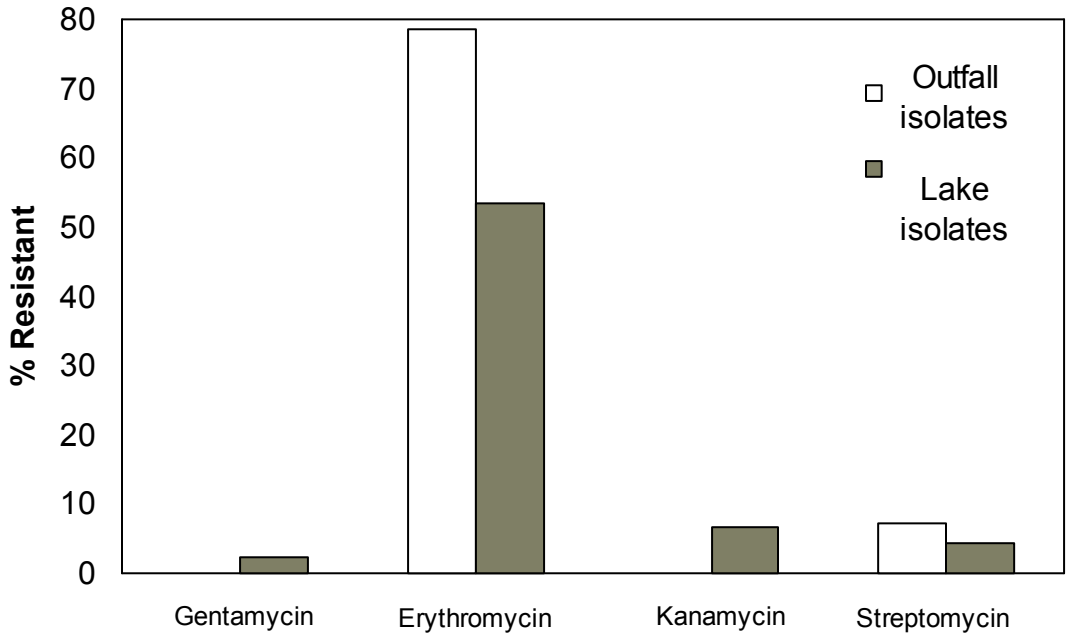
As noted above, most of the higher antimicrobial resistances from the lakes surveyed were resistances to β-lactams. These lakes are presumed to exhibit no apparent evidence of selective pressure from the presence of antimicrobial drugs. This poses the question of how these resistances were established and maintained. A possible answer might be the natural evolution of genes intended for other purposes (Baquero and Blázquez, 1997; Davies, 1994). For example, some mechanisms of resistance to aminoglycosides are due to a chromosomal 2'-*N*-acetyltransferase, which has been shown to be involved with maintenance of peptidoglycan structure

FIGURE 19: Resistances of water isolates to β-lactams and cefaclor*.



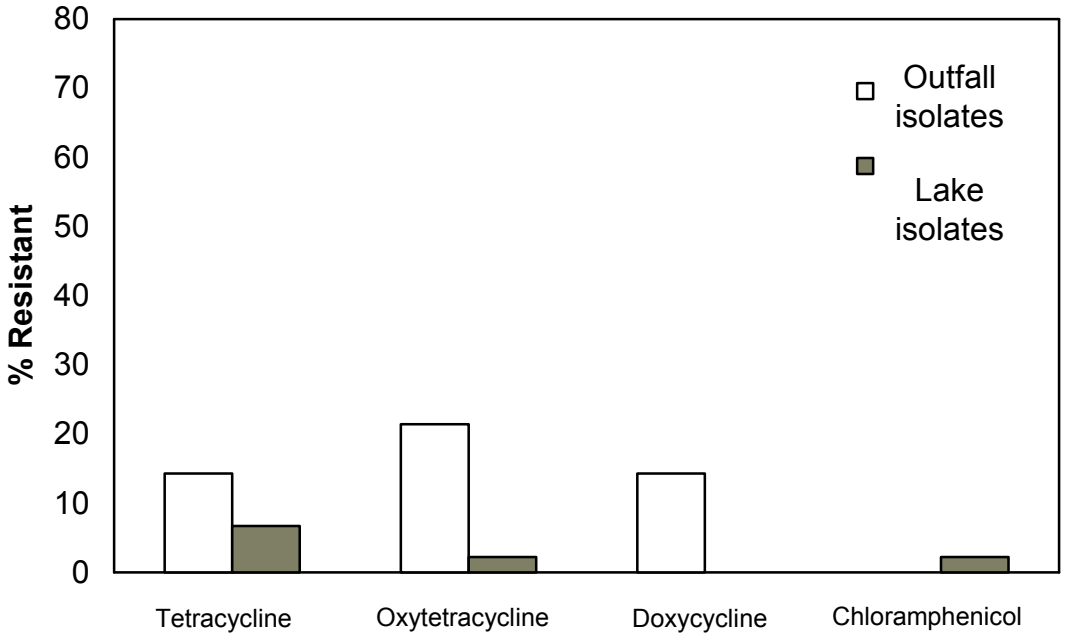
*Intermediate resistances were not considered.

FIGURE 20: Resistances of water isolates to aminoglycosides*.



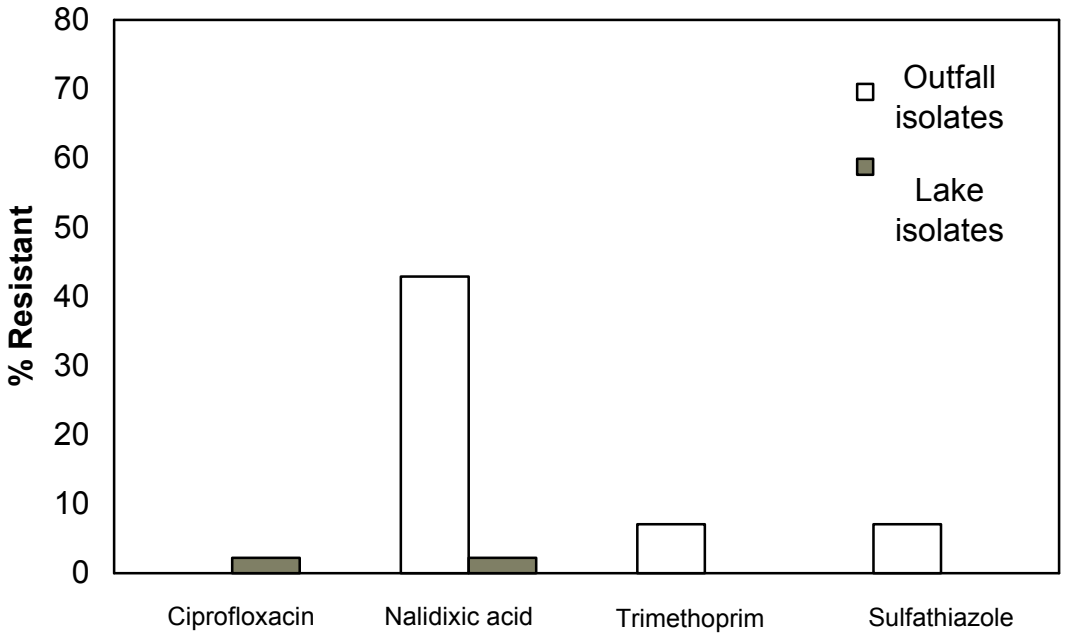
*Intermediate resistances were not considered.

FIGURE 21: Resistances of water isolates to tetracyclines and chloramphenicol*.



*Intermediate resistances were not considered.

FIGURE 22: Resistances of water isolates to fluoroquinolone, quinolone, trimethoprim, and sulfonamide antimicrobials*.



*Intermediate resistances were not considered.

(Payie *et al.*, 1995). A previous report revealed evidence for chromosomal β-lactamases from environmental isolates, suggesting a natural source for transferable resistances to penicillins, cephalothin and cefturoxime (Humeniuk *et al.*, 2002). The genes listed above likely evolved before the antibiotic era (Baquero and Blázquez, 1997), and suggest antibiotic-resistance does occur outside of antibiotic-induced selective pressure.

A striking resistance to the fluoroquinolone, ciprofloxacin, was discovered from an isolate from American Horse Lake, strain AHW1G3. The MIC of ciprofloxacin of this isolate was found to be 400 μg ml^{-1} (Liou *et al*., unpublished data). This result was in contrast to the MIC reported for clinical isolates such as *Streptococcus pneumoniae* (MIC for >99% of strains <4 μg ml^{-1}) (Sahm *et al.*, 2002), and *Bacillus anthracis* (MIC ranges from 0.06-2 μg ml^{-1}) (Bryskier, 2002). Although rare, other ciprofloxacin-resistant bacteria have been isolated from environmental sources (Waldenström *et al.*, 2005; Ash *et al.*, 2002; Goñi-Urriza *et al*., 2000; Edge and Hill, 2005). The study by Ash *et al.* (2002) found Gram-positive and Gram-negative river isolates resistant to ciprofloxacin, but no MIC data was reported for this resistance. A ciprofloxacin-resistant strain of *Aeromonas* was isolated from river water samples (Goñi-Urriza *et al*., 2000). The reported MIC of this isolate (4 μg ml^{-1}) was again markedly less than strain AHW1G3. A ciprofloxacin-resistant strain of *Campylobacter jejuni* was isolated from a wild bird (Waldenström *et al.*, 2005). This isolate had a reported MIC of 128 μg ml^{-1}, still less than strain AHW1G3. Ciprofloxacin-resistant strains of

Campylobacter have been found in agriculture in the United States (MICs >32 and 24 μg ml^{-1}) (Sato *et al.*, 2004). Previous studies have shown that in poultry flocks, ciprofloxacin-resistant *Campylobacter* species can be isolated in high numbers following treatment with fluoroquinolones (Humphrey *et al.*, 2005; Griggs *et al.*, 2005). This would support the idea that fluoroquinolone treatment rapidly selects for resistant strains, however no apparent history of ciprofloxacin treatment or contamination was associated with the environmental studies (Ash *et al.*, 2002; Goñi-Urriza *et al*., 2000; Waldenström *et al.*, 2005), with the study of Sato *et al.* (2004), or with this study. Edge and Hill (2005) recently found ciprofloxacin-resistant strains of *E. coli* from wastewater. Although the MICs were not determined in this study, the strains of *E. coli* exhibited resistance to ciprofloxacin to at least 20 μg ml^{-1} (Edge and Hill, 2005).

Since fluoroquinolone antimicrobials are synthetic, bacteria have never previously encountered this drug. Bacterial enzymatic inactivation of fluoroquinolones is not likely to occur, and modification of the target site is the most common mechanism for resistance to fluoroquinolones (Schmitz, *et al.*, 2002). The primary mechanism for bacterial fluoroquinolone resistance are point mutations in the genes that code for the A and B subunits of DNA gyrase (Yoshida *et al.*, 1990; Yoshida *et al*., 1991; Wang *et al.*, 1993). Point mutations in the quinolone resistance-determining region of the *gyrA* gene are considered the primary cause of fluoroquinolone resistance (Giraud *et al.*, 1999), however mutations in both the *gyrA* and *gyrB* genes have been shown

to cause high-level resistance to ciprofloxacin (MIC 32 μg ml^{-1}) (Heisig, 1993). Similarly, point mutations in genes encoding DNA topoisomerase IV (*parC* and *parE*) have been shown to confer resistance to ciprofloxacin (Ferrero *et al.*, 1994). It appears that certain species have higher frequencies of mutations in the DNA gyrase gene that select for ciprofloxacin-resistant strains in the presence of ciprofloxacin (Griggs *et al.*, 2005). Ciprofloxacin-resistance may also be enhanced by the overexpression of multidrug efflux pumps (Pumbwe *et al.*, 2004), which might explain the presence of ciprofloxacin-resistance in MAR bacteria. There is no solid evidence for transferable high-level ciprofloxacin resistance between strains. However, very low levels of plasmid-transferable ciprofloxacin resistance have been documented (Martínez-Martínez, *et al.*, 1998; Cheung *et al.*, 2005; Wang *et al.*, 2003; 2004). These reports show that very low levels (MIC 0.0064-0.25 μg ml^{-1}) of ciprofloxacin resistance are conferred in recipient strains. This low level resistance is conferred by protein Qnr, which may protect DNA gyrase from quinolones (Martínez-Martínez, *et al.*, 1998; Tran *et al.*, 2005). However, this type of quinolone resistance appears to be rare among clinical isolates screened (Jacoby *et al.*, 2003).

This report found the existance of high levels of antimicrobial resistance in numerically-dominant heterotrophs and presumptive coliforms from rural lakes in sparsely-populated areas that have little human impact, and no apparent antibiotic input. How these resistances were established and were maintained remains a mystery, however these findings agree with

the belief that natural reservoirs of antimicrobial resistances exist.

Future work, determination of mechanism of ciprofloxacin resistance and further environmental investigation. *Microbacterium* strain AHW1G3 exhibited an unusually high resistance (400 μg ml^{-1}) to the fluoroquinolone, ciprofloxacin. Such a high level of resistance to ciprofloxacin has not been encountered in the literature. This observation raises the question of what mechanism(s) are involved in this level of resistance. The mechanism(s) of this level of resistance could be investigated by employing several known techniques. First, the genes in strain AHW1G3 encoding the subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) can be amplified by PCR using degenerate primers targeting the conserved sequences of these genes (Muñoz and De La Campa, 1996). The PCR product can be sequenced and analyzed for common point mutations responsible for fluoroquinolone resistance in high G+C Gram-positive cocci (Schmitz *et al*., 2002). Since the overexpression of efflux pumps also contribute to ciprofloxacin resistance (Pumbwe *et al.*, 2004; Schmitz *et al.*, 2002), the contribution to ciprofloxacin resistance in strain AHW1G3 by efflux pumps can be investigated using reserpine, an inhibitor of Gram-positive efflux pumps (Markham, 1999). In addition, expression of the known genes encoding efflux pumps could be measured in strain AHW1G3 as well as in other strains including those that exhibit lower levels of ciprofloxacin resistance. The expression the genes encoding efflux pumps in the presence of ciprofloxacin relative to a housekeeping gene could be measured with

reverse-transcriptase PCR (RT-PCR) assays (Eaves *et al.*, 2004; Plumbe *et al*., 2004). This research would provide evidence whether the expression of efflux pumps contribute to the high-level ciprofloxacin resistance in strain AHW1G3. Ciprofloxacin resistance in strain AHW1G3 is probably due to mutations in the DNA gyrase or topoisomerase IV genes, however the level of overexpression of efflux pumps is expected to correlate with the high level of ciprofloxacin resistance in this organism.

In addition to the research outlined above, further environmental investigations of American Horse Lake should be conducted. A screening of the lake and the Canadian River tributaries upstream and downstream for verification of the absence of fluoroquinolone antimicrobials should be conducted. A survey of the lake and tributaries for bacteria resistant to ciprofloxacin should then be conducted. The media used should contain ciprofloxacin from 0.5 to 300 μg ml^{-1} to collect organisms that are resistant to both high-levels and low-levels of this drug. After initial isolation, all strains collected should be identified, and MICs of their resistance to fluoroquinolones should be determined. This data would determine if any correlations exist between bacterial species and levels of fluoroquinolone resistance. If correlations between species and level of resistance are determined, then the mechanisms involved should be investigated using the methods outlined above. This research would determine whether the observed high level of ciprofloxacin resistance may be intrinsic in certain bacterial species or groups.

APPENDIX

Phenotypic Characterization of Two

Cloacibacterium-like Strains

Two additional strains (NRS10 and NRS19) that possess similar characteristics to *Cloacibacterium* were isolated from municipal wastewater from Norman (Cleveland Co., OK). These two strains were initially screened for similarities to *Cloacibacterium* strain NRS1^T by the same methods listed in Chapter One. The results of the partial characterization of strains NRS10 and NRS19 are listed below.

Strains NRS10 and NRS19 were Gram-negative, catalase and oxidase positive, non-spore forming rods. Both strains produced carotenoid-type pigments, and the KOH test for flexirubin-type pigments was negative for both strains. Neither exhibited gliding motility as observed with a wet mount. Both strains hydrolyzed starch, esculin and weakly hydrolyzed DNA and gelatin. Neither strain hydrolyzed casein, urea, citrate, chitin, pectin, alginate, uric acid, xanthine, or hypoxanthine. Both strains were methyl red and Voges-Proskauer negative, and nitrate was not reduced. Neither strain degraded cellulose or agar. Both strains grew at temperatures from 23ºC to 36ºC. Strain NRS19 also grew at 18ºC, however strain NRS10 did not. The only result from strains NRS10 and NRS19 that differs from the *Cloacibacterium* strains was with casein hydrolysis. All *Cloacibacterium* strains characterized in Chapter One hydrolyzed casein.

Strain NRS10 showed no resistance to any of the antibiotics tested. Strain NRS19 was resistant to erythromycin, nalidixic acid, and kanamycin, which was similar to the antibiotic resistance profiles of NRS32 and MRS14.

BIOLOG analysis did not produce an identification for either strain.

Both strains utilized the following: α-cyclodextrin, dextrin, glycogen, tween 40, tween 80, D-galactose, cellobiose, gentabiose, α-D-glucose, maltose, α-ketovaleric acid, L-alanylglycine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, L-threonine, inosine, uridine, thymidine, glycerol, D,L-α-glycerol phosphate and α-D-glucose-1-phosphate. Differences between the two strains are summarized in TABLE 17. None utilized the following: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, l-erythritol, D-fructose, L-fucose, *m*-inositol, α-D-lactose, lactulose, D-mannitol,, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, xylitol, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, D,L-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol. The BIOLOG data from NRS10 and NRS19 were consistent with the *Cloacibacterium* strains described in Chapter One.

The results of the FAME analysis were also consistant with those of

TABLE 17: Differences between strains NRS10 and NRS19 from BIOLOG analysis.

Substrate	NRS10	NRS19
D-arabitol	+	–
D-trehalose	+	–
Turanose	+	–
Pyruvic acid methyl ester	–	+
Succinic acid mono-methyl ester	–	+
L-asparagine	–	+
L-aspartic acid	–	+
Urocanic acid	+	–
D-glucose-6-phosphate	+	–

the described *Cloacibacterium* strains, although strain NRS10 exhibited slightly less consistency with the other strains (TABLE 18). Strains NRS10 and NRS19 exhibited a predominance of 15:0 *iso* (21-38%), 15:1 *iso* (unknown unsaturation position) (4-9%), 13:0 *iso* (2-9%) and 17:0 *iso* 3-OH (6%).

From the partial phenotypic results, strains NRS10 and NRS19 are likely strains of *Cloacibacterium*, although strain NRS10 is likely a different species than the other strains.

TABLE 18: Cellular fatty acid composition of strains NRS10 and NRS19.

	NRS10	NRS19
$C_{12:0\ 3\text{-}OH}$	1.2	1.7
$C_{13:0\ iso}$	2.1	9.3
$C_{13:0\ anteiso}$		1.1
$C_{13:1\ at\ 12\text{-}13}$	1.2	
$C_{14:0}$	2.6	
$C_{14:0\ iso}$	4.0	3.1
$C_{15:0}$	2.9	
$C_{15:0\ iso}$	20.7	38.0
$C_{15:0\ anteiso}$	11.6	5.4
$C_{15:0\ 2\text{-}OH}$	1.5	
$C_{15:0\ iso\ 3\text{-}OH}$	2.5	4.0
$C_{15:1\ iso\ F}$*	4.4	9.4
$C_{15:1\ anteiso\ A}$*	7.2	5.7
$C_{16:0}$	5.9	
$C_{16:0\ iso}$	3.0	
$C_{16:0\ 3\text{-}OH}$	6.6	
$C_{16:0\ iso\ 3\text{-}OH}$	3.8	1.8
$C_{16:1\ iso\ H}$	1.1	
$C_{16:1\ (\omega 5c)}$	2.7	
$C_{17:0\ iso\ 2\text{-}OH}$	2.0	
$C_{17:0\ iso\ 3\text{-}OH}$	5.8	6.2

*Unknown unsaturation position.

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