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HALOPHILIC AND HALOTOLERANT BACTERIA FROM RIVER WATERS AND SHALLOW GROUNDWATER ALONG THE ROUGE RIVER OF SOUTHEASTERN MICHIGAN

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ABSTRACT

The use of sodium chloride to melt highway and road snow is believed to have a significant effect on the groundwater ecosystem of the rivers where the salt from the roads drain. As the river composition changes, the bacterial population also changes to favour those bacteria that are more suited to the higher salt concentrations. In this experiment, we surveyed the cultivable salt-loving organisms (halophiles) on three sites that encompass the Rouge River (Lotz; site 1, Lilly, site; 8, and Ford Field, site 9). A total of 125 isolates were surveyed. Representative isolates of distinct morphologies were subjected to physiological test, using API strips and identified by 16 rDNA sequence analysis. The 16S rDNA sequences were analyzed and compared with sequences from Genbank. Results indicated that the SSU rRNA sequences of the bacterial isolates were similar to six major genera, *Bacillus, Staphylococcus, Halobacillus, Paenabacillus*, *Halomonas*, and *Clostridium.* Half of the isolates sequenced were similar to *Bacillus* spp. The API assay showed that the majority of the isolates were positive for the enzymes tryptophane deaminase, gelatinase and β-galactosidase. Indole production, acetoin production and citrate utilization were not observed for any isolates. Fermentation of carbohydrates was observed for very few isolates. The primary enzyme found in all isolates was arginine dihydrolase, which might be an indicator of the presence of such enzyme in halophilic and halotolerant bacteria present in the Rouge River.

Keywords: Enzymes, 16S rDNA, *Halomonas*, *Halobacillus*, *Bacillus*, halophiles

INTRODUCTION

The use of sodium chloride to melt highway and road snow is also believed to have a significant effect on the health of the Rouge River. Salts are readily mobilized by rain and meltwaters; hence they can easily enter rivers, lakes, and shallow groundwater systems where they can cause serious degradation of water quality [1-6]. Studies have shown that a high percentage of deicing salts can be removed as surface run-off and subsequently delivered to rivers and streams [5-6]. Groundwater contamination by road de-icing chemicals has been described many authors. For instance, in Massachussetts, Illinois, and Wisconsin the use of chloride on state highways was held responsible for chloride concentrations in local groundwaters exceeding the drinking water standard of 250 mg l^{-1} [1-3]. In Southeastern Michigan, chloride concentrations as high as 2500 mg l^{-1} have been recorded from sections of the Rouge River that received direct run-off [4]. This concentration is 30 times higher than comparative upstream concentrations [5]. Previous studies have indicated that road salt may have negative impacts on the flora and fauna of aquatic systems and it has been shown that different concentrations of NaCl can affect normal osmoregulatory and physiological processes of individual aquatic invertebrates [7-8].

As deicing salts are removed as surface run-off and subsequently delivered to river, the ecology and composition of the river changes. The microbial populations also change, to allow growth of those microbes that are more suited to the higher salt concentrations. Hence, halophilic and halotolerant bacteria are likely to be found at the site. Salt normally means NaCl, and the distinction between tolerance for salt and requirement for salt should be noted. There are several categories of halotolerant microbes: non-tolerant, those which tolerate only a small concentration of salt (about 1% w/v); slightly tolerant, tolerating up to 6-8%; moderately tolerant, up to 18-20%; and extremely tolerant, those microbes that grow over the whole range of salt concentrations from zero

up to saturation [9]. Halophilic microorganisms are those that require salt for growth: non-halophilic, those that are often stimulated in their growth by a small amount of salt (about 1% in the growth medium); slightly halophilic, grow optimally in the presence of 2-3% NaCl; moderately halophilic, grow best somewhere in the range of 5-10% NaCl (w/v); and extremely halophilic grow optimally at NaCl concentrations greater than 10% (w/v) [9].

Halophilic bacteria have the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Research on the halophilic and halotolerant bacteria often seems to be less glamorous than the study of the archaea, with their unique adaptations, including a highly saline cytoplasm, specialized salt-requiring proteins, and the unique light-driven proton and chloride pumps bacteriorhodopsin and halorhodopsin [10]. Halophilic bacteria were often neglected, even though they inhabit a wide range of habitats such as saline lakes, saltern ponds, desert and hypersaline soils, and salted foods, a range much less restricted than the habitats in which the halophilic archaea thrive [11-12]. Although they are less exciting at first glance than the extreme halophiles, the moderately halophilic bacteria and solute-tolerant microorganisms in general, pose quite sufficiently interesting questions, especially those implied by their ability to grow over wide ranges of solute concentrations.

Recently, there have been indications that halophilic and halotolerant bacteria may have greater potential in degradation of pollutants than was previously assumed. For instance, *Marinobacter hydrocarbonoclasticus* degraded a variety of aliphatic aromatic hydrocarbons [13]. A halotolerant *Streptomyces* sp., isolated from an oil field in Russia, degraded crude petroleum [14]. To date, microbiological studies of river waters and shallow groundwater wells along the Rouge River have focused on the enumeration on indicator bacteria that impact surface water quality [15]. The work described here represents one of the first studies to describe the population of halophilic and halotolerant bacteria in the Rouge River. Ultimately, the isolates presented here may help us understand their potential roles in bioremediation.

MATERIALS AND METHODS

Site Description and Sample Collection

Three sites (Lotz, site mw-1; Lilly, site mw-8; and Ford Field, site mw-9) were surveyed along the Rouge River that passes through a heavily commercial and urbanized part of Southeast Michigan (Figure 1). Well locations selected were generally immediately down gradient of major intersections. For example, the sites at Lotz (site mw-1) and Lilly (site mw-8), represent the locations of major intersections along Michigan avenue where land use impacts were likely to occur. Although the Ford Field site (site mw-9) is further north of Michigan Avenue, it is located just east of Ford Field, a large recreational site used by the City of Dearborn for sporting and other events. Each of the nine location is also located immediately down-gradient of one or more combined sewer overflows (CSO) sites. At each of the three well locations, surface water (river water) and groundwater samples were collected.

River water samples were obtained by wading into the

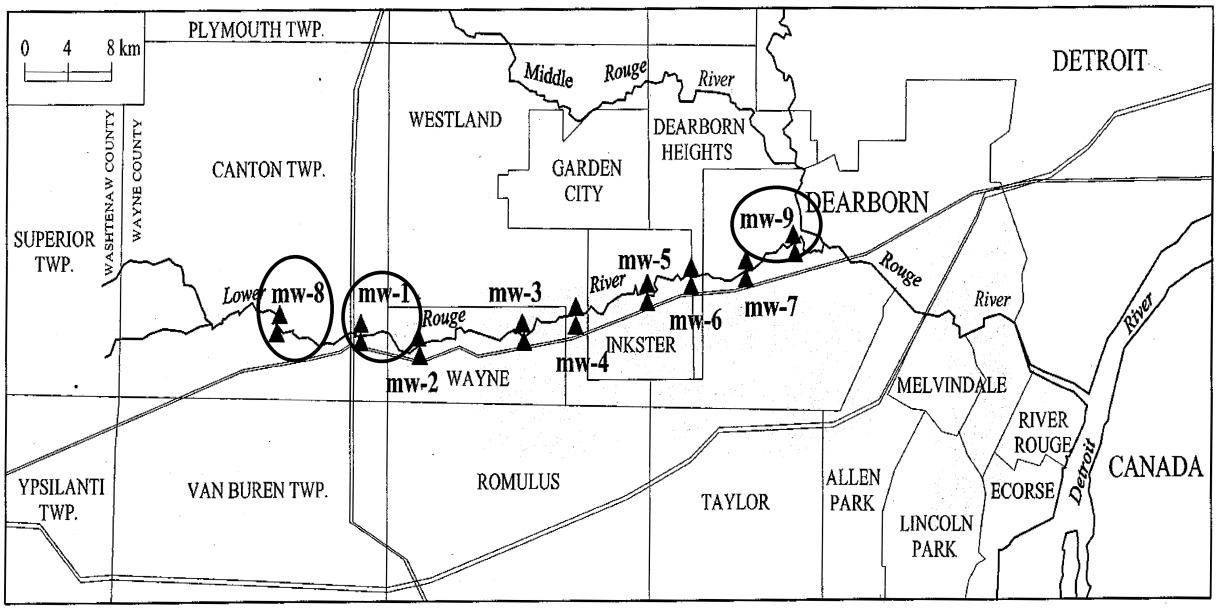

Figure 1. Location of sampling sites along the lower branch of the Rouge River. The ▲ symbols indicate the locations of the groundwater wells. Three wells are installed in each of these wells. The encircled sites (site mw-1, Lotz; site mw-8, Lilly and site mw-9, Ford Field) are associated with the sites selected for this study.

river at mid-stream, and manually lowering a sterile glass bottle to a depth approximately equivalent to 0.6 of the depth of the river. At sample sites where the river depth was too great to wade safely (>1.5 m), samples were collected by suspending the sample bottle on a telescoping aluminum pole and collecting water sample from a depth of approximately 1 m. Groundwater samples were collected using a low-flow purging pump. Low-flow purging pumps water from a well at a very low rate, minimizing agitation and mixing with stagnant water contained in solid casing regions of the well. All water samples were stored in sterilized media bottles, transported to the laboratory in a cooler, and then stored in a climate-controlled cold room. Samples were analyzed within 8 h of collection to prevent bacterial death.

Groundwater and river water temperatures ranged from 11.4-15.6ºC (Table 1). The pH values varied between 7.22 and 7.94. Dissolved oxygen concentrations of groundwater samples was significantly lower (0-6 mg l^{-1}) than the river water samples (12-20 mg l^{-1}). Site 1 generally had higher electrical conductivity than sites 8 and 9. The electrical conductivity of river water samples was relatively lower (1107-1378 μS) than that of the groundwater samples (1357-3999 μS) (Table 1).

Enrichment and Isolation

Direct plating and liquid enrichment were used to isolate halotolerant aerobic heterotrophic organisms from groundwater samples. Enrichments in SP medium [16], nutrient broth (Difco), and R2A medium (Difco) containing 10 and 20% NaCl were performed at room temperature. The 30-ml water samples inoculated in the field were aseptically transferred to 250-ml Erlenmeyer flasks containing 70 ml culture medium and were incubated on a rotary shaking platform at 200 rpm. Aliquots (100 μl) were plated after 48 h. One ml of the liquid enrichment cultures was transferred to a fresh liquid medium after 1 week and 100-μl aliquots were again plated after 24 and 48 h. Groundwater and river water aliquots were also spread directly on the surface of plates. Plates were kept for several weeks and representative colony types were collected to capture slow-growing organisms. Colonies arising on the plates were selected for isolation based on gross morphological and physiological features, differing in pigmentation, size, margin and rate of growth. When sufficient growth was obtained, plates were removed and colonies were enumerated. Immediately following enumeration, unique colonies were aseptically transferred to a fresh agar and isolated using the streak plate method. Each isolate was subjected to at least three successive streak plating to ensure purity. The isolates were maintained on agar slants at room temperature and as 50% glycerol stocks at-80ºC. The groundwater and river samples were also analyzed for population size of total aerobic heterotrophs using the dilution agar-plate method.

Morphological, Physiological and Biochemical Tests

Gram-staining characteristics and cell morphologies were determined by standard methods [17]. Motility was assessed by examining wet mounts of 24-h cultures at 1000X and by stab inoculation of Sulfur-Indole-Motility medium (SIM; BBL). Physiological characterization of strains was based on API 20E testing (BioMereux, Vitek, Inc.). Test results were obtained as specified by the manufacturer. API 20E strip descriptions are as follows: ONPG (β-galactosidase), ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (orthinine decarboxylase), CIT (citrate utilization), H_2S (sulfide production), URE (urease), TDA (tryptophane deaminase), IND (indole production), VP (Voges-Proskauer reaction), GEL (gelatin liquefaction), ARA (arabiose fermentation), GLU (glucose fermentation), MAN (mannitol fermentation), SAC (sucrose fermentation), AMY (amygdalin fermentation), RHA (rhamhose fermentation), MEL (melbiose fermentation), and INO (inosol fermentation).

PCR, DNA Sequencing and Phylogenetic Analysis

Isolates were subcultured on 0.1 X tryptic soy agar (TSA). DNA extracts from each isolate were prepared using. DNEasy kit (Qiagen, Inc., Valencia, CA). The genomic DNA

Table 1. Field data obtained from the three different sites.

Sites		Dissolved oxygen (mg l^{-1})	pH	Temperature (ºC)	Electrical conductivity (μS)
Site 1	1-gw-1	0	7.82	13.8	3999
	1-gw-2	4	7.93	13.5	2216
	1-sw	12	7.87	15.1	1378
Site 8	8-gw-1	5	7.23	15.6	2088
	8-gw-2	2	7.22	12.0	2455
	8-sw	20	7.22	15.5	1288
Site 9	9-gw-1	6	7.61	11.4	1447
	9-gw-2	2	7.94	11.8	1357
	9-sw	17	7.56	14.8	1107

gw=groundwater; sw=river water

was used as the target of PCR amplification of complete 16S rDNA fragments using bacterial primers FD1 and 1506 [18]. PCR was performed in an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) as 50-µl reactions containing 1x PCR buffer (50 mM KCl, 10 mM Tris, 0.1% Triton X-100, pH 9.0), 1mM dNTP's, 1.5 mM $MgCl_2$, 1 µM of each primer, 4 µg bovine serum albumin (BSA), and 2.5U *Taq*. The thermal cycling protocol used included initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 1 min. A final extension step at 72°C for 7 min was also used. The PCR amplicons were checked by electrophoresis on 1% agarose gels. Three amplicons were pooled for each set of 16S rDNA sequencing reactions in order to maximize sequencing template concentration and to allow for the detection of possible microheterogeneity in any rDNA gene copies. Amplicons were purified with Montage PCR 96 filter plates (Millipore Corporation. Bedford, MA). Flanking and internal primers 350r, 519f, 788f, 925r and 1099f [18] were used for direct sequencing of the amplicons. DNA sequencing was performed with ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA), and the ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems, Foster, CA). All sequence fragments generated from a given template were edited against electropherograms and then assembled into contigs using SeqMan (Lasergene DNASTAR, Inc, Madison, WI). For most sequences, two to four overlapping fragments (from both coding and noncoding strand) were used to assemble the contigs. Constextual 16S rDNA sequences for all unknown sequences were identified from published databases using BLAST [19], and by comparison with sequences deposited in the Ribosomal Database Project [20]. All sequences were manually aligned using MegAlign (Lasergene DNASTAR, Inc, Madison, WI). Phylogenetic and molecular evolutionary analyses were constructed with distance matrices and the neighbor-joining method with MEGA version 3.0 [21].

Accession Numbers

Sequences have been submitted to Genbank and appear with accession numbers: DQ981795 (1-gw1-su4-2); DQ981796 (1-gw1-su4-4); DQ981797 (1-gw1-su4-5); DQ981798 (1-gw2-su4-10); DQ981799 (1_gw1-su4-18); DQ981800 (1-gw1-su4-26); DQ981801 (1-gw1-su5-2); DQ981802 (1-gw1-su5-3); DQ981803 (1-gw2-su4-12); DQ981804 (1-gw2-su4-210); DQ981805 (1-gw2-su5-2); DQ981806 (1-sw-su5-1); DQ981807 (1-sw-su5-2); DQ981808 (1-sw-su5-3); DQ981809 (8-gw1-su4-6); DQ981810 (8-gw1-su4-410); DQ981811 (8-gw1-su5-2); DQ981812 (8-gw1-su5-3); DQ981813 (8-gw1-su5-4); DQ981814 (8-gw2-su4-1); DQ981815 (8-gw2-SU4-5); DQ981816 (8-gw2-su5-2); DQ981817 (8-gw2-su5-3); DQ981818 (8-sw-su5-1); DQ981819 (8-sw-su5-2); DQ981820 (8-sw-su5-3); DQ981821 (8-sw-su5-4); DQ981822 (8-sw-su5-5); DQ981823 (9-gw1-su4-1); DQ981824 (9-gw1-su5-1); DQ981825 (9-gw1-su5-2); DQ981826 (9-gw1-su5-3); DQ981827 (9-gw2-su5-1); DQ981828 (9-gw2-su5-3); DQ981829 (9-gw2-su5-4); DQ981830 (9-gw3-su4-1); DQ981831 (9-gw3-su4-4); DQ981832 (9-sw-su5-1); DQ981833 (9-sw-su5-2).

RESULTS

Enrichments and Isolation

The cultivation of microbes in combination of 16S rDNA approach gave insight into the culturable composition of halophilic and halotolerant bacteria from the river waters and shallow groundwater wells of the Rouge River. Culturing efforts yielded 125 bacterial isolates. Our results have shown significant recovery of halotolerant/halophilic bacteria in site 1 (Lotz; where the electrical conductivity values were highest), and least recovery in site 9 (Ford Field; where the electrical conductivity values were lowest) (Figure 2). Overall, the greatest number of colonies was found in the ground water samples. The number of colonies recovered from the river water (surface water) samples ranges between 1 and 5. The data we have completed shows the greatest concentration of halophiles to be found in the Lilley Rd gw1 site. We have also observed six different types of cellular morphologies (long rods, short rods, v-shaped rods, streptobacilli, cocci and spirilli) six various types of colony morphologies (filamentous, circular, lobate, rhizoid, filiform and undulate) on streak plates. The majority of the isolates were from Gram-positive taxa. Thirty-four isolates were gram negative and 29 were gram positive. The majority of the isolates were non-motile rods, spirilli or cocci. Most of the isolates (80%) reported here were retrieved from SP medium (10% saline). Dilution plating experiments were used to quantify the culturable heterotrophic bacteria in the river water and groundwater samples. Cell densities of total heterotrophic bacteria at the time of inoculation yielded the following results (site 1, 2.65-7.75 x 10^2 CFU ml^{-1}; site 8, 1.25-1.4 x 10^2 CFU ml^{-1}; and site 9, 1.75 x 10^2-4.40 x 10^3 CFU ml^{-1}). The microbial numbers were low across all samples and the variations between samples were relative small (≤ one order of magnitude). In general, the river samples had a greater number of total aerobic heterotrophs than groundwater samples. The greatest total aerobic heterotroph count was found in river water samples from site 9 (9-sw3) (Figure 3).

Phylogenetic Analysis of Isolates

The rDNA sequences of 39 unique isolates were obtained and phylogenetically analyzed: 14 were obtained from site 1 (mw-1, Lotz), 14 were isolated from site 8 (mw-8, Lilly) and 11 were obtained from site 9 (mw-9, Ford Field). Phylogenetic analysis of 16S rDNA sequences revealed that the isolates displayed close relationships to several bacterial species of various groups (Figure 4). The 16S rDNA sequences clustered to two lineages of bacteria: α and γ-*Proteobacteria* and *Firmicutes* (low G+C, mostly gram-positive bacteria). BLAST searches revealed that the sequences of the isolates that fell in γ-*Proteobacteria* were closely related (99% related) to a number of *Halomonas* sp. (Figure 4; Table 2).

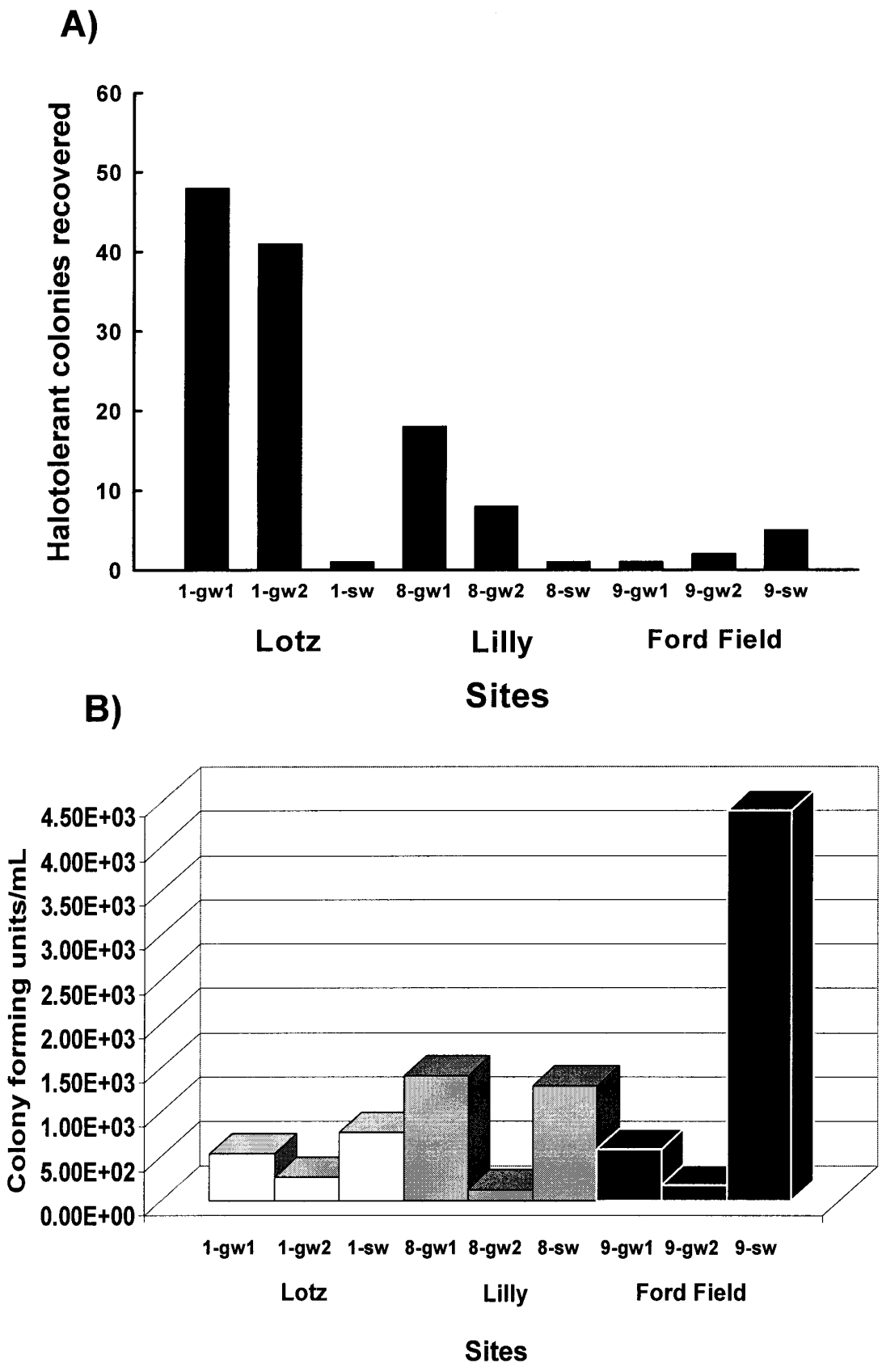

Figure 2. Number of halotolerant/halophilic bacterial colonies recovered (A), and total heterotrophic bacterial counts of groundwater from ground water and river water samples collected at three different sites of the Rouge river (B). gw=groundwater; sw=river water.

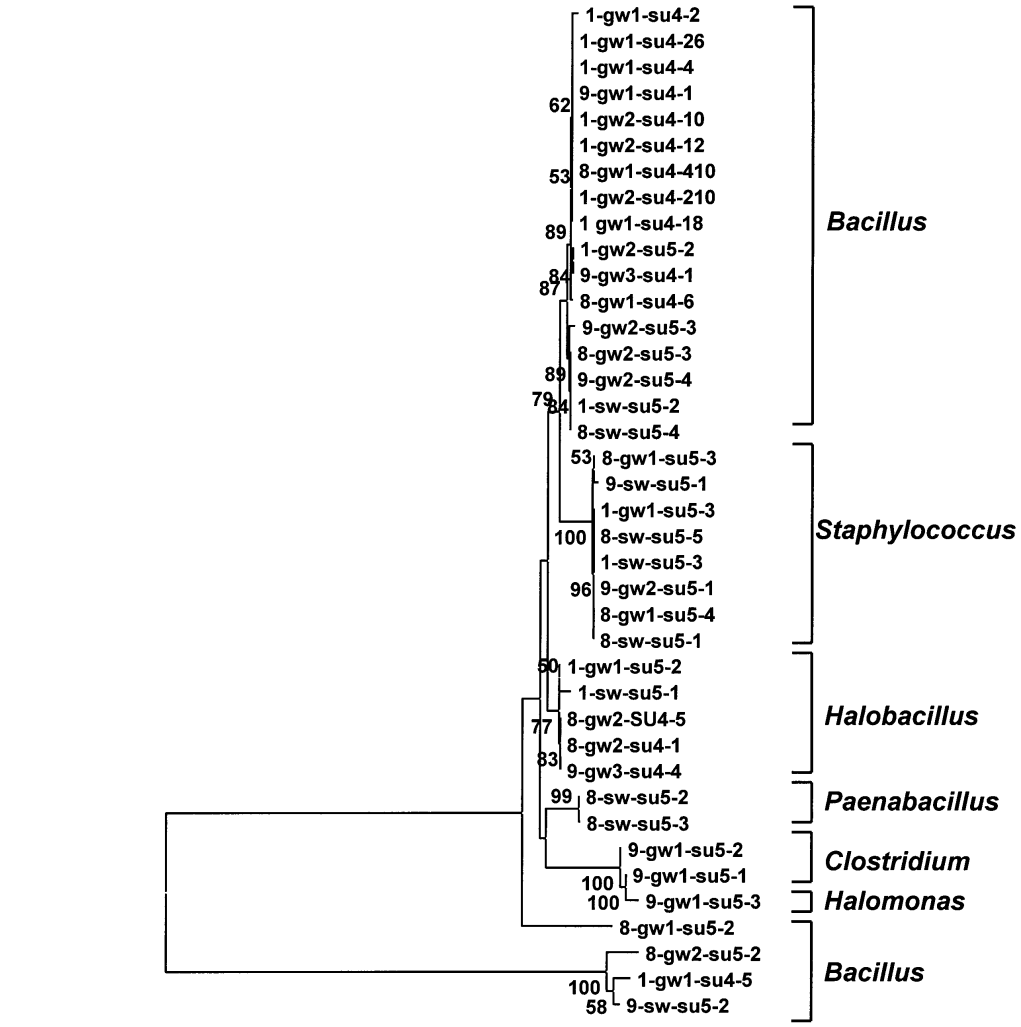

Figure 3. Phylogenetic relationships of bacterial isolates from groundwater and river water samples and reference sequence from Genbank. Phylogenetic trees were constructed in MEGA with distance matrices and the neighbor-joining method. The scale bar indicates the expected number of changes per sequence position. The accession numbers for reference sequences are listed in Table 2.

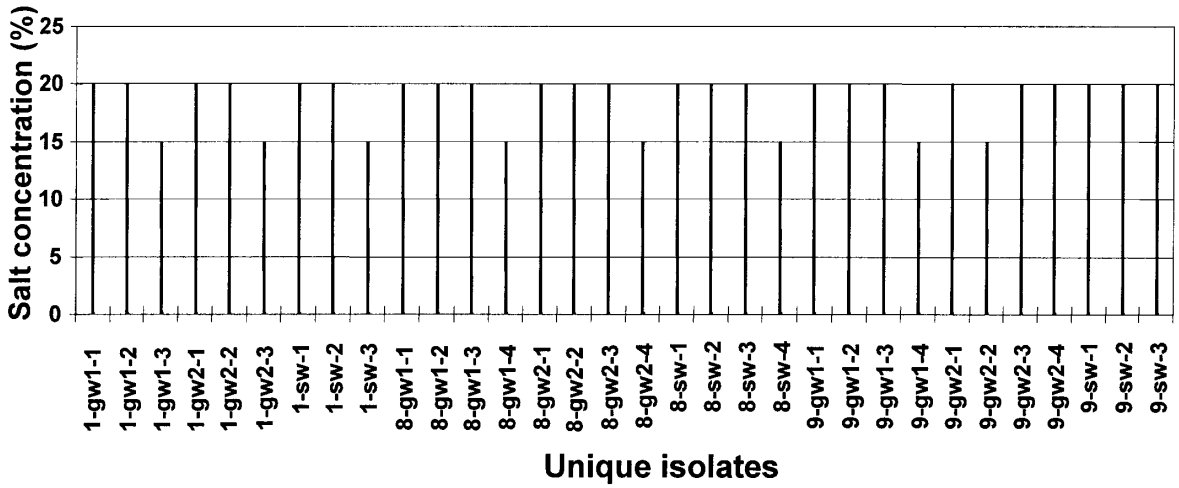

Figure 4. Salt tolerance of representative groundwater and river water isolates. The data presented for each isolate indicates salt concentrations at which visible growth occurred. gw=groundwater; sw=river water.

Table 2. Identification of isolates using 16S rDNA sequence analysis.

Isolate No.	Nearest relative in Genbank	Phylogenetic affiliation (phyla[a]/family)	% 16S RDNA identity
1-gw1-su4-2	*Bacillus* sp. SD-B1 (AB189316)	*Bacillaceae* (1)	98%
1-gw1-su4-4	*Bacillus* sp. MO12 (AY553105)	*Bacillaceae* (1)	99%
1-gw1-su4-5	*Bacillus licheniformis* strain GXN151 (AY291582)	*Bacillaceae* (1)	99%
1-gw2-su4-10	*Bacillus licheniformis* CICC 10219 (AY786999)	*Bacillaceae* (1)	100%
1_gw1-su4-18	*Bacillus* sp. GSP63 (AY553106)	*Bacillaceae* (1)	99%
1-gw1-su4-26	*Bacillus licheniformis* YB-42 (AY601721)	*Bacillaceae* (1)	99%
1-gw1-su5-2	*Halobacillus trueperi* strain GSP062 (DQ157162)	*Bacillaceae* (1)	99%
1-gw1-su5-3	*Staphylococcus epidermidis* (AJ717377)	*Staphylococcaceae* (1)	99%
1-gw2-su4-12	*Bacillus* sp. (AJ000648)	*Bacillaceae* (1)	99%
1-gw2-su4-210	*Bacillus* sp. MO9 (AY553102)	*Bacillaceae* (1)	99%
1-gw2-su5-2	*Bacillus subtilis* MO2 (AY553095)	*Bacillaceae* (1)	99%
8-gw1-su4-6	*Bacillus licheniformis* CICC10181 (AY842871)	*Bacillaceae* (1)	99%
8-gw1-su4-410	*Bacillus licheniformis* GSP30 (AY505509)	*Bacillaceae* (1)	99%
8-gw1-su5-2	*Halomonas boliviensis* LC1 (AY245449)	*Halomonadaceae* (2)	99%
8-gw1-su5-3	*Staphylococcus* sp. BBF1 (AM158916)	*Staphylococcaceae* (1)	99%
8-gw1-su5-4	*Staphylococcaceae bacterium* KVDn (DQ490408)	*Staphylococcaceae* (1)	99%
8-gw2-su4-1	*Halobacillus* sp. GSP35 (AY553078)	*Bacillaceae* (1)	99%
8-gw2-su4-5	*Halobacillus* sp. NT N168 (AB167053)	*Bacillaceae* (1)	98%
8-gw2-su5-2	*Bacillus* sp.(AJ842963)	*Bacillaceae* (1)	99%
8-gw2-su5-3	*Bacillus* sp. MH07 (AY690701)	*Bacillaceae* (1)	98%
9-gw1-su4-1	*Bacillus licheniformis* (BLI582722)	*Bacillaceae* (1)	99%
9-gw1-su5-1	*Clostridium sporogenes* (DQ278865)	*Clostridiaceae* (1)	97%
9-gw1-su5-2	*Clostridium sporogenes* (AY442816)	*Clostridiaceae* (1)	99%
9-gw1-su5-3	*Clostridium sporogenes* (X68189)	*Clostridiaceae* (1)	93%
9-gw2-su5-1	*Staphylococcus* sp. (X86635)	*Staphylococcaceae* (1)	99%
9-gw2-su5-3	*Bacillus pumilus* strain KL-052 (AY030327)	*Bacillaceae* (1)	99%
9-gw2-su5-4	*Bacillus subtilis* WL-6 (DQ198162)	*Bacillaceae* (1)	99%
9-gw3-su4-1	*Bacillus licheniformis* strain CICC 1021 (AY786999)	*Bacillaceae* (1)	99%
9-gw3-su4-4	*Halobacillus* sp. GSP43 (AY505520)	*Bacillaceae* (1)	99%
1-sw-su5-1	*Halobacillus* sp. CNJ931 PL04 (DQ448799)	*Bacillaceae* (1)	98%
1-sw-su5-2	*Bacillus licheniformis* CICC10103 (DQ212969)	*Bacillaceae* (1)	99%
1-sw-su5-3	Low G+C bacterial isolateHTA563 (AB002641)	*Staphylococcaceae* (1)	99%
8-sw-su5-1	*Staphylococcus* sp. MO28 (AY553115)	*Staphylococcaceae* (1)	99%
8-sw-su5-2	*Paenibacillus* sp. SB45-2B (AF395029)	*Paenibacillaceae* (1)	97%
8-sw-su5-3	*Paenibacillus* sp. P163 (AM183351)	*Paenibacillaceae* (1)	97%
8-sw-su5-4	*Bacillus subtilis* KL-077 (AY030331)	*Bacillaceae* (1)	100%
8-sw-su5-5	*Staphylococcus* epidermidis KL-096 (AY030342)	*Staphylococcaceae* (1)	99%
9-sw-su5-1	*Staphylococcus* sp. H780 (AB177644)	*Staphylococcaceae* (1)	99%
9-sw-su5-2	*Bacillus licheniformis* B425 (DQ523501)	*Bacillaceae* (1)	99%

[a](1)= *Firmicutes*; (2)= *Proteobacteria* (γ); gw=groundwater; sw=river water

The 16S rDNA gene sequences of the isolates that fell into the phylum *Firmicutes* demonstrated very close relationship (98-99%) with 16S rDNA gene sequences of several *Bacillus*, *Halobacillus*, *Clostridium*, *Paenibacillus*, and *Staphylococcus* spp., which represented approximately 97% of the total number of unique isolates sequenced. The majority of the 16S rDNA sequences were recovered from groundwater samples (29 isolates; 81% of the unique isolates). Bacterial species found from groundwater samples were also found in the river samples, with the exception of *Clostidium sporogenes* and *Paenibacillus* spp., which were found only in groundwater and river samples, respectively (Table 2).

Physiological Analysis of Selected Isolates

Phenotypic analyses using API strips were performed on 15 selected isolates: 1-gw1-su-26, *Bacillus licheniformis* YB-42; 1-gw1-su-5, *Bacillus licheniformis* GXN151; 1-gw1-su-18,

Bacillus sp. GSP63; 1-gw2-su-10, *Bacillus licheniformis* CICC 10219; 1-gw2-su-210, *Bacillus* sp.; 1-gw2-su-12, *Bacillus* sp.; 1-sw-su5-2, *Bacillus licheniformis* CICC10103; 8=1-sw-su5-1, *Halobacillus* sp. CNJ931; =1-gw1-sp4, *Bacillus* sp MO12; 1-gw1-su5-2, *Halobacillus trueperi* YB-42=9-sw-su5-1, *Clostidium sporogenes*; 9-sw-su5-1, *Staphylococcus* sp.; 8-gw2-su4-1, *Halobacillus* sp; 8-gw2-su4-5, *Halobacillus* sp. NT N168; and 9-gw1-su4-1, *Bacillus licheniformis* BLI582722. All of the 15 isolates were arginine dihydrolase (ADH) positive (Table 3). The majority of the isolates showed positive results for tryptophane deaminase (TDA), gelatinase (GEL) and β-galactosidase (ONPG). Indole production, acetoin production and citrate utilization were not observed for any isolates. Fermentation of carbohydrates was observed for very few isolates. Glucose fermentation (GLU) was observed for three isolates (1-gw1-su5-2, 9-sw-su5-1, 9-sw-su5-1); sucrose fermentation (SAC) for four isolates (1-gw1-su5-2, 9-gw1-su5-1, 9-sw-su5-1, and 8-gw2-su4-5), and mannitol fermentation (MAN) for one isolate (9-sw-su5-1). The primary enzyme found in these bacterial groups was the arginine dihydrolase, which might be an indicator of the presence of such an enzyme in halophilic and halotolerant bacteria present in the Rouge River.

Salt Tolerance and Requirements of Unique Isolates

All 39 unique bacterial isolates were tested for their requirements and abilities for growth at different salinities (Figure 4). The data presented for each isolate indicates the salinities at which visible growth occurred. The vast majority of the unique bacterial isolates were tolerant of high salinities (10% or greater), but did not require these high salinities for growth. Twenty-two isolates required 5% salinity and 12 isolates required 10% salinity. Only five isolates required 15% salinity. Many of the isolates exhibited wide ranges of tolerance greater than 15% salinity (Figure 5).

Table 3. API 20E results on selected isolates.

	Selected isolates‡														
Enzymatic Activity/substrate†	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Tryptophane deaminase (TDA)	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Gelatinase (GEL)	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+
Acetoin production (VP)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-galactosidase (ONPG)	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+
Arginine dihydrolase (ADH)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization (CIT)	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
Indole production (IND)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (arabinose; ARA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (mannitol; MAN)	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Fermentation/ Oxidation (glucose; GLU)	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Fermentation/ Oxidation (sucrose; SAC)	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
Fermentation/ Oxidation (amygdalin; AMY)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (rhamnose; RHA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (sorbitol; SOR)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (melibiose; MEL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation/ Oxidation (inositol; INO)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase (ODC)	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
Lysine decarboxylase (LDC)	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
Urease (URE)	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Sodium thiosulfate production (H_2S)	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-

† +, positive result; -, negative result

‡ (**1**)=1-gw1-su-26, *Bacillus licheniformis* YB-42; (**2**)=1-gw1-su-5, *Bacillus licheniformis* GXN151; (**3**)=1-gw1-su-18, *Bacillus* sp. GSP63; (**4**)=1-gw2-su-10, *Bacillus licheniformis* CICC 10219; (**5**)=1-gw2-su-210, *Bacillus* sp.; (**6**)=1-gw2-su-12, *Bacillus* sp.; (**7**)=1-sw-su5-2, *Bacillus licheniformis* CICC10103; 8=1-sw-su5-1, *Halobacillus* sp. CNJ931; (**9**)=1-gw1-sp4, *Bacillus* sp MO12; (**10**)= 1-gw1-su5-2, *Halobacillus trueperi* YB-42 (**11**)=9-gw1-su5-1, *Clostidium sporogenes*; (**12**)=9-sw-su5-1, *Staphylococcus* sp.; (**13**)=8-gw2-su4-1, *Halobacillus* sp; (**14**)= 8-gw2-su4-5, *Halobacillus* sp. NT N168; (**15**)=9-gw1-su4-1, *Bacillus licheniformis* BLI582722.

DISCUSSION

The present study demonstrated the presence of halophilic and halotolerant bacteria in river water and groundwater samples. The initial enrichment and isolation experiments yielded 125 isolates that represent six different genera (*Bacillus, Staphylococcus, Halobacillus, Paenabacillus, Halomonas*, and *Clostridium*). The high percentage of deicing salts removed as surface run-off and subsequently delivered to rivers and streams may have selected for halotolerant strains. The development of communities rich in saline genera (i.e. *Halobacillus* and *Halomonas*) may have occurred over time after many years of salt use. It is also not surprising to find those organisms reported in saline environment in the river water and groundwater samples. The salt tolerance of the halophilic bacteria isolated in this study should allow them to thrive in marine aquatic environments. For instance, several *Bacillus*, *Staphylococcus*, *Halomonas*, *Paenebacillus* and *Clostridium* spp., are well known for their broad salt tolerance, being able to tolerate salinities of 10% NaCl or even greater. Most of these bacterial isolates are spore formers. The capacity of these bacteria to produce spores contributes to their resistance to a broad range of physiological stresses such as salinity. It was remarkable that a number of halotolerant/halophilic groups belonging to *Bacillus*, *Halobacillus*, *Staphylococcus* and *Halomonas* found in this study, were previously found in saline environments such as the Salt Plains National Wildlife Refuge, Great Salt Plains of Oklahoma [22], Bolivian hypersaline lake [23], deep-sea sediments [24-25], and tropical marine sediments [26]. These bacterial groups were found both in the river water and groundwater samples. The culture collection also includes several isolates that are closely related to bacteria found in aquatic systems. In the case of actively growing microbial communities where there is no speculation about whether they have been dormant over long periods of geological time, the way in which communities may have developed, either from a native prokaryotic biota or by input from outside, is not easy to dissect. Only future investigations on microbial diversity will help to throw light on this question. It is possible however, that the bacterial population in the groundwater and river water responded to differences in the local environment (i.e. increased salinity), and slowly changed the parent population. Speculation on the origin and dynamics of microbial communities in river and groundwater systems is complicated because of the complexity of the microbial communities and the methodological difficulties associated with microbial ecological studies. For example, it is not easy to accurately analyze the distribution of organisms in a particular environment. Although it is now possible to gain a better insight into the distribution of organisms in the natural environment, the resolution of the 16S rDNA approach is such that one can not easily determine whether one is dealing with a single species or several closely related species, or even to what extent there may be different subpopulations of the same species in the same environment. At present, we certainly do not understand the ecological significance of such complex microbial populations. Although the present study attempted to examine individual strains in the laboratory, different methods are needed to study the same organisms in complex populations as well as to study their interactions.

Analysis of the 16S rDNA sequences revealed that members belonging to *Bacillus* dominated the culture collection (Table 2). Members of the genus *Bacillus* have been isolated from groundwater system by Chapelle et al. [27]. Several species of *Bacillus* spp. are important degraders of organic pollutants. For example, *Bacillus cereus* has been found to degrade 1,3-dichlorobenzene derived from town-gas industrial influent [28]; *Bacillus subtilis* has been used to degrade p-aminobenze from textile industry wastewater [29]; and *Bacillus licheniformis* has been shown to degrade organic hydrocarbons in soil [30]. These organisms play an important role in the fate of many groundwater contaminants (both organic and inorganic); although future studies are needed that deal with the response of these bacteria to organic and inorganic contamination from river water and groundwater. A wide variety of pollutants including metals, polychlorinated biphenyls, polyaromatic hydrocarbons, and bacteria were cited as the major sources of contamination [15, 31]. The degradation of the Rouge River is representative of that found in many urbanized and industrialized areas within the Great Lakes Basin. Combined sewer overflows (CSOs), urban storm water discharges, nonpoint source pollution, and municipal and industrial discharges all contribute to the contamination of the Rouge River. This study demonstrated that the river water and groundwater harbor a variety of halotolerant and halophilic bacteria that may have potential in bioremediation of organic contaminants at the site. These microorganisms may also play important roles in the fate of many groundwater contaminants (both organic and inorganic). However, future studies are needed that deal with the response of these bacteria to organic and inorganic contamination from river water and groundwater.

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