Vol. 48: 131–140, 2007 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published July 10

Abundance dynamics and sequence variation of neomycin phosphotransferase gene (*nptII*) homologs in river water

Bin Zhu*

National Water Research Institute, Environment Canada, 11 Innovation Blvd, Saskatoon, Saskatchewan S7N 3H5, Canada

ABSTRACT: Monitoring the abundance and dynamics of antibiotic resistance genes is essential for assessing their potential effects on environmental and human health. The objectives of the present study were to investigate: (1) the abundance of neomycin phosphotransferase (*nptII*) gene homologs in water samples collected monthly from the South Saskatchewan River (Canada) for 24 mo, and (2) sequence variation of the cloned *nptII* gene homologs retrieved from the river. DNA from river microbial communities was used to transform a natural competent *Pseudomonas stutzeri* strain containing a truncated *nptII* gene on a plasmid (*P. stutzeri* pMR7). Of the 24 water samples, the recovery of kanamycin resistant (Km^R) transformants from DNA of 4 samples indicated the presence of *nptII* gene homologous sequences. However, the natural transformation process required ~3.2×10^4 copies of the *nptII* gene to recover a single Km^R transformant. To overcome the limitation of detection, a real-time PCR assay using SYBR Green I was developed to quantify the abundance of *nptII* gene homologous sequences in river microbial communities. The results showed that *nptII* gene homologous sequences were present in the river, and their abundance varied over the course of this study, ranging from undetectable levels to 4.36×10^6 copies l^{-1} water. Furthermore, *nptII* gene homologous sequences amplified from river microbial community DNA were cloned, and unique clones were sequenced. Comparison of the nucleotide and deduced amino acid sequences of the cloned fragments to those of the *nptII* gene on transposon Tn5 showed that they had over 96% homology.

KEY WORDS: Neomycin phosphotransferase gene · *nptII* · Abundance dynamics · Water samples · Homologous sequence · Real-time PCR

INTRODUCTION

Among the mechanisms conferring bacterial antibiotic resistance, antibiotic resistance genes are particularly important because: (1) genetic elements coding for antibiotic resistance can be inherited from generation to generation and (2) bacteria can transfer them to other members of their own species and to other species via horizontal gene transfer (Davies 1994, Lorenz & Wackernagel 1994). Meanwhile, antibiotic resistance genes are thought to evolve rapidly in response to antibiotic use, as evidenced by the ineffectiveness of some antibiotics (Davies 1994). This has led to the termination of clinical and veterinary use of many antibiotics since the discovery of penicillin in the 1920s. Intriguingly, Sundin & Bender (1996) showed that antibiotic resistance genes can persist even after the antibiotic use has diminished, implying that factors other than direct selection are also involved in the maintenance of these genes in natural environments.

Resistance to kanamycin (a member of the aminoglycoside family of antibiotics) in environmental bacteria is common (van Elsas 1992, Leff et al. 1993b). A number of mechanisms of kanamycin resistance have been identified in bacteria, one of which is the neomycin phosphotransferase (*nptII*) gene (Beck et al. 1982). Based on screening for kanamycin resistance (Km^R) in the culturable fraction of bacteria in environmental samples, previous studies indicated that only a small number of Km^R isolates carried the *nptII* gene; e.g. of

*bin.zhu@ec.gc.ca

the 350 Km^R bacterial isolates collected from water and soil samples by Smalla et al. (1993), only 3 isolates contained the *nptII* gene. Similarly, Leff et al. (1993a) detected 3 isolates carrying the *nptII* gene after screening 184 Km^R colonies isolated from river water samples.

It has been estimated that over 99% of bacteria in the environment are non-culturable in the laboratory (Amann et al. 1995). Thus, monitoring the abundance of the *nptII* gene in natural bacterial communities is a challenge because the *nptII* gene may be carried by non-culturable bacteria. To overcome this obstacle, molecular techniques have been exploited to investigate the prevalence of *nptII* gene homologous sequences in natural bacterial populations, with the involvement of microbial community DNA extraction and probe-based Southern blot hybridization (Leff et al. 1993a, Smalla et al. 1993). In recent years, real-time PCR has been widely used in quantifying bacterial genes including antibiotic resistance genes (Brennan & Samuel 2003, Smith et al. 2004). Compared with conventional PCR-based assays, real-time PCR offers major advantages, including the large dynamic range of quantification and the exclusion of post-PCR manipulations, thus greatly reducing the risk of carry-over contamination. Meanwhile, real-time PCR can be performed on only minimal amounts of DNA and has a high-throughput capacity (Sharkey et al. 2004).

Most of the known antibiotic resistance genes have been discovered in clinical and veterinary bacterial strains, whereas their abundance and diversity in natural environments are not well characterized (Sèveno et al. 2002, Riesenfeld et al. 2004). In the present study, we described the quantification of *nptII* gene homologous sequences in water samples collected monthly from a river over a period of 24 mo. First, we monitored the presence of *nptII* gene homologous sequences in river microbial community DNA by testing their transforming ability with a competent *Pseudomonas stutzeri* strain harboring plasmid pMR7 (Meier & Wackernagel 2003). Then we applied a real-time PCR assay using SYBR Green I to investigate the dynamics of abundance of *nptII* gene homologous sequences in river water. Further, homologous sequences of the *nptII* gene retrieved from river microbial communities were cloned and sequenced; their nucleotide and deduced amino acid sequences were compared to those of the *nptII* gene on transposon Tn5.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas stutzeri* LO15 is a natural competent strain (Graupner et al. 2000). It was transformed to harbor a plasmid (pMR7) that contains the truncated *nptII* gene with a 10 bp deletion (de Vries & Wackernagel 1998), designated *P. stutzeri* pMR7 (Meier & Wackernagel 2003). *P. stutzeri* pMR7 is sensitive to kanamycin. When cells of *P. stutzeri* pMR7 take up extracellular DNA carrying the *nptII* gene, the truncated gene can be restored through homologous recombination, thereby restoring the bacterial kanamycin resistance. Plasmid pNS1 (6.0 kb) harbored in *Escherichia coli* K-12 has the *nptII* gene (Sikorski et al. 2002).

Collection and filtration of water samples. The Saskatchewan River is Canada's fourth largest river, formed at the confluence of the North and South Saskatchewan Rivers more than 1200 km downstream from its glacial origins. This 8th order river crosses much of the prairie regions of central Canada, with the drainage encompassing approximately 364 000 km^2 (Rosenberg et al. 2005). In the present study, we collected river water samples monthly from the South Saskatchewan River, Saskatchewan, Canada, at one location during 2003 and 2004. A total of 24 water samples were obtained. At the time of sample collection, water temperatures ranged from 0.1°C (in the winter months) to 21.1°C (in the summer months). The pH was slightly above neutral (pH range, 6.43 to 8.50; median pH = 7.72). Subsurface water (10 l) was collected from the river and immediately transported to the laboratory. If ice covered the river in winter, it was broken with an auger to collect water. Subsamples (1 l river water) were filtered on a Millipore Durapore filter (pore size, 0.22 µm; 47 mm in diameter) to collect microbial biomass; 3 filters were collected for each sample. The filters were stored at −80°C until DNA extraction.

DNA extraction. Frozen filters were thawed and cut into small pieces using a sterile razor blade prior to DNA extraction. Community DNA was extracted and purified from each filter using the Fast Soil DNA extraction kit (Qbiogene) and Beadbeater (Savant), following the manufacturers' instructions. Total DNA was eluted in 50 µl double-distilled water (ddH_2O) (25 µl were used for natural transformation; 25 µl and its serial 10-fold dilutions were used for PCR and real-time PCR). Plasmid DNA of pNS1, pMR7 and *Pseudomonas stutzeri* pMR7 Km^R transformants was extracted from cells grown overnight in Luria-Bertani (LB) liquid culture (37°C, shaking at 250 rpm) by the plasmid midi kit (Qiagen) following the manufacturer's instruction. DNA was quantified on a spectrofluorometer.

Natural transformation of *Pseudomonas stutzeri* pMR7. Transformation efficiency was tested based on the natural competent capacity of *P. stutzeri* pMR7 with plasmid pNS1 DNA. For transforming *P. stutzeri* pMR7 with DNA from river water, 5 µl of microbial community DNA was used as DNA donor for each

transformation. Negative control was performed identically except for the omission of DNA. Transformation procedure was similar to the method described by Meier & Wackernagel (2003), with minor modification. Briefly, cells of *P. stutzeri* pMR7 grown overnight in LB with streptomycin (100 µg ml^{-1}) were washed twice with sterile 0.9% NaCl and adjusted to a density of ~1.0×10^9 cells ml^{-1}. An aliquot (50 µl) of cell suspension was then mixed with 5 µl of river microbial community DNA. The mixtures were spotted onto one LB agar plate. After incubation for 20 h at 28°C, the agar piece with the spot of cells was transferred to a glass tube containing 1 ml of sterile 0.9% NaCl. The tube was vortexed to suspend the cells from the agar. Appropriate dilutions of the resuspended cells were plated on LB plates with kanamycin (50 µg ml^{-1}) and incubated at 28°C for 3 d to determine the number of Km^R colonies. The total number of bacterial colonies on LB plates was determined by serial dilutions. The transformation frequency was expressed as the percentage of Km^R transformants over total viable recipient cells.

PCR analysis. The specific primers targeting the *nptII* gene used for PCR analysis are listed in Table 1. By using Primer Express software (Applied Biosystems), the primer set (nptII-532F and nptII-737R) was designed to flank the 10 bp deletion of the *nptII* gene. Hence, they can be used to confirm the restoration of the *nptII* gene in Km^R transformants. Their specificity to the *nptII* gene was verified by searching the GenBank database. All primers were purchased from Invitrogen. Amplification was conducted in a 20 µl reaction containing 4 µl of DNA template (10 ng $µl^{-1}$), 1 × PCR buffer (Promega), 0.1 µM of each primer, 1 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs) and ddH_2O. The amplification program was as follows: 1 cycle of 95°C for 4 min; 30 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min, with a final cycle of 72°C for 7 min. The PCR products were separated by electrophoresis in 2% w/v agarose in 1 × Tris-acetate-EDTA (TAE) for 2.5 h at 103 V. For the detection of the *nptII* gene in microbial community DNA by PCR, plasmid pNS1 was used as a positive control. After electrophoresis, the gels were visualized by ethidium bromide staining and documented in a digital gel documentation system.

Table 1. PCR primers used in this study. F: forward primer; R: reverse primer. Positions represent the open reading frame of the *nptII* gene on Tn5 transposon. Source: 1 = de Vries & Wackernagel (1998), 2 = present study

Primer	Sequence (5′ to 3′)	Position	Source
nptII-F	gaa caa gat gga ttg cac gc	7–26	1
nptII-R	gat gtt tcg ctt ggt ggt c	400–418	1
nptII-532F	atg ccc gac ggc gag gat ct	532–551	2
nptII-737R	ata ccg taa agc acg agg aag cg	715–737	2

Real-time PCR analysis. During real-time PCR, the fractional cycle in which the increase in the fluorescence generated by the accumulation exceeds 10 standard deviations of the mean baseline fluorescence, with a selected range of cycles, is referred to as the threshold cycle (C_t). The C_t is inversely proportional to the starting amount of DNA (the initial quantity of a target gene), allowing the generation of standard curves by using samples containing serial 10-fold dilutions of target genes. The primer set of nptII-F and nptII-R was used in real-time PCR. To establish a standard curve, copies of the *nptII* gene contained in plasmid pNS1 were calculated after DNA quantification and standards were prepared by serially diluting purified DNA of plasmid pNS1. The real time amplification plot was developed when the copy numbers used ranged from 1.0×10^7 to 10 copies per reaction. Briefly, river microbial community DNA and its serial 10-fold dilutions were used as DNA templates, DNA (4 µl) was added to a reaction mixture containing 0.15 µM of each primer, 12.5 µl of 2 × SYBR Green QPCR Master Mix (Stratagene), and sterile ddH_2O in a final volume of 25 µl. Real-time PCR was carried out with the SYBR Green (with Dissociation Curve) program on the Mx4000 Multiplex Quantitative PCR System (Stratagene). Cycling parameters were at 95°C for 10 min to denature, 40 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, followed by a melting curve analysis. The real-time PCR assay included a standard curve of 7 serial 10-fold dilution points of pNS1 (ranging from 1.0×10^7 to 10 copies of the *nptII* gene), and all reactions were performed in triplicate and the median C_t value was used for analysis. The melting curve analysis was performed to verify the correct product by its specific melting temperature (T_m). It consisted of a denaturation step at 95°C for 1 min, lowered to 55°C for 30 s, and followed by 40 cycles of heating the samples to 95°C with continuous reading of fluorescence.

Cloning *nptII* gene homologous fragments. For cloning homologous sequences of the *nptII* gene from river microbial communities, the primer set of nptII-F and nptII-737R was chosen because it covered the major portion of the *nptII* gene and excluded a conserved region (located at the end of the open reading frame of the *nptII* gene) that was shared by other aminolyocoside phosphotransferase sub-families (Shaw et al. 1993). DNA from the water samples in which *nptII* gene homologous sequences were detected by real-time PCR was combined and PCR was run as described above. After electrophoresis on an agarose gel, amplified DNA was excised and purified by a Qiaquick gel extraction kit (Qiagen). Subsequently,

purified PCR products were ligated into the pDrive cloning vector, and then introduced into competent *Escherichia coli* cells by heat shock transformation following the manufacturer's instructions (Qiagen). Clones containing the correct insert were confirmed by PCR amplification with the universal primers T7 and sP6. Subsequently, the amplified fragments were digested by restriction enzymes to identify unique clones, which were detected by restriction fragment length polymorphism (RFLP) analysis first with *Msp*I plus *Rsa*I, and for clones not resolved with *Hha*I plus *Hae*III. Based on digestion patterns, plasmid DNA was purified for unique inserts using a Qiagen plasmid mini kit and the concentration of plasmid DNA was determined by a spectrophotometer prior to sequencing.

DNA sequencing and phylogenetic analysis. DNA sequences were determined on both strands of the purified plasmid DNA as template by automated fluorescent *Taq* cycle sequencing using ABI 377 Sequencers (Applied Biosystems). Similarity searches against sequence database were performed using an updated version of the BLAST program at the National Center for Biotechnology Information server (www.ncbi.nlm.nih.gov). The phylogenetic relationship was constructed using the maximum likelihood program in the PAUP4.0 package (http://paup.csit.fsu.edu) after *nptII* gene homologous sequence and their amino acid alignments were calculated using ClustalW (Thompson et al. 1994). The unrooted phylogenetic consensus tree was generated using Treeview (Page 1996).

Nucleotide sequence accession number. The nucleotide sequences reported in the present study have been submitted to the GenBank sequence database and assigned the accession numbers DQ449894 to DQ449904.

RESULTS

Natural transformation of *Pseudomonas stutzeri* pMR7 with river microbial community DNA

To detect the potential presence of *nptII* gene homologous sequences contained in river microbial communities, we used river microbial DNA to transform *Pseudomonas stutzeri* pMR7, which is sensitive to kanamycin because of the deletion (10 bp) of the *nptII* gene on plasmid pMR7. The results showed that Km^R transformants were recovered from 4 water samples with a transformation frequency range of 1.48 to 5.13×10^{-7} (Table 2). Meanwhile, no Km^R colonies were detected when *P. stutzeri* pMR7 cells were cultured without plasmid DNA as negative control. Further, to confirm that kanamycin resistance shown by Km^R transformant colonies was attributable to the restoration of the truncated *nptII* gene, we extracted plasmid DNA from Km^R transformants and amplified with the primer set of nptII-532F and nptII-737R (flanking the 10 bp deletion of the *nptII* gene on plasmid pMR7). The result showed that a 206 bp fragment from the *nptII* gene was amplified (Fig. 1), indicating that the 10 bp deletion of the *nptII* gene on plasmid pMR7 was restored. Together with the recovery of Km^R transformants, this result demonstrated that *nptII* gene homologous sequences were present in river microbial communities and that they could restore the biological activity of the truncated *nptII* gene in *P. stutzeri* pMR7 by homologous recombination.

Table 2. Number of kanamycin-resistant (Km^R) transformants recovered from transforming *Pseudomonas stutzeri* pMR7 with river microbial community DNA extracted from 1 l of water, expressed as the mean of 3 filters ± SD. The detection limit is ca. 3.0×10^{-8}

Date	Amount of DNA (µg)	Km^R transformants	Transformation frequency ($\times 10^{-7}$)
2003			
Feb 12	18.25 ± 1.23	48.7 ± 12.5	1.48 ± 0.31
Dec 11	22.90 ± 1.19	36.6 ± 7.5	5.13 ± 0.55
2004			
Sep 13	22.05 ± 1.86	32.1 ± 7.3	3.11 ± 0.63
Dec 7	16.90 ± 1.47	56.2 ± 10.8	3.15 ± 0.47

Reproducibility of real-time PCR assay

After testing the transformation efficiency of *Pseudomonas stutzeri* pMR7 with plasmid pNS1 DNA, we observed that a minimum of 3.2×10^4 copies of the *nptII* gene were needed to obtain a single Km^R transformant (Fig. 2). This limitation of detection could underestimate the abundance of *nptII* gene homolo-

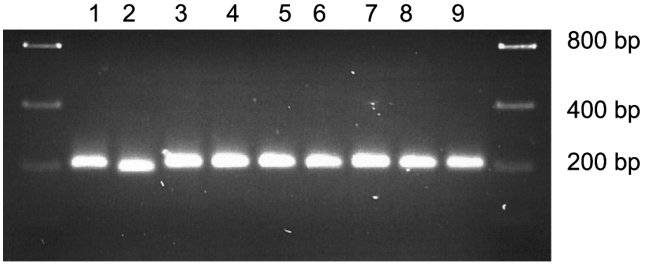

Fig. 1. Amplification of the specific fragment containing the 10 bp deletion in the *nptII* gene on plasmid pMR7 and its kanamycin-resistant (Km^R) transformants. After *Pseudomonas stutzeri* pMR7 was transformed with DNA from river microbial communities, plasmid DNA from individual Km^R transformants was amplified with the primer set of nptII-532F and nptII-737R (flanking the 10 bp deletion of the *nptII* gene). Lane 1: plasmid pNS1; Lane 2: plasmid pMR7; Lanes 3 to 9: 7 individual Km^R transformants

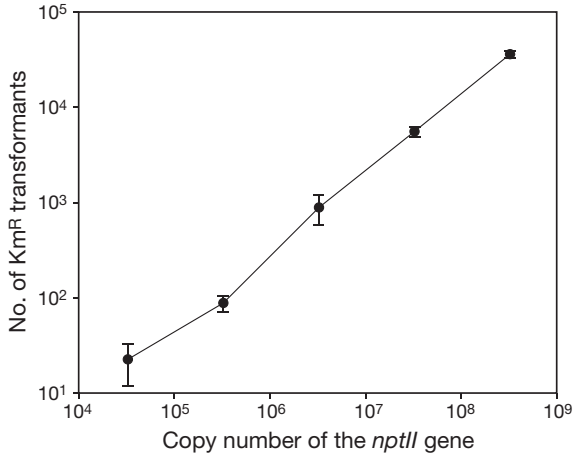

Fig. 2. Transformation efficiency of *Pseudomonas stutzeri* pMR7 with the indicated copies of the *nptII* gene carried by plasmid pNS1. Data (±SD) are the mean of 3 independent experiments

gous sequences in river water samples. Therefore, we applied a real-time PCR assay using SYBR Green I to quantify the abundance of *nptII* gene homologous sequences. Prior to application of real-time PCR, a BLAST search in the ENTREZ database was performed to confirm the specificity of the primer set (nptII-F and nptII-R). Subsequently, the quantification sensitivity of real-time PCR was conducted by using serially 10-fold diluted DNA of plasmid pNS1 containing the *nptII* gene (equivalent to 10 to 1.0×10^7 copies of target). The results demonstrated that a typical amplification plot (Fig. 3A) and a high correlation in the standard curve ($R^2 = 0.997$) (Fig. 3B) were generated. The melting curve analysis showed a single peak (T_m = 87.5°C), confirming the specificity of the amplified PCR fragment (Fig. 3C). Further, the reproducibility of the real-time PCR assay was assessed by measuring C_t values for 4 replicates of the standards (Table 3). The results represented independent dilution series and different PCR runs. The mean C_t, standard deviation, and percent coefficient of variation (% CV) were calculated for each template concentration. The results showed low variability, with % CVs ranging from 0.81 to 2.52%, indicating that the assay was efficiently reproducible.

Abundance dynamics of *nptII* gene homologous sequences in river water over 24 mo

Real-time PCR was applied to quantify the abundance of *nptII* gene homologous sequences present in river water samples collected over 24 mo. For each sample, river microbial community DNA and its serially diluted concentrations (typically 10^{-1} to 10^{-2}) were run by real-time PCR to determine the copy number of *nptII* gene homologous sequences. After real-time PCR, the specificity of the amplified fragments generated from river community DNA was confirmed by the dissociation curve analyses, which had a T_m of 87.5°C (data not shown). The results showed that the abundance of *nptII* gene homologous sequences varied considerably over the course of this study (Table 4). Although *nptII* gene homologous sequences were not detected in most of the water samples, they were present in 9 samples. The abundance was as high as 4.36×10^6 copies l^{-1} water in the sample collected on February 12, 2003, and 4.60×10^5 in the sample of September 13, 2004.

PCR amplification and restriction digestion of the *nptII* gene homologous fragments

To investigate the potential genetic variation of *nptII* gene homologous sequences in microbial communities of the river, we combined microbial community DNA from the 9 water samples in which *nptII* gene homologous sequences were detected. A major portion of the *nptII* gene was amplified from the combined DNA with the primer set of nptII-F and nptII-737R for cloning, representing 731 bp of the neomycin phosphotransferase coding region (792 bp). In total, 122 clones containing the correct insert were obtained. After digestion with the 2 rounds of restriction enzyme combinations, 11 clones were identified as unique sequences based upon their digestion pattern.

Phylogenetic relationship among the *nptII* gene homologous sequences

Homology searches in GenBank using the BLAST program revealed that all 11 clones showed high levels of sequence identity (>97.9%) to that of the *nptII* gene (GenBank accession V00618) on Tn5 (Fig. 4), with most having 4 to 6 nucleotide substitutions, deletions, or insertions at various nucleotide positions. Clone K025 had the most nucleotide changes (15 of 731) compared to that of the *nptII* gene. This result indicates that there is sequence variation among the cloned fragments retrieved from the river.

Conservation of the deduced amino acid sequence of the *nptII* gene fragments

The cloned 731 bp fragment represented 243 of the total 264 amino acids of the neomycin phosphotransferase encoded by the *nptII* gene, designated APH(3′)-

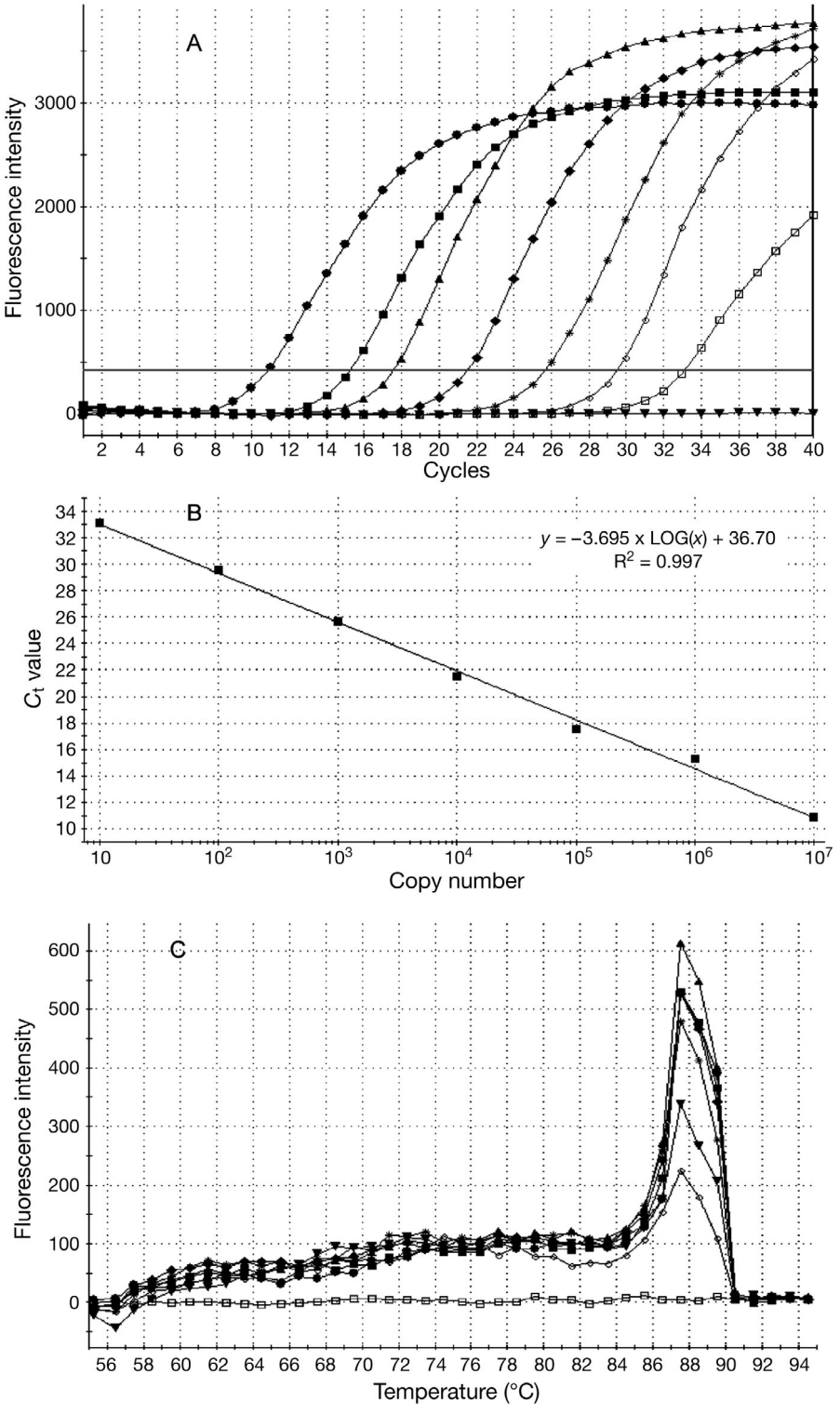

Fig. 3. Establishment of the standard curve for quantifying the *nptII* gene. (A) Amplification plots of the *nptII* gene standards. The copy number of the *nptII* gene is (from left to right) 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2 and 10, with a non-template control showing no amplification. (B) Standard curve was generated from the threshold (C_t) values of the amplification plots with Stratagene Mx 4000. This curve represents the standard curve only; no unknowns are represented. (C) Dissociation curve analysis shows a melting point of 87.5°C

IIa (Shaw et al. 1993). Subsequently, the deduced amino acid of the cloned fragments was aligned and compared with the amino acid sequence of the *nptII* gene (Fig. 5). The results showed that these sequences shared a high similarity with that of the *nptII* gene (>96% identity). Clone K025 shared a 96% identity and 98% similarity with that encoded by the *nptII* gene.

DISCUSSION

Previous studies have demonstrated that although only a small portion of culturable Km^R bacterial strains carried the *nptII* gene, the presence of *nptII* gene homologous sequences in DNA extracted from natural microbial communities was detected using Southern blot analyses with the *nptII* gene as probes (Leff et al. 1993a, Smalla et al. 1993). To assess the abundance of *nptII* gene homologous sequences in environments, their abundance in both culturable and non-culturable bacterial populations should be known (Smalla et al. 1993, Sèveno et al. 2002). The real-time PCR assay used in the present study proved useful for quantifying the abundance of *nptII* gene homologous sequences in microbial communities. It allowed the specific amplification of the *nptII* gene DNA over a 7-log DNA concentration range. The specificity of the assay was confirmed by a search for primer-specific sequences with the BLAST program and melting curve analyses. Taken together, it can be applied to quantify the abundance of *nptII* gene homologous sequences when a large number of environmental samples need to be screened.

For bacterial kanamycin resistance, both spatial and temporal differences in the frequency of the *nptII* gene were observed in some aquatic environments, suggesting that a large number of samples should be studied to obtain a representative gene frequency in an ecosystem (Leff et al. 1993a, McArthur & Tuckfield 2000). Variation of the abundance of *nptII* gene homologous sequences detected in the river system

Table 3. Summary of copy number of *nptII* gene and threshold cycle (C_t) values that were generated from 4 different real-time PCR runs performed on 4 separate dilution series of the *nptII* gene contained in plasmid pNS1 DNA. Mean C_t: average C_t value of the 4 independent real-time PCR runs; CV: ratio between SD and the mean of the repeated measurements multiplied by 100

Copy number	Mean C_t	SD	% CV
10^7	10.94	0.35	2.17
10^6	15.39	0.19	1.12
10^5	17.73	0.15	0.88
10^4	21.45	0.14	0.81
10^3	25.91	0.26	1.47
10^2	29.35	0.31	1.19
10^1	32.65	0.44	2.52

over a period of 24 mo was observed in this study. Of the 24 water samples, 9 showed the presence of *nptII* gene homologous sequences, with copy number ranging from 1.26×10^3 to 4.36×10^6 l^{-1} water, implying temporal changes in the abundance of *nptII* gene homologous sequences in the river system. Smalla et al. (1993) suggested that the occurrence of the *nptII* gene in freshwater environments might be due to discharges of sewage, animal manure and municipal wastewater that entered to open waters in a largely uncontrolled fashion. Obviously, these sources may contain bacteria carrying *nptII* gene homologous sequences or impose a strong selection pressure (McArthur & Tuckfield 2000). Meanwhile, Leff et al. (1993a) reported the non-random occurrence of the *nptII* gene in a pristine stream with bank sediments that had a higher abundance than channel sediments or submerged leaf surfaces. Therefore, in addition to agricultural, medical, or sewage sources, some biogeochemical processes and selective factors may be associated with the occurrence frequency of different antibiotic resistance

Table 4. Copy number of *nptII* gene homologous sequences in 1 l of water collected monthly from the South Saskatchewan River in 2003 and 2004. For each water sample, data are expressed as the mean of triplicate real-time PCR runs ± SD. ND: not detected ($<1.0 \times 10^3$ copies l^{-1} water)

Date	Copy number
2003	
Jan 8	ND
Feb 12	$4.36 \pm 0.42 \times 10^6$
Mar 21	$2.51 \pm 0.79 \times 10^3$
Apr 2	ND
May 12	ND
Jun 28	ND
Jul 16	ND
Aug 11	ND
Sep 10	ND
Oct 10	$2.15 \pm 0.54 \times 10^3$
Nov 13	ND
Dec 11	$3.14 \pm 0.81 \times 10^5$
2004	
Jan 8	$1.69 \pm 0.47 \times 10^3$
Feb 9	ND
Mar 8	$4.92 \pm 0.87 \times 10^3$
Apr 18	ND
May 7	ND
Jun 9	ND
Jul 12	ND
Aug 6	ND
Sep 13	$4.60 \pm 0.72 \times 10^5$
Oct 14	ND
Nov 10	$1.26 \pm 0.36 \times 10^3$
Dec 7	$6.85 \pm 0.52 \times 10^4$

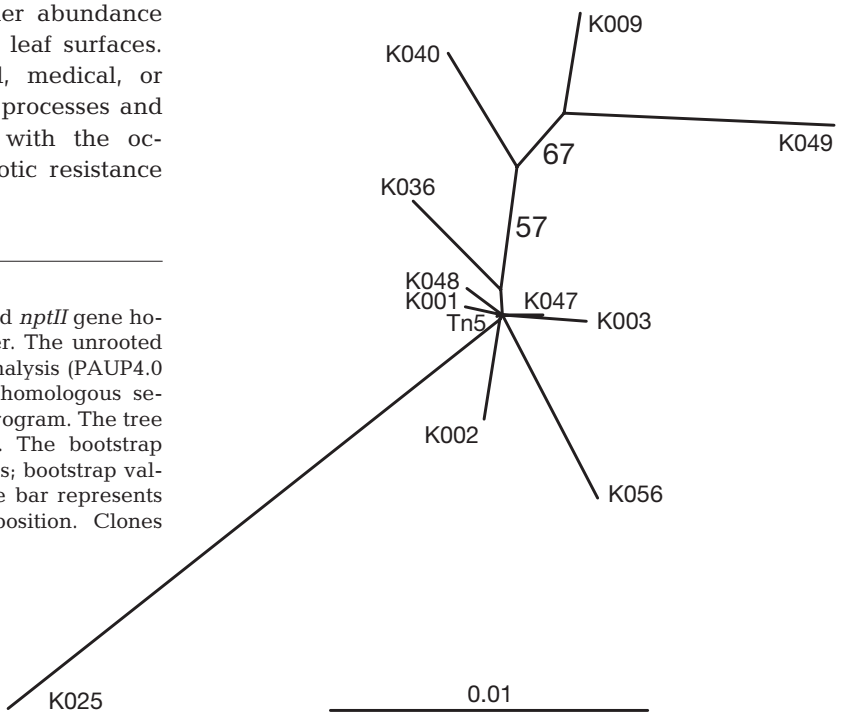

Fig. 4. Phylogenetic relationship of the cloned *nptII* gene homologous sequences retrieved from the river. The unrooted tree was constructed using the parsimony analysis (PAUP4.0 package) after the alignment of the *nptII* homologous sequence was calculated using the ClustalW program. The tree was derived using the Treeview program. The bootstrap analysis was conducted using 1000 replicates; bootstrap values >50 are shown on internal nodes. Scale bar represents 0.01 nucleotide chances per nucleotide position. Clones K001, K002, K003, K009, K025, K036, K040, K047, K048, K049, and K056 correspond to GenBank accession numbers DQ449898, DQ449903, DQ449900, DQ449894, DQ 449904, DQ449897, DQ-449896, DQ449901, DQ449899, DQ449895, and DQ-449902, respectively

```
K036            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDVAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K040            -LDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 59
Tn5             EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K002            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K048            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K001            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRTVLFVKTDLSGALNELQD 60
K047            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K009            -DDGLHAGSPAAWVERLFGHDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 59
K049            IQIGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K003            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
K056            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSARGRPVLFVKTDLSGALNELQD 60
K025            EQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQD 60
X90856          AATSMPPQAPSTWADYLAGYRWRGQGEGCSAATVHRLEAARRPTLFVKQEVLSAHAELPA 60
AJ278514        VALDEVSELKNLLSPLLDECTFEEVEYGQSDARVIRVLFPDRNTAYLKYASGSSAQEILQ 60
                   .  .         *    :     * * . * *:    * . ::*    .:  *:

K036            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K040            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 115
Tn5             EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K002            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K048            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K001            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K047            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K009            EAARLSWLATTGVPCAAVLDVVTEAGRDRLLLGEVPGQDLLS----SHLAPAEKVSIMAD 115
K049            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K003            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVPIMAD 116
K056            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
K025            EAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS----SHLAPAEKVSIMAD 116
X90856          EIARLRWLHGAGIDCPQVLNETQSDGRQWLLMSAMPGDTLSALAQRDELEPERLVRLVAA 120
AJ278514        EHQRTRWLRTR-ALVPEVISYVSTSTVTILLTKALIGHNAAD---AADADPVIVVAEMAR 116
                *  *  **       . *:. .       **   : *.         .  *   *  :*

K036            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K040            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 175
Tn5             AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K002            AMRRLHTLDPATCPFDRQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K048            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K001            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K047            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K009            AMRRLHTLDPATCPFVHQAKHRIERARTQMEAGLVDQDDLDEEHQGLAPAELFARLKARM 175
K049            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K003            AMRRLHTLDPATCPFNHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARM 176
K056            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAGLFAGLKARM 176
K025            AMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGPAPAELFARLKARM 176
X90856          ALRRLHDLDPAACPFDHRLERRLDTVRQRVEAGLVDEADFDDDHRGRSATELYRLLLDRR 180
AJ278514        ALRDLHSISPDDCPFDERLHLRLKLASGRLEAGLVDEEDFDHARQGMLARDVYEQLFIQM 176
                *:* ** :.*  *** .: . *:. .  ::******: *:*. ::*  .  ::  *  :
                ____________                ____________________

K036            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLG-ADRYQDIALATRDIAEELGGEWAD 235
K040            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 235
Tn5             PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K002            PDGEDLVVTHGDACLPNIMVENGRFSGFNDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K048            PDGEDLVETHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K001            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K047            PDGEDLVVTHGDACLPNIMVEIGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K009            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 235
K049            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGQLGVADRYQDIALATRDIAEELGGEWAD 236
K003            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K056            PDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWAD 236
K025            PDGEDLVVTHGDACLPNIMVENGRFSGFID-GRLGVADRYQDIALATRDIAEELGGERAD 235
X90856          PAVEDLVVAHGDACLPNLLAEGRRFSGFIDCGRLGVADRHQDLALAARDIEAELGAAWAE 240
AJ278514        PGAEQLVVTHGDACPENFIFQGNAFVGFIDCGRVGLADKYQDLALASRNIDAVFGPELTN 236
                *  *:** :*****  *:: :   * ** * *::* **::**:***:*:*   :*   ::

K036            RRLVLYG 242
K040            RRLVLYG 242
Tn5             RFLVLYG 243
K002            R-LVLYG 242
K048            R-LVLYG 242
K001            R-LVLYG 242
K047            R-LVLYG 242
K009            R-LVLYG 241
K049            R-LVLYG 242
K003            R-LVLYG 242
K056            R-LVLYG 242
K025            R-LVLIT 241
X90856          AFLVEYG 247
AJ278514        QFFIEYG 243
                  ::
```

Fig. 5. Comparison of the deduced amino acid sequences encoded by the *nptII* gene homologs and their closest relative, the *nptII* gene [APH(3′)-IIa] on Tn5. Also included are 2 members (EMBL accession X90856 and GenBank accession AJ278514) of the APH(3′)-IIb, characterized in *Pseudomonas aeruginosa* and *Achromobacter xylosoxydans*, respectively. Identical residues are each indicated by an asterisk; conservative amino acid substitutions are each indicated by a colon; and semi-conservative amino acid substitutions are each indicated by a dot. The 2 of the 3 highly conserved motifs that are known to be involved in catalytic activity of the APH enzymes are overlined

traits in some environments (Sundin & Bender 1996, McArthur & Tuckfield 2000). For instance, metal resistance genes and antibiotic resistance genes were often found on the same plasmids or mobile genetic elements in natural microbial communities (Wireman et al. 1997, Yurieva et al. 1997), implying that antibiotic resistance under natural conditions is made more complex by frequent genetic association with metal resistance genes (McArthur & Tuckfield 2000). Future studies on this genetic association would shed light on the persistence and evolution of antibiotic resistance genes in natural environments.

Cultured microorganisms, especially clinical bacterial strains, have been the major source of most of the known antibiotic resistance genes. In recent years, a few studies have demonstrated that natural environments may harbor a reservoir of antibiotic resistance genes (Sèveno et al. 2002, Riesenfeld et al. 2004), emphasizing that antibiotic resistance genes and their diversity in natural environments need to be characterized. Meanwhile, Waters & Davies (1997) suggested that sequence analysis of genetic elements conferring potential antibiotic resistance in microbial communities would be valuable both in predicting mechanisms of resistance likely encountered in subsequent clinical use and in understanding the evolution of bacterial antibiotic resistance. For instance, previous studies have identified conserved sequences within the phosphotransferase coding region by investigating aminoglycoside-resistant mutants with alterations in amino acid sequences and changes in the resistance profiles (Yenofsky et al. 1990). For the amino acid sequence of aminoglycoside resistance genes of the APH(3′)-II type, there were 3 highly conserved motifs identified to be involved in the catalytic activity of the APH enzymes (Shaw et al. 1993). On comparison with the *nptII* gene, APH(3′)-IIa, and 2 members (EMBL accession X90856 and GenBank accession AJ278514) of the APH (3′)-IIb, 2 of the 3 motifs were located within the cloned region of the *nptII* gene homologous fragments (Fig. 5). It was evident that these 2 enzyme-binding sites were highly conserved among the cloned sequences, supporting the opinion that the conserved regions of the neomycin phosphotransferase-coding sequence were important enzyme-binding domains.

The results from the real-time PCR analyses demonstrated that *nptII* gene homologous sequences were present in microbial communities of the South Saskatchewan River and that their abundance varied over the course of a 2 yr period. Meanwhile, the comparison of the nucleotide and deduced amino acid sequences of the cloned *nptII* homologous fragments to those of the *nptII* gene on transposon Tn5 showed that they had high levels of homology (>96%). Further studies to quantify the abundance of *nptII* gene homologous sequences at different spatial scales in the river will generate a representative gene frequency in the ecosystem and shed light on the mechanisms underlying the persistence of the antibiotic resistance gene.

Acknowledgements. The research was financially supported by the Canadian Environmental Protection Act (CEPA) and the National Water Research Institute, Environment Canada. The author is grateful to Dr. J. Maguire for his insightful comments on an earlier version of the manuscript and to the 3 anonymous reviewers for their thoughtful reviews, which resulted in a much-improved manuscript. The author also thanks Dr. W. Wackernagel for kindly providing the marker-rescue system and J. Roy for technical assistance.

LITERATURE CITED

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Brennan RE, Samuel JE (2003) Evaluation of *Coxiella burnetii* antibiotic susceptibilities by real-time PCR assay. J Clin Microbiol 41:1869–1874

Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264:375–382

de Vries J, Wackernagel W (1998) Detection of *nptII* (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. Mol Gen Genet 257:606–613

Graupner S, Frey V, Hashemi R, Lorenz MG, Brandes G, Wackernagel W (2000) Type IV pilus genes *pilA* and *pilC* of *Pseudomonas stutzeri* are required for natural genetic transformation, and *pilA* can be replaced by corresponding genes from nontransformable species. J Bacteriol 182:2184–2190

Leff LG, Dana JR, McArthur V, Shimkets LJ (1993a) Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl Environ Microbiol 59:4417–4421

Leff LG, McArthur JV, Shimkets LG (1993b) Spatial and temporal variability of antibiotic resistance in freshwater bacterial assemblages. FEMS Microbiol Ecol 13:135–144

Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 58:563–602

McArthur JV, Tuckfield RC (2000) Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl Environ Microbiol 66:3722–3726

Meier P, Wackernagel W (2003) Monitoring the spread of recombinant DNA from field plots with transgenic sugar beet plants by PCR and natural transformation of *Pseudomonas stutzeri*. Transgenic Res 12:293–304

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comp Appl Biosciences 12:357–358

Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6:981–989

Rosenberg DM, Chambers PA, Culp J, Franzin WG and 5 others (2005) Nelson and Churchill River Basins. In: Benke

AC, Cushing CE (eds) Rivers of North America. Elsevier Academic Press, Burlington, MA

Sèveno NA, Kallifidas D, Smalla K, van Elsas JD, Collard JM, Karagouni AD, Wellington EMH (2002) Occurrence and reservoirs of antibiotic resistance genes in the environment. Rev Med Microbiol 13:15–27

Sharkey FH, Banat IM, Marchant R (2004) Detection and quantification of gene expression in environmental bacteriology. Appl Environ Microbiol 70:3795–3806

Shaw KJ, Rather PN, Hare RS, Miller GH (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev 57:138–163

Sikorski J, Teschner N, Wackernagel W (2002) Highly different levels of natural transformation are associated with genomic subgroups within a local population of *Pseudomonas stutzeri* from soil. Appl Environ Microbiol 68:865–873

Smalla K, van Overbeek LS, Pukall R, van Elsas JD (1993) Prevalence of *nptII* and Tn5 in kanamycin-resistant bacteria from different environments. FEMS Microbiol Ecol 13:47–58

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004) Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. Appl Environ Microbiol 70:7372–7377

Sundin GW, Bender CL (1996) Dissemination of the strA-strB streptomycin resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. Mol Ecol 5:133–143

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

van Elsas JD (1992) Antibiotic resistance gene transfer in the environment: an overview. In: Wellington EMH, van Elsas JD (eds) Genetic interactions between microorganisms in natural environments. Pergamon Press, London, p 17–39

Waters B, Davies J (1997) Amino acid variation in the GYrA subunit of bacteria potentially associated natural resistance to fluoroquinolone antibiotics. Antimicrob Agents Chemother 41:2766–2769

Wireman J, Liebert CA, Smith T, Summers AO (1997) Association of mercury resistance with antibiotic resistance in gram-negative fecal bacteria of primates. Appl Environ Microbiol 63:4494–4503

Yenofsky RL, Fine M, Pellow JW (1990) A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure. Proc Natl Acad Sci USA 87:3435–3439

Yurieva OG, Kholodii G, Minakhin L, Gorlenko Z, Kalyaeva E, Mindlin S, Nikiforov V (1997) Intercontinental spread of promiscuous mercury resistance transposons in environmental bacteria. Mol Microbiol 24:321–329

Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA

Submitted: March 30, 2006; Accepted: January 23, 2007
Proofs received from author(s): June 29, 2007