The Journal of Microbiology, October 2005, p.383-390
Vol. 43, No. 5

Diversity of Denitrifying Bacteria Isolated from Daejeon Sewage Treatment Plant

Young-Woon Lim[1], Soon-Ae Lee[2], Seung Bum Kim[3], Hae-Young Yong[2], Seon-Hee Yeon[2], Yong-Keun Park[4], Dong-Woo Jeong[2] and Jin-Sook Park[2,*]

[1]Department of Wood Science, University of British Columbia, 2900-2424 Main Mall, Vancouver, B.C. V6T 1Z4, Canada
[2]Department of Biotechnology, Hannam University, 133 Ojung-dong, Daeduk-gu, Daejeon 306-791, Republic of Korea
[3]Department of Microbiology, School of Bioscience and Biotechnology, Chungnam National University, 220 Gung-dong, Yusong-gu, Daejeon 305-764, Republic of Korea
[4]Laboratory of Microbial Genetics, School of Life Science and Biotechnology, Korea University, 5 Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea

(Received March 31, 2005 / Accepted August 8, 2005)

The diversity of the denitrifying bacterial populations in Daejeon Sewage Treatment Plant was examined using a culture-dependent approach. Of the three hundred and seventy six bacterial colonies selected randomly from agar plates, thirty-nine strains that showed denitrifying activity were selected and subjected to further analysis. According to the morphological and biochemical properties, the thirty nine isolates were divided into seven groups. This grouping was supported by an unweighted pair group method, using an arithmetic mean (UPGMA) analysis with fatty acid profiles. Restriction pattern analysis of 16S rDNA with four endonucleases (*Alu*I, *Bst*UI, *Msp*I and *Rsa*I) again revealed seven distinct groups, consistent with those defined from the morphological and biochemical properties and fatty acid profiles. Through the phylogenetic analysis using the 16S rDNA partial sequences, the main denitrifying microbial populations were found to be members of the phylum, *Proteobacteria*; in particular, classes *Gammaproteobacteria* (*Aeromonas*, *Klebsiella* and *Enterobacter*) and *Betaproteobacteria* (*Acidovorax*, *Burkholderia* and *Comamonas*), with *Firmicutes*, represented by *Bacillus*, also comprised a major group.

Key words: ARDRA, denitrifying bacteria, sewage treatment plant

The nitrification and denitrification processes in wastewater treatment plants reduce the nitrogen content in wastewater. Nitrification is the biological oxidation of ammonia to nitrate. Denitrification is the reduction of nitrate to nitrogen gas via nitrite, nitric oxide and nitrous oxide. Denitrifying bacteria play a key role in the removal of nitrogen compounds from wastewater (Knowles, 1982). Denitrification by bacteria in sewage treatment plant is of particular interest, as nitrates and nitrites are hazardous to human health (Kempster *et al.*, 1997), and more importantly, a main cause of eutrophication (Gray, 1990). Bacteria capable of denitrification can be easily isolated from sediment, soil and aquatic environments. *Pseudomonas* species are generally presumed to be the predominant microorganisms through which denitrification is achieved (Lazarova *et al.*, 1992; Janda *et al.*, 1998). However, recent studies have shown that various species, including *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium* and *Hyphomicrobium,* as well as *Pseudomonas,* are responsible for denitrification in soil (Zumft, 1992; Otlanabo, 1993; Chèneby, *et al.*, 2000). Therefore, it seems unlikely that only *Pseudomonas* species are responsible for the denitrification that occurs in such incredibly diverse microbial consortia as exist in sewage treatment plants.

Many nitrate reducing bacteria only have the enzymatic ability to reduce nitrates to nitrites, with no further reduction of the nitrites (Glass *et al.*, 1997). According to Rheinheimer (1985), most denitrifying bacteria in aquatic systems are capable of complete denitrification. As a whole, the full effect of complete denitrification on the nitrogen removal in sewage treatment plants remains to be determined and; thus, a more intensive understanding of denitrifying bacteria is essential, as this may be one of the next steps for optimizing the nitrogen removal efficiency of sewage treatment plants. Therefore, the analysis of the microbial community structure related to nitrogen removal is a prerequisite for understanding the nitrogen removal process.

A culture dependent approach has been used for the

* To whom correspondence should be addressed.
(Tel) 82-42-629-7498; (Fax) 82-42-629-8355
(E-mail) jspark@hannam.ac.kr

study of the diversity of denitrifying populations in wastewater treatment plan ts (Krogulska and Mycielski, 1984; Patureau *et al.*, 2000; Drysdale *et al.*, 2001). The information derived from 16S rDNA sequences facilitates not only the identification of bacteria in environmental samples, but also a more complete understanding of microbial phylogeny (Woese, 1987; Chèneby *et al.*, 2000). Amplified rDNA restriction analysis (ARDRA) has also been an extremely valuable tool for assessing the diversity from large amounts of data (Cho *et al.*, 2003; Yoon *et al.*, 2003; Yeon *et al.*, 2005).

In this study, the isolated denitrifying bacteria were identified and characterized, using their morphological and biochemical properties, cellular fatty acid composition and 16S rDNA sequencing.

Materials and Methods

Sampling and isolation of denitrifying bacteria

Sewage water samples were taken from inflow and effluent regions of the Daejeon Sewage Treatment Plant, Daejeon, Republic of Korea, during spring, 2001 and fall, 2002. Samples (100 μl each) were diluted within the range 10^{-1} to 10^{-7}, spread onto 1/10 nutrient broth (Difco, USA) and incubated at 30°C. All colonies appearing on the plates over a period of 3 weeks were characterized by several criteria related to their morphology, and individual colonies streaked onto 1/10 nutrient broth for isolation. Subsequently, denitrifying bacteria were selected by checking for the presence of nitrite or nitrate by the addition of 0.2% sulphanilamide and 0.1% α-naphthyleneamine.

Morphological and biochemical characterization

The denitrifying bacteria were characterized using colony pigment, Gram-straining, catalase, oxidase, indole production, glucose acidification, arginine dihydrolase, urease, esculin hydrolysis, geletine, hydrolysis and â-galactosidase tests. Additional tests were performed using API Kits (BioMerieux, France) to determine the ability of the bacteria to utilize glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, caproate, adipate, malate, citrate and phenyl-acetate. Isolated denitrifying bacteria were tentatively identified to the genus level by examining their phenotypic and biochemical characteristics, as described previously (Krieg and Holt, 1984; Sneath *et al.*, 1986; Holt *et al.*, 1994). DNA was extracted by the method of Saito and Miura, 1963), and the G+C contents determined by reversed-phase high-performance liquid

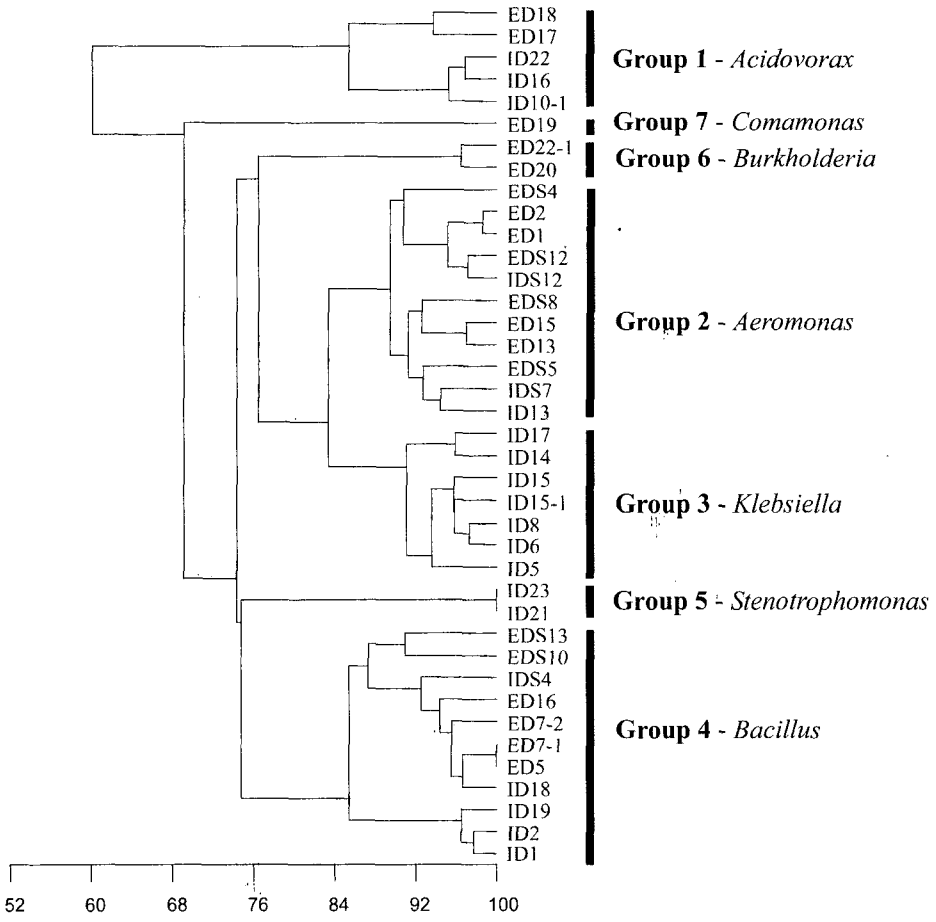

Fig. 1. Dendrogram showing the relationships between the denitrifying bacterial isolates based on the biochemical characters.

chromatography (Tamaoka and Komagata, 1984). Similarity between strains was estimated from the combined endonuclease restriction data, using the Multi-Variate Statistical Package (MVSP, Version 3.1). The UPGMA method was used to build the similarity dendrogram from the percentage similarity matrix using the MVSP software.

Table 1. Morphological characteristics, G+C contents (mol %) and biochemical characteristics of the denitrifying bacterial isolates

Group	Isolates[1]	Gram	Shape[2]	G+C (%)	Biochemical Characteristics[3]								
					OXI	IND	GLU	ADH	URE	ARA	MNE	CIT	PAC
1	ID10-1	–	R	64	+	–	–	–	–	–	–	–	–
	ID16	–	R	64	+	–	–	–	–	–	–	–	–
	ID22	–	R	60	+	–	–	–	–	–	–	–	–
	ED18	–	SR	63	+	–	–	–	–	–	–	–	–
	ED17	–	SR	66	+	–	–	–	–	–	–	–	–
2	ID13	–	SR	59	+	+	+	+	–	+	+	–	–
	IDS7	–	SR	66	+	+	+	+	–	+	+	+	–
	IDS12	–	SR	59	+	+	+	+	–	+	+	–	–
	ED1	–	SR	57	+	–	+	–	–	+	+	+	–
	ED2	–	SR	58	+	–	+	–	–	+	+	+	–
	ED13	–	SR	55	+	+	+	+	–	-	+	+	–
	ED15	–	SR	57	+	+	+	+	–	-	+	+	–
	EDS4	–	SR	65	+	+	+	+	+	+	+	+	–
	EDS5	–	SR	54	+	+	+	+	–	+	+	–	–
	EDS8	–	SR	60	+	–	+	–	–	+	+	+	–
	EDS12	–	SR	57	+	–	+	+	–	+	+	–	–
3	ID5	–	SR	52	–	–	+	+	+	+	+	+	+
	ID15	–	SR	54	–	–	+	–	–	+	+	+	+
	ID6	–	SR	57	–	–	+	–	+	+	+	+	+
	ID8	–	SR	58	–	–	+	–	–	+	+	+	+
	ID14	–	SR	54	–	–	+	+	–	+	+	+	–
	ID17	–	SR	57	–	–	+	+	–	+	+	+	–
	ID15-1	–	SR	57	–	–	+	–	+	+	+	+	–
4	IDS4	+	CR	38	–	–	–	+	+	+	–	+	–
	EDS10	+	CR	35	–	–	–	+	+	-	–	–	–
	EDS13	+	CR	37	–	–	–	+	–	-	–	–	–
	ID1	+	R	48	–	–	–	–	–	-	+	–	–
	ID2	+	R	44	–	–	–	–	–	-	+	–	–
	ID19	+	R	38	–	–	–	–	–	-	+	–	–
	ID18	+	R	39	–	–	–	+	+	+	+	+	–
	ED5	+	R	42	–	–	–	+	+	+	+	+	–
	ED7-1	+	R	39	–	–	–	+	+	+	+	+	–
	ED7-2	+	R	47	–	–	–	+	+	+	+	+	–
	ED16	+	R	37	–	–	–	+	+	+	+	+	–
5	ID21	–	R	38	–	–	–	–	+	–	–	–	–
	ID23	–	R	40	–	–	–	–	+	–	–	–	–
6	ED20	–	R	66	–	–	–	–	–	–	+	+	+
	ED22-1	–	R	64	–	–	–	–	–	–	+	+	+
7	ED19	–	R	63	–	–	–	–	+	+	+	–	–

+, positive; –, negative.

[1]ID, inflow (2001); ED, efflux (2001); EDS, efflux (2002)

[2]R, rod; SR, short-rod; CR, chained-rod.

[3]OXI, oxidase; IND, indole production; GLU, glucose acidification; ADH, arginine dihydrolase; URE, urease; ARA, arabinose; MNE, mannose; CIT, citrate; PAC, phenyl-acetate.

Cellular fatty acid profile

After an incubation period of 24 to 48 h on Trypticase Soy Broth agar plates at 30°C, the bacterial biomass was harvested, saponified, methylated and analyzed by capillary gas-liquid chromatography employing the Sherlock Microbial Identification System (MIDI Inc., USA). The similarities between isolates were computed, with similarity indices from 0.5 to 0.9 considered reliable for identifying individual species.

Amplified rDNA restriction analysis (ARDRA) and 16S rDNA sequencing

The 16S rDNA gene was amplified using the primer set fd1 (5'-AGA GTT TGA TCM TGG CTC AG-3') (Weisburg *et al.*, 1991) and 1492r (5'-TAC GGH TAC CTT GTT ACG ACT T-3') (Lane, 1991). PCR and restriction fragment analyses were performed, as previously described by Chèneby *et al.* (2000.). Briefly, after amplification, the PCR products (10 μl) were digested with the restriction endonucleases, *Alu*I, *Msp*I, and *Rsa*I, for 16 h at 37°C, and *Bst*UI for 4 h at 60°C. The fragments obtained were then separated on a 2.5% (w/v) agarose gel in 0.5× TBE buffer. DNA fragments were sized by reference to a DNA molecular marker (1kb plus maker, Gibco BRL, USA). Restriction profiles were classified according to the presence or absence of digested fragments for each enzyme.

One to five representatives from each group were chosen for 16S rDNA sequencing, using the primer set 341f (5'-CCT ACG GGA GGCAGC AG-3') (Muyzer *et al.*, 1988.) and 926r (5'-CCG TCA ATT CMT TTR AGT TT -3') (Lane, 1991). Sequencing was performed using the dideoxy terminator sequencing method, with an ABI model 310 (version 3.0) automated sequencer. The nucleotide sequence data generated in this study have been deposited in the GenBank DNA sequence database. The 16S rRNA gene sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1997), and finally optimized using the PHYDIT program version 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/). Maximum parsi-

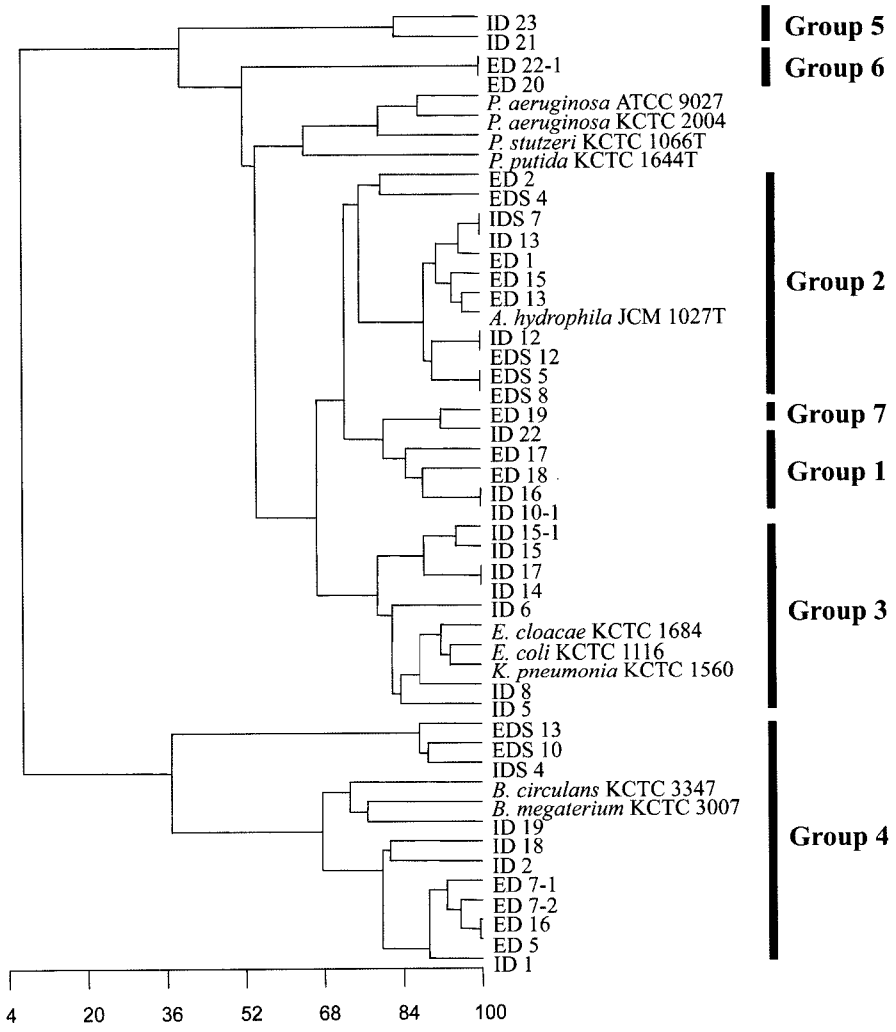

Fig. 2. Dendrogram of the denitrifying bacterial isolates from sewage water based on the fatty acid profiles.

mony analyses were performed using PAUP*4.0b4a (Swofford, 1999). Heuristic searches, using tree-bisection reconnection, with 100 random sequence additions were employed. The strength to support the branches of trees was evaluated through 1000 replicates of the bootstrap resampling (simple addition sequence, TBR swapping and MAXTREES unrestricted) (Hillis and Bull, 1993).

Results

Isolation and morphological and biochemical characterization of denitrifying bacteria

Out of 227 and 149 colonies isolated in 2001 and 2002, respectively, 39 isolates capable of denitrification were identified. According to the colony morphology and biochemical characteristics, the isolates were divided into seven distinct groups. Examples of selective characters are shown in Table 1. A dendrogram was constructed using the MVSP program based on the morphological and biochemical characteristics (Fig. 1). Groups 1, 2, 3 and 4 contained 5, 11, 7 and 11 isolates, respectively, while groups 5, 6 and 7 included 2, 2 and 1 isolate, respectively. The strains in Group 4 were Gram positive, with those in all other groups being Gram negative. The morphological and biochemical properties enabled assignment of each group to generic level (Fig. 1). Groups 1 to 7 were identified as *Acidovorax, Aeromonas, Klebsiella, Bacillus, Stenotrophomonas, Burkholderia* and *Comamonas* respectively. Members of *Betaproteobacteria* (*Acidovorax*, *Burkholderia* and *Comamonas*) and *Gammaproteobacteria* (*Aeromonas*, *Klebsiella* and *Stenotrophomonas*) were found to be the predominant denitrifying bacterial taxa. The DNA base compositions for each group are presented in Table 1. These data were consistent with the assignment of each group to their corresponding genera.

Cellular fatty acid profile

The predominant saturated and unsaturated fatty acids in all denitrifying isolates, with the exception of those in Group 4, were *n*-hexadecanoic acid (16:0) and *cis*-11-octadecenoic acid (18:1 ω7*c*). In contrast, *n*-pentadecanoic acid (15:0) and 12-methyltetradecanoic acid (anteiso-15:0) were the major saturated and unsaturated fatty acids in Group 4. According to the cellular fatty acid

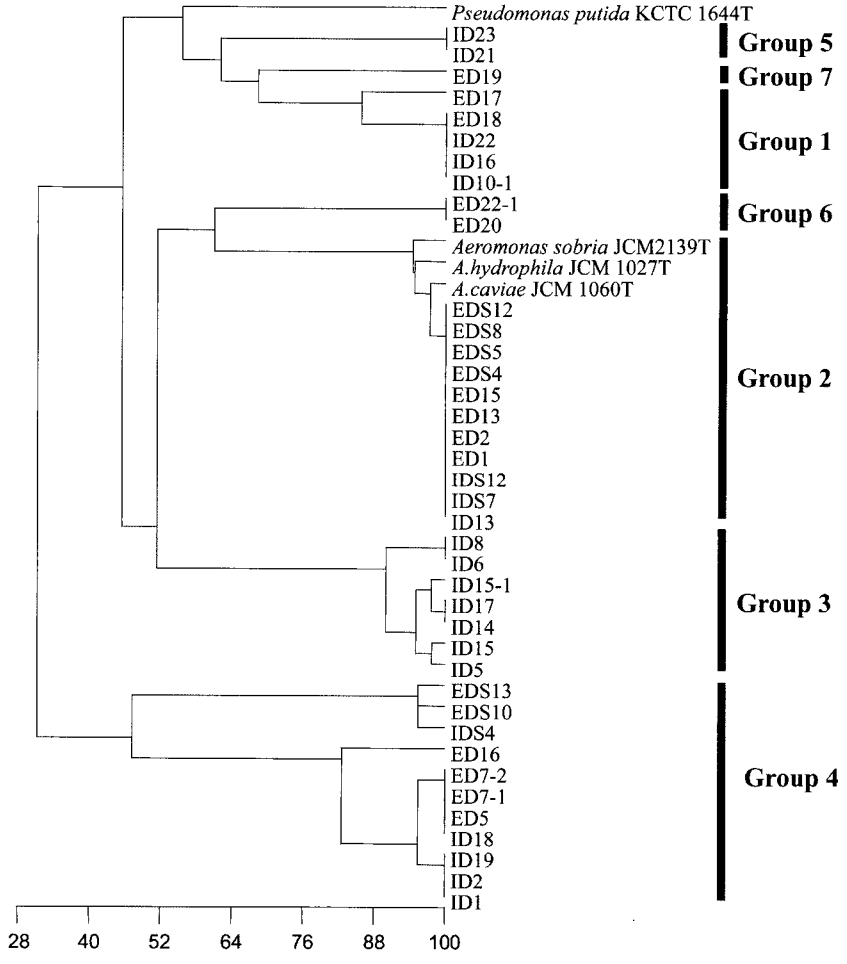

Fig. 3. UPGMA dendrogram obtained after restriction of the amplified 16S rDNA gene with four different enzymes (*Alu*1, *Bst* U1, *Msp*I and *Rsa*I).

profiles, the thirty nine isolates were again divided into seven groups (Fig. 2), as the strain composition of each group was identical to that found from the phenotypic properties. The sole exception was strain ID22, which belonged to Group 1 (*Acidovorax* group) in the phenotypic analysis, but fell into Group 7 (*Comamonas* group) from the fatty acid analysis. The clustering pattern between the groups was different from that based on the phenotypic properties. Group 4 (*Bacillus*) was separated from the other groups, with a similarity level of 36%, but *Betaproteobacteria* and *Gammaproteobacteria* were mixed within a big clade (Fig. 2).

ARDRA and 16S rDNA sequence analysis

All isolates yielded the same sized bands, ca. 1.5kb after amplification with the fd1 and 1492r primer set. ARDRA with *Alu*I digestion resulted in the highest number of bands. Denitrifying isolates could be divided into 7 ARDRA groups after digestion with the four restriction endonucleases (Fig. 3). Although the branching patterns of the dendrogram were different, the groupings were congruent with those found from the phenotypic properties and fatty acid profiles (Fig. 3).

Fourteen representative isolates from each of the seven groups were selected for 16S rDNA partial sequencing. Approximately 580 nucleotides were sequenced from each amplified 16S rDNA using the 341f and 926r primer set. The deposited nucleotide sequences are represented in the phylogenetic tree shown in Fig. 4. A parsimony analysis yielded the 48 most parsimonious trees, and example of which is shown in Fig. 4. From the phylogenetic tree, it is apparent that the seven major groups can be consistently associated with the genera identified from the morphological- and biochemical-properties and fatty acid profiles (Fig. 4). However, the strains in Group 5, which were initially assigned as *Stenotrophomonas*, were clustered with some enteric bacteria instead, namely *Enterobacter*, *Pantoea agglomerans* and *Salmonella enterica*. From the above results, it was evident that the denitrifying populations in the wastewater treatment system encompassed a wide variety of bacterial genera, mainly those belonging to the *Betaproteobacteria* and *Gammaproteobacteria*.

Discussion

Wastewater treatment processes are dependent on microbial activities for the removal of inorganic nitrogen or phosphorus. Denitrifying bacteria are important for the removal of nitrogen compounds from wastewater, where both denitrification and nitrification processes are combined (Janda *et al.*, 1998). Analysis of the microbial com-

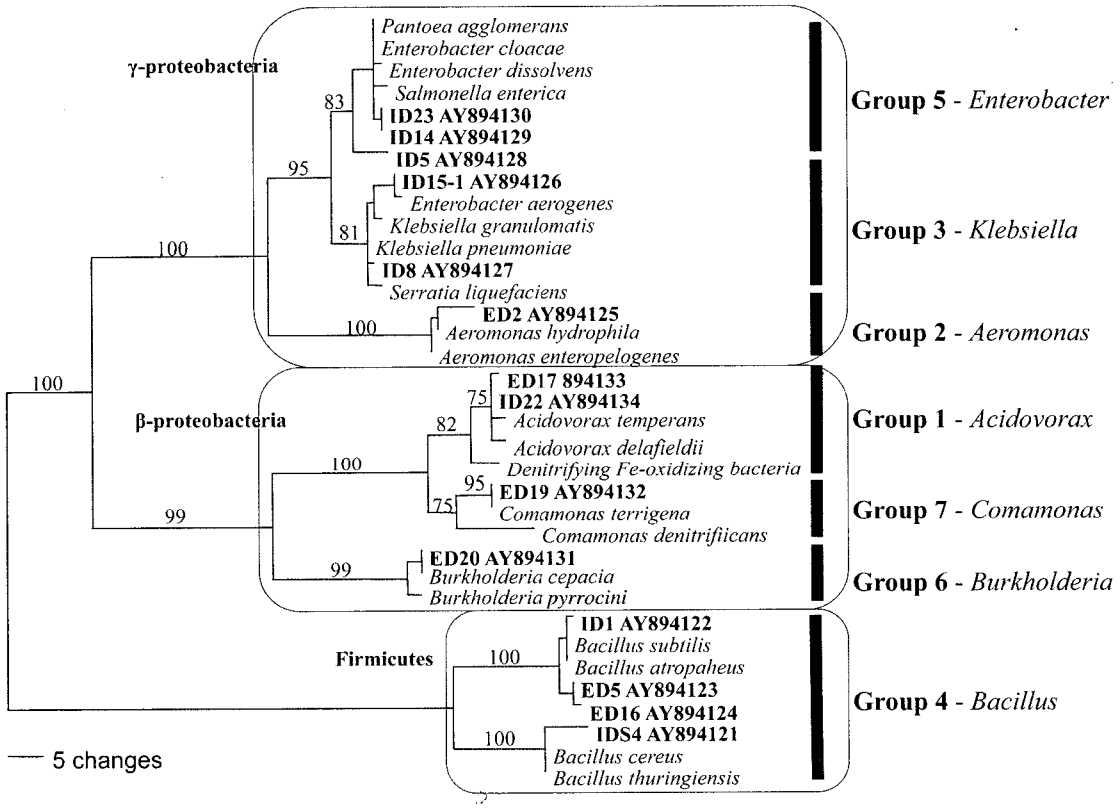

Fig. 4. The most parsimonious tree comparing the 16S rDNA sequences of the isolates to reference sequences retrieved from GenBank. Bootstrap support values are indicated (when greater than 70%) at the based of the corresponding branches. Taxa originating from the representative isolates of denitrifying bacteria are in bold. Tree length=322 steps, consistency index=0.8043, retention index=0.9634.

munity structure related to denitrification and nitrification; thus, will be important for understanding the nitrogen removal process. However, there have only been a few researches on the microbial community related to the treatment processes that occur within domestic sewage treatment plants (Ahn *et al.*, 2003, Jeon *et al.*, 2003, Lee *et al.*, 2004), and even fewer studies on the diversity of denitrifying bacteria (Kim *et al.*, 2001, Lee *et al.*, 2005). This study is the first extensive study on the culturable diversity of denitrifying bacteria found within a domestic sewage treatment plant.

In the last decade, a set of molecular tools have been developed and applied for the investigation of the microbial community composition and dynamics in activated sludge systems, in both cultivation dependent and independent manners (Pike and Carrington, 1972; Wagner *et al.*, 1993; Juretschko *et al.*, 2002). Denitrifying bacteria are also widely distributed among taxonomic and phylogenetic varieties of microorganisms (Tiedje, 1988). A number of bacterial taxa involved in denitrification within wastewater treatment systems have been reported in Korea, including *Pseudomonas*, *Arthrobacter*, *Staphylococcus* and *Bacillus* (Kim *et al.*, 2001; Lee *et al.*, 2005). Despite the limited sampling, our study clearly revealed the broad diversity of bacteria involved in denitrification. Based on their biochemical characteristics and fatty acid profiles, the thirty nine isolates fell into seven groups, corresponding to the genera *Acidovorax, Aeromonas, Klebsiella, Bacillus, Stenotrophomonas, Burkholderia* and *Comamonas*. The identification was identical to those found from ARDRA and 16S rDNA sequencing, with the exception of Group 5. The members of Group 5 were identified as *Stenotrophomonas* due to their morphological and biochemical properties and cellular fatty acid composition, but showed high sequence similarities with enteric bacteria. Interestingly, no denitrifying isolates were found to belong to *Pseudomonas*, which was contrary to expectation. The groupings based on the three different methods employed in this study were consistent with each other, although the relative relationships between the groups were different. Although 16S rDNA is commonly used for analyses of the diversity of denitrifying bacteria populations (Magnusson *et al.*, 1998; Chèneby *et al.*, 2000), the level of acceptable variation in the 16S rRNA gene is low, which is because of the functionality of this gene. In contrast, the biochemical characteristics and fatty acid profiles provided higher resolution between strains. Whether differences exist in the denitrifying capacity between the groups remains to be examined. This study revealed that the entire denitrification process might include activities contributed to by a number of different bacterial groups.

Acknowledgements

This research was funded by the Daejeon Environmental Technology Development Center, under the Research Development Program (2001), and the assistance of which the authors would like to acknowledge.

References

Ahn, I. S., M.W. Kim, H.J. La, K.M. Choi, and J.C. Kwon. 2003. Bacterial community composition of activated sludge relative to type and efficiency of municipal wastewater treatment plants. *J. Microbiol. Biotechnol.* 13, 15-21.

Chèneby, D., L. Philippot, A. Hartmann, C. Henault, and J.-C. Germon. 2000. 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soil. *FEMS Microbiol. Ecol.* 34, 121-128.

Cho, H.B., J.K. Lee, and Y.K. Choi. 2003. The genetic diversity analysis of the bacterial community in groundwater by denaturing gradient gel electrophoresis (DGGE). *J. Microbiol.* 41, 327-334.

Drysdale, G.D., H.C. Kasan, and F. Bux. 2001. Assessment of denitrification by the ordinary heterotrophic organisms in an NDBEPR activated sludge system. *Water Sci. Technol.* 43, 147-54.

Glass, C., J.A. Silverstein, and L. Denton. 1997. Bacterial populations in activated sludge denitrifying high nitrate waste reflect pH differences, p. 377-380 *Proc. 2nd Int. Conf. on Microorganisms in Activated Sludge and Biofilm Processes*, Berkeley, Califonia.

Gray, N.F. 1990. Activated Sludge: Theory and Practice. Oxford University Press, New York, New York.

Hillis, D.M. and J.J. Bull. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182-192.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams. 1994. Bergey's Manual of Determinative Bacteriology. The Williams & Wilkins, Baltimore, Maryland.

Janda, V., J. Rudovsky, J. Wanner, and K. Marha 1998. *In situ* denitrification of drinking water. *Water Sci. Technol.* 20, 215-219.

Jeon, C.O., S.H. Woo, and J.M. Park. 2003. Microbial communities of activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose. *J. Microbiol. Biotechnol.* 13, 385-393.

Juretschko, S., A. Loy, A. Lehner, and M. Wagner. 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst. Appl. Microbiol.* 25, 84-99.

Kempster, P.L., H.R. van Vliet, and A. Kuhn. 1997. The need for guidelines to bridge the gap between ideal drinking water quality and that quality which is practically achievable and acceptable. *Water SA.* 23, 163-167.

Kim, J.K., S.K. Kim, and S.H. Kim. 2001. Characterization of immobilized denitrifying bacteria isolated from municipal sewage. *J. Microbiol. Biotechnol.* 11, 756-762.

Knowles, R. 1982. Denitrification. *Microbiol. Rev.* 46, 43-70.

Krieg, N.R. and J.G. Holt. 1984. Bergey's Manual of Systematic Bacteriology, vol. 1. Williams & Wilkins, Baltimore, USA.

Krogulska, B. and R. Mycielski. 1984. Bacterial microflora participating in the removal of nitrogen from industrial wastewaters by nitrification and denitrifaction. *Acta Microbiol. Pol.* 33, 67-76.

Lane, D.J. 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E.

Stackebrandt and M. Goodfellow (eds.), Nucleic Acid Techniques in Bacterial Systematics, John Wiley and Sons, New York, New York.

Lazarova, V.Z., B. Capdeville, and L. Nikolov. 1992. Biofilm performance of a fluidized bed biofilm reactor for drinking water denitrification. *Water Sci. Technol.* 26, 555-566.

Lee, S.Y., H.G. Kim, J.B. Park, and Y.K. Park. 2004. Denaturing gradient gel electrophoresis analysis of bacterial populations in 5-stage biological nutrient removal process with step feed system for wastewater treatment. *J. Microbiol.* 42, 1-8.

Lee, S.Y., S.H. Lee, and Y.K. Park. 2005. New *nir*S-harboring denitrifying bacteria isolated from activated sludge and their denitrifying functions in various cultures. *J. Microbiol. Biotechnol.* 15, 14-21.

Magnusson, G., H. Edin, and G. Dalhammar. 1998. Characterization of efficient denitrifying bacteria strains isolated from activated sludge by 16S-rDNA analysis. *Wat. Sci. Tech.* 38, 63-68.

Muyzer, G.,T. Brinkhoff, U. Nubel, C. Santegoeds, H. Schafer, and C. Wawer. 1988. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 1-27. *In* ADL. Akkermans, van Elsas JD and de Bruijin, FJ (eds.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Nederlands.

Otlanabo, N.L. 1993. Denitrification of ground water for potable purposes. WRC report No. 403/1/93.

Patureau, D., E. Zumstein, J.P. Delgenes, and R. Moletta. 2000. Aerobic Denitrifiers Isolated from Diverse Natural and Managed Ecosystems. *Microb. Ecol.* 39, 145-152.

Pike, E.B. and E.G. Carrington. 1972. Recent developments in the study of bacteria in the activated-sludge process. *Water Pollut. Control* 71, 583-605.

Rheinheimer, G. 1985. Aquatic Microbiology, 3rd ed. VEB Gustav Fischer Verlag: Jena.

Saito, H. and K. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* 72, 619-629.

Sneath, P.H.A., N.S. Mair, M.E. Sharpe, and J.G. Holt. 1986. Bergey's Manual of Systematic Bacteriology, vol. 2. Williams & Wilkins, Baltimore, Maryland.

Swofford, D.L. 1999. *PAUP*: Phylogenetic Analysis Using Parsimony* (* and other methods), version 4.0b4a. Sinauer Associates, Sunderland, Massachusetts.

Tamaoka, J. and K. Komagata. 1984. Determination of DNA base composition by reversed high-performance liquid chromatography. *FEMS Microbiol. Lett.* 25, 125-128.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc. Acids Res.* 24, 4876-4882.

Tiedje, J.M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. *In* A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms. J. Wiley & Sons, Inc., New York, New York.

Wagner, M., R. Amann, P. Kämpfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K.H. Schleifer. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* 59, 1520-1525.

Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.T. Lane. 1991. 16S ribosomal DNA amplification of phylogenetic study. *J. Bacteriol.* 173, 697-703.

Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* 51, 221-271.

Yeon, S.H., W.J. Jeong, and J.S. Park. 2005. The diversity of culturable organotrophic bacteria from local solar salterns. *J. Microbiol.* 43, 1-10.

Yoon, Y.J., K.H. Im, Y.H. Koh, S.K. Kim, and J.W. Kim. 2003. Genotyping of six pathogenic *Vibrio* species based on RFLP of 16S rDNAs for rapid identification. *J. Microbiol.* 41, 312-319.

Zumft, W.G. 1992. The denitrifying prokaryotes, p. 554-582. *In* A. Balows, H.G. Trüper, M. Dworkim, W. Harder and K.H. Schleifer (eds.), The Prokaryotes. Springer-verlag, New York, New York.