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Molecular identification of nitrifying bacteria in activated sludge

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Abstract
Biological nitrification is a microbiological process which ammonia is oxidized to nitrate by ammonia-oxidizing bacteria (AOB). The nitrification is widely used in wastewater treatment process as the first step to remove nitrogen from the wastewater. We were identified the nitrifying bacteria in activated sludge collected from different municipal wastewater treatment systems using a molecular technique. The wastewater treatment plants differed in both wastewater characteristics and operating parameters; such as influent organic matter, ammonium, hydraulic retention time, and solids retention time. Biochemical tests as well as 16S rRNA gene study performed. The molecular technique targeting of the 16S in the sludge of the wastewater treatment plants were examined. Two different nitrifying bacteria were isolated from the municipal activated sludge. The results have indicated the two isolates to be *Nitrobacter* and *Nitrospira*. Rapid identification of the nitrifiyers seems to be one of the most important results in this technique.

Keywords: Molecular method, Nitrification, Nitrifying bacteria, Isolation, Identification

1. Introduction
Traditionally, the detection of bacteria in water, wastewater, and other environmental samples is restricted by the ability to culture such organisms from complex environmental samples. In recent years, the application of molecular techniques to the study of natural and engineered environmental systems has increased our insight interaction of microorganisms present in complex environments. Also, the molecular methods have supplied the means for examining microbial diversity and detecting specific organisms without the need for cultivation [1]. These innovative techniques have also been applied to detection and identification and enumeration of organisms from water and environmental samples[2]. The nitrifying bacteria are typical examples of fastidious bacteria. They are slowly growing bacteria and are difficult to enumerate by cultivation-dependent methods [3]. Nitrification is a two-step process performed by two different functional bacterial groups. AOB_s are responsible for the conversion of ammonia to nitrite, whereas NOB_s convert nitrite to nitrate as part of the two-step process of nitrification [4]. The Nitrifying bacteria are found in a variety of their shapes, including rods, curved rods, spheres, spirals, and lobular forms. These bacteria are gram-negetive and either heterotrophic, chemolithotrophic or facultative chemolithotrophic [5]. Among AOB_s, *Nitrosomonas europaea* has been the most widely studied species. Moreover, *Nitrobacter* has been the Nitrite-Oxidizing Bacteria (NOB) genus most commonly identified from water. Recent observations indicate that *Nitrospira* often are the dominant NOB in activated-sludge systems [6]. The AOB present in environments belong to a monophyletic group from the ß subclass of *Proteobacteria*, it is possible to investigate the whole ammonia oxidizing population with molecular tools by targeting part of the ammonium monooxygenase (amoA) gene [7]. More recently, progress in molecular ecology has enabled the successful application of 16S rRNA in identification of different bacteria. A variety of recently developed molecular techniques made it possible to explore the nitrifying bacteria in the environment and to enhance our knowledge of its functioning. In this study, the aim was to investigate the identification of nitrifying bacteria from the activated sludge. The 16S rRNA approach has been used to analyze communities of nitrifying bacteria in municipal activated sludge. A further objective was to introduce the molecular technique as a method for identification of environmental bacteria.

2. Materials and methods

Grab samples of mixed liquor were collected from the four municipal wastewater treatment plants in Tehran, Iran. The samples were stored in an icy cooler and immediately transported to the laboratory for further experiments. Ammonium, nitrite, nitrate, chemical oxygen demand and dissolved oxygen were determined using standard methods [8]. The flocs dispersion was made with 30 ml aliquots by a 5 min agitation in sterile tubes with glass beads (diameter 3_{mm}). For bacterial isolation, serial dilutions within the range of 10^4 -10^8 were plated on nitrifying bacteria medium. The positive tubes were inoculated to agar medium and after growing, colonies (mixed and single colony) were transferred to different concentrations of ammonium in basal medium (up to 1000 mg/l of ammonium as $(NH_4)_2SO_4$). The basal salt's medium contained per liter of distilled water: K_2HPO_4, 0.9 g; KH_2PO_4, 0.45 g; NH_4Cl, 0.45 g; $MgSO_4$, 0.2 g; $CaCl_2$_$2H_2O$, 0.02 g; $FeCl_3$, 0.005 g; and trace elements solution, 1 ml containing (mg/l): H_3BO_3, 400; $ZnSO_4$_$7H_2O$, 400; $CoCl_2$, 50; $NiCl_2$_$6H_2O$, 200; Na_2MoO_4_$2H_2O$, 300; $CuSO_4$-$5H_2O$, 10; $MnSO_4$-H_2O,500. Different kinds of colonies could be distinguished on the medium. Removal of ammonium rate in the media under the batch tests was checked with different isolated bacteria. The grown samples were frequently taken throughout the batch experiments for the determination of nitrogen species (nitrite and nitrate) following standard procedures. The cultures that converted high amount of ammonia were chosen for final and specific identification. Initial schemes were performed with biochemical tests as suggested by Bergey˙s Manual of Determinative Bacteriology [9]. We applied PCR to the samples based on the 16S rRNA gene for AOB and NOB bacteria. The method of Clavo *et al*. [10] was used for the extraction of DNA from sediments. Substances DNA purification was with Cinagen DNA extraction kit as specified by the manufacturer. All amplification reactions were performed in 0.2-ml thin walled microtubes, with the reaction mixture covered by a layer of sterile paraffin oil, in a thermal cycler (Eppendrof, Co). The reaction mixtures (100 ml) contained PCR buffer (100 mM Tris-HCl [pH 8.8], 15 mM MgCl2, 500 mM KCl, 1% Triton X-100, 0.1% [V/V] gelatin), 200 mM each deoxynucleoside triphosphate, 20 pM each primer, 50 ng of template DNA, and 1 U of Taq polymerase (Cinagen, Iran). Primers *amoA* and *amoB* were used to amplify 491-bp and 500-bp fragments of 16S rRNA of AOB (Table 1).

Table 1: PCR primers used in the experiments

Primer	Sequence (5′–3′)
amoA-f	GGGGTTTCTACTGGTGGT
amoA-r*	CCCCTCKGGAAAGCCTTCTTC
amoB-f*	TGGTAYGACATKAWATGG
amoB-r	ACGGGGCAAGAACATCGG
Eub338-f	ACTCCTACGGGAGGCAGC
NIT3-r	CCTGTGCTCCATGCTCCG
Ntspa0685M-r	CGGGAATTCCGCGCTC

The DNA samples were subjected to PCR amplification by direct application of *Nitrosomonas* and *Nitrosospira* primers. PCR was performed in a total volume 50 µl in 0.2 ml PCR tubes using a DNA thermocycler (Eppendorf). The following composition was used for a single reaction: 1× PCR buffer, 0.4 mM of each primer, 3 µl of template DNA, and 2.5 U of *Taq* DNA polymerase. Moreover, the PCR conditions were as follows: 5 min at 95°C followed by 35 cycles of 30s at 94°C, 1 min at [49°C (for primer *amoB*), 60°C (*amoA*) and 56°C, and 1 min 72°C, followed by 7 min final extraction at 72°C. The PCR was run with initial denaturation of the DNAs at 94°C for 3 min followed by 30 cycles of 30 s at 94°C (denaturation), 1 min at 46°C (Annaling), and 1 min at 73°C (Extention) along with negative control. A negative control consisting of reaction mixture without template was included in every PCR reaction. Aliquots (10 µl) of the PCR products were electrophoresed in 1.2 % agarose gels by using standard electrophoresis procedures.

3. Results and discussion

In order to identify the nitrifying bacteria in municipal wastewater treatment plants, a series of batch tests were performed using activated sludge taken from different municipal wastewater treatment plants in Tehran, Iran. Because of very slow growing and difficult to culture of nitrifying bacteria, the molecular method was used for

the nitrifying bacteria. Different studies have been performed to isolate and identify bacteria from activated sludge plants [11]. Used PCR method targeting the 16S rRNA gene and group specific functional genes, *amoA* and *amoB* primers, to investigate genera *Nitrosomonas* and *Nitrosospira* and genera *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*, respectively. The PCR result of molecular analysis is shown in Figure 1.

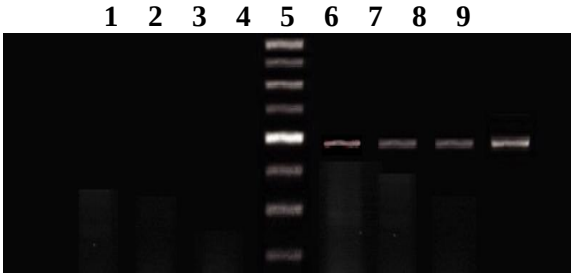

Figure 1: PCR amplification of the specific 500-bp *amoB* fragment from wastewater treatment plants

The results indicated that *Nitrosomonas* bacteria and *Nitrosococcus* bacteria found in the most wastewater treatment plants. Similarly, Mendum *et al*. [12] used *amoA* primer to identified *Nitrosomonas europaea*. According to physiological properties on ammonium affinity, members of *Nitrosospira spp.* and/or *Nitrosomonas* clusters are the prevailing AOB in the environment with low ammonium, whereas *Nitrosomonas* cluster is dominant in the environment that is rich in ammonium [13]. The same results were observed in Ekbatan municipal wastewater treatment plant with low ammonium concentration. The identification of *Nitrobacter* spp. using NIT3 primer was proposed by Kelly *et al.* [14]. Traditionally, *Nitrobacter* have been considered the most important bacteria for nitrite oxidation. However, current observations indicate that *Nitrospira* often are the dominant NOB in activated sludge systems. In addition, *Nitrospira* reportedly have high affinity towards oxygen [3]. Therefore, used Ntspa0685M primer to identification of *Nitrospira* spp. in our experiments. The results shows *Nitrospira* sp. identified in the most samples. Juretschko *et al.* [15] reported the same results using industrial wastewater with high amounts of ammonia. Also, Siripong and E. Rittmann [3] observed coexisting *Nitrobacter* and *Nitrospira* genera for nitrite-oxidizing bacteria. The low temperature may enhance the diversity of this NOB genus. Spieck *et al.* [16] detected *Nitrobacter* and *Nitrospira* in samples from activated-sludge system receiving municipal wastewater. With respect to NOB, several studies have indicated that nitrite concentration is the major factor controlling the competition between *Nitrospira* and *Nitrobacter* [17]. For comparing the molecular method and culture method, we used the culture method according to Bergey´s Manual of Determinative Bacteriology [9]. The cell shape, motility, utilization of urea and growing on anaerobic condition were investigated [18]. In all samples utilization of urea and growing on anaerobic condition were negative but motility was positive. Moreover, all bacteria were gram-negative. These results also show that the AOB and NOB coexisted in the same system. The coexistence of various nitrifiers having different growth and survival characters is an example of functional redundancy, which may be valuable for maintaining the stability and performance of nitrifying bioreactors.

Conclusion

Investigated bacterial populations relevant to nitrification in four municipal wastewater treatment plants. *Nitrosomonas* and *Nitrosococcus* bacteria are isolated from the most wastewater treatment plants. The observed diverse nitrifying populations prevailing in the plants are related to niche differentiation concerning ammonium concentrations and system operation. This investigation showed that the molecular technique represents a convenient and effective tool to detect nitrifying bacteria in environmental. This technique can especially be applied as a rapid screening method for studies in which many environmental samples have to be monitored for differences in the composition of the nitrifying bacteria.

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References

1. Gilbride, K. A., Lee, D. Y., Beaudette, L. A., *J. Microbiol. Methods* 66 (2006) 1.
2. Rezaee, A., Godini, H., Dehestani, S., Kaviani, S., *Iran. J. Environ. Health. Sci. Eng*, 7(2010) 313.
3. Siripong, S., Rittmann, B. E., (2007) *Water Res*, 41(2007) 1110.
4. Beman, J.M., Sachdeva, R., Fuhrman, J.A., *Environ. Microbiol*., 12 (2010) 1282.
5. Limpiyakorn, T. Shinohara, Y., Kurisu, F. Yagi, O. , *FEMS* Microbiol. *Ecol*, 54(2005) 205.
6. Junier, P., Kim, O.-S., Hadas, O., Imhoff, J.F., Witzel, K.P. , *Appl. Environ. Microbiol*. 36(2008) 18.
7. Caffrey, J.M., Bano, N., Kalanetra, K., Hollibaugh, J.T., The ISME J., 1(2007) 660.
8. APHA-AWWA-WPCF. (21st ed) American Public Health Association, Washington, DC (2005).
9. Holt J. G. Krieg N. R. Sneath P. H. A. Staley, J. T. Williams, S. T., 9th Ed., USA: Williams & wilkins, 1994.
10. Calvo, L. Gil, L. J., *J. Microbiol. Methods*. 57 (2004) 69.
11. Fong. K.P.Y, Tan. H.M, *World J. Microbiol. Biotechnol*., 16 (2000) 441.
12. Mendum, T.A. Sockett, R.E. Hirsch, P.R., *Appl. Environ. Microbiol*, 65 (1999) 4155.
13. Whang, L.-M., Chien, I-C., Yuan, S.-L. and Wu, Y.-J., (2009) *Chemosphere* 75(2009) 234–242.
14. Kelly, J. J., Siripong, S., McCormack, J., Janus, L. R., Urakawa, H. El Fantroussic, S. Noble, P. A., Sappelsa, L. Rittman, B. E., Stahl, D. A., *Water Res*., 39 (2005) 3229.
15. Juretschko, S. Timmermann, G., Schmid, M. Schleifer, K. H. Pommerening-Roser, Koops, A. and Wagner, M., *Appl. Environ. Microbiol*., 64(1998) 3042.
16. Spieck, E., Hartwig, C., McCormac, I., Maixner, F., Wagner, M., Lipski, A., Daims, H., *Environ. Microbiol*, 8(2006) 405.
17. Ce, A.L. and Garnier, B. J., *Water Res*. 39(2005) 4979.
18. Koops, H.P., Andreas, P.R., *FEMS Microbiol. Ecol.*, 37(2001) 1.