Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2018

www.elsevier.com/locate/jbiosc

Novel heterotrophic nitrogen removal and assimilation characteristic of the newly isolated bacterium *Pseudomonas stutzeri* AD-1

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Received 31 October 2017; accepted 13 March 2018
Available online xxx

AD-1, an aerobic denitrifier, was isolated from activated sludge and identified as *Pseudomonas stutzeri*. AD-1 completely removed NO_3^- or NO_2^- and removed 99.5% of NH_4^+ during individual culturing in a broth medium with an initial nitrogen concentration of approximately 50 mg L^{-1}. Results showed that larger amounts of nitrogen were removed through assimilation by the bacteria. And when NH_4^+ was used as the sole nitrogen source in the culture medium, neither NO_2^- nor NO_3^- was detected, thus indicating that AD-1 may not be a heterotrophic nitrifier. Only trace amount of N_2O was detected during the denitrification process. Single factor experiments indicated that the optimal culture conditions for AD-1 were: a carbon-nitrogen ratio (C/N) of 15, a temperature of 25°C and sodium succinate or glucose as a carbon source. In conclusion, due to the ability of AD-1 to utilize nitrogen of different forms with high efficiencies for its growth while producing only trace emissions of N_2O, the bacterium had outstanding potential to use in the bioremediation of high-nitrogen-containing wastewaters. Meanwhile, it may also be a proper candidate for biotreatment of high concentration organic wastewater.

[**Key words:** Aerobic denitrification; Bacteria assimilation; *Pseudomonas*; Nitrogen removal; Trace N_2O emission]

Over decades, the excessive fixation of nitrogen especially NO_3^- and NH_4^+, has been a serious problem for aquatic ecosystems, and this has led to a series of environmental consequences (1). To control and remediate nitrogen pollutants in water, the efficient and low-cost technology used to remove nitrogen is biological nitrification and denitrification, which involves two separate steps. Nitrification is the oxidation of NH_4^+ to NO_3^- via NO_2^- under aerobic conditions, while denitrification indicates that NO_3^- is converted into N_2O or N_2 via NO_2^- and NO successively as electron receptors under completely anoxic conditions (2,3). The two steps require different oxygen conditions. Even though aerobic nitrifiers are sensitive to organic compounds (4), anaerobic denitrifiers consume large amounts of organic materials (5). Therefore, these phenomena impose restrictions on the applications of biological nitrification and denitrification.

To date, the methods adopted for nitrogen removal based on biological nitrification and denitrification usually involve the following processes: short-cut nitrification and denitrification, anaerobic ammonia oxidation, completely autotrophic nitrogen removal over nitrite process, and an oxygen-limited autotrophic nitrification–denitrification process (6). Mechanisms of aerobic denitrification have been of great concern since their first detection in the 1980s (7). It was proven that a group of bacteria known as aerobic denitrifiers could carry out denitrification when exposed to oxygen, which provided evidence for the possibility of simultaneously carrying out both nitrification and denitrification. Aerobic denitrifiers could utilize NO_3^- as an electron receptor for their growth in the presence of oxygen. This is based on the fact that the enzyme that catalyzes NO_3^- restoration is a periplasmic enzyme instead of being membrane-bound (5,8). Thus, it is not sensitive to oxygen (9) and is encoded by the periplasmic nitrate reductase gene (NAP). Therefore, aerobic denitrifiers were the primary subjects of research due to their better adaptability to the aquatic environment (10), as well as on the basis of their cost benefit.

Although aerobic denitrifiers are currently well-known, studies on them often focus on their mechanism of NO_3^- metabolism. Therefore, there has been little research on the removal of NH_4^+, particularly by denitrifiers (11). However, NH_4^+ along with its oxidation to NO_2^-, which is widely distributed in industrial wastewaters, can be harmful for the hydrosphere (12). It is clearly harmful for the aquaculture industry, since NO_2^- can oxidize ferrous ion to iron ion, thus weakening the oxygen carrying ability of hemoglobin resulting in a high mortality rate of aquatic animals (13). Currently, some research has focused on heterotrophic nitrification-aerobic denitrification that targets its potential applications for both NH_4^+ and NO_3^- removal. While the link between the two processes may not be universal (14), Joo et al. (15) has demonstrated a new and efficient pathway for the removal of NH_4^+ by *Alcaligenes faecalis* No. 4. The N_2 production is thought to via the hydroxylamine that was generated by NH_4^+oxidation, thus clearly distinguishing it from other types of microorganisms. Nitrogen

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https://doi.org/10.1016/j.jbiosc.2018.03.010

Please cite this article in press as: Qing, H., et al., Novel heterotrophic nitrogen removal and assimilation characteristic of the newly isolated bacterium *Pseudomonas stutzeri* AD-1, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.03.010

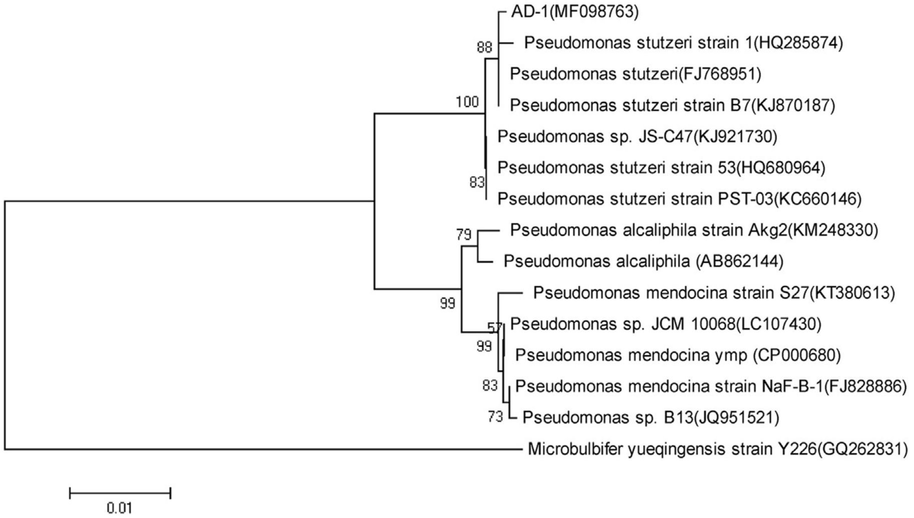

FIG. 1. Neighbor-Joining phylogenetic tree based on the 16S rRNA gene of AD-1 and other selected sequences, using *Microbulbifer yueqingensis* strain Y226 as an outgroup. The numbers on the branches indicate bootstrap values based on 1000 replications. 0.02 denotes the genetic distance.

assimilatory removal pathway has been proved to be of great significance due to its high efficiency in removal of both NH_4^+ and NO_3^-. Indeed, a study done with *Vibrio* sp. Y1-5 (16) had showed high assimilatory ability of NH_4^+ without the loss of nitrogen during the removal process. Therefore, both applied and basic research studies on the response of aerobic denitrifiers to NH_4^+ and NO_2^- are still required for efficient wastewater purification.

In this study, an aerobic denitrifier termed *Pseudomonas stutzeri* AD-1 was used to remove NO_3^-, NO_2^- and NH_4^+ separately through an experimental laboratory procedure. Results showed that NO_3^- was completely removed within 24 h along with emission of minute quantity of N_2O generated from aerobic denitrification pathway. NO_2^- and NH_4^+ were consumed within one day without the detection of NO_3^-, and assimilatory was the main nitrogen removal pathway. The goal of this study is to provide useful data for further applied research on the bioremediation of high-nitrogen-containing wastewaters.

MATERIALS AND METHODS

Enrichment and isolation of aerobic denitrifiers The activated sludge was obtained from the Tang-Xun-Hu sewage treatment works in Wuhan, Hubei Province, China. The samples were placed in bottles, sealed and immediately transported to the laboratory. The bottles were then left undisturbed for 1 h for the sludge and upper liquid to separately settle. The liquid was carefully decanted and discarded, and the sludge was then incubated in 500 mL conical flasks with 100 mL sterilized denitrification broth medium (DBM). DBM contained the following reagents per liter: 5.0 g of KNO_3; 16.67 g of sodium succinate dibasic hexahydrate; 1.5 g of KH_2PO_4; 10.55 g of $Na_2HPO_4 \cdot 12H_2O$; 0.1 g of $MgSO_4$; 0.3 g of NH_4Cl and 2 mL of trace element solution. The final pH was adjusted to 7.0. The components of the trace element solution were as follows per liter: 1.8 g of $FeCl_2 \cdot 4H_2O$; 0.25 g of $CoCl_2 \cdot 6H_2O$; 0.01 g of $NiCl_2 \cdot 6H_2O$; 0.01 g of $CuCl_2 \cdot 2H_2O$; 0.70 g of $MnCl_2 \cdot 4H_2O$; 0.5 g of $ZnCl_2$; 0.5 g of H_3BO_3; 0.03 g of $Na_2MoO_4 \cdot 2H_2O$ and 0.01 g of $Na_2SeO_3 \cdot 5H_2O$. Culture flasks were then sealed with a breathable sealing membrane and shaken in a rotary shaker at 30°C and 160 rpm. A five mL suspension was incubated into 100 mL fresh DBM after every 4 days for 4 consecutive times. The suspension was subjected to 10–fold serial dilutions and then spread on BTB agar plates. BTB agar plates contained the following reagents per liter: 1.0 g of KNO_3; 8.5 g of trisodium citrate dehydrate; 1.0 g of KH_2PO_4; 0.05 g of $FeSO_4 \cdot 7H_2O$; 0.2 g of $CaCl_2$; 1.0 g of $MgSO_4 \cdot 7H_20$; 1 mL of 1% bromothymol blue; and 20 g of agar. The pH was maintained at 7.0–7.3. The BTB plates were then cultured in a 30°C temperature chemostat until blue-ringed colonies formed. Each single colony was separately tested using denitrification test medium (DTM), which was similar to DBM but used NO_3^--N as the sole N source at 50 mg L^{-1}. After comparing the NO_3^- removal rates, the colony with the highest efficiency was marked AD-1 and cultured in DBM for further research.

Identification of AD-1 Amplification of the 16S rRNA gene was conducted by PCR using bacterial universal primers 8F (AGAGTTTGATCCTGGCTCAG)/1492R (GGTTACCTTGTTACGACTT), subjected to electrophoresis in 1% agarose gels, and then visualized using ethidium bromide staining. The PCR amplification occurred in a total volume of 50 μL and contained the following components: 25 μL of 2 × Es Taq MasterMix (CW Biotechnology Company, Beijing. China), 1 μL of template, 1 μL of each primer and 22 μL of sterilized DD H_2O. The PCR conditions were as follows: 94°C for 7 min; 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; then 72°C for 4 min. The PCR products were sequenced by the I-congene Biotechnical Company (Wuhan, China). After online analysis of the sequencing results using the Basic Local Alignment Search Tool (BLAST) on the NCBI, several related sequences were selected to construct phylogenetic tree. The construction of the phylogenetic tree was carried out using the Neighbor-Joining (NJ) method with the BIOEDIT and MEGA5.1 software.

For NAP gene analysis, the primers were NAP1 (TCTGGACCATGGGCTTCAA CCA) and NAP2 (ACGACGACCGGCCAGCGCAG). The PCR procedures were as follows: 95°C for 7 min; 37 cycles at 95°C for 30 s, 59°C for 30 s and 72°C for 1 min; and 72°C for 4 min.

Nitrogen removal characteristics of AD-1 Broth medium with several different forms of nitride was used to test AD-1, to characterize the response of this isolates. The source of nitrogen per liter for this study was as follows: 1. 50 mg L^{-1} of NO_3^--N; 2. 50 mg L^{-1} of NO_2^--N; 3.50 mg L^{-1} of NH_4^+-N; 4. 50 mg L^{-1} of NH_4^+-N and 50 mg L^{-1} of NO_2^--N. Other components in one liter of the broth medium were as follows: 16.67 g of sodium succinate dibasic hexahydrate; 1.5 g of KH_2PO_4; 5.27 g of $Na_2HPO_4 \cdot 12H_2O$; 0.1 g of $MgSO_4$; and 2 mL trace element solution. The isolates were pre-cultured at 30°C and 160 rpm in a rotary shaker to the exponential phase, and then 4 mL bacterial suspension was centrifuged at 8000 rpm for 7 min, followed by three washed with sterilized DD H_2O, and then re-suspended to 4 mL. The suspension was inoculated into flasks of 500 mL with a working volume of 200 mL to obtain 2% inoculum. During the cultivation, the bacterial suspensions were sampled periodically to determinate the optical density (OD_{600}), then filtered through 0.22 μm membrane filters to determine the NO_3^-, NO_2^-, NH_4^+ and dissolved total nitrogen (DTN). The same process was conducted to determine the suspended total nitrogen (TN) prior to filtration at the beginning and the end (60 h) of culture. TN, DTN, NO_3^-, NO_2^-, NH_4^+, NH_2OH and intracellular nitrogen was detected to test nitrogen balance by the consumption of NO_3^- at the beginning and the end.

Detection of nitrous oxide One milliliter bacterial suspension as described was inoculated into a 1 L bottle with 100 mL DTM. The bottles were tightly sealed with a gas-impermeable rubber plug and then cultured in a rotary shaker. For comparison, the blank DTM without inoculation was used as the control. For the determination of N_2O, headspace air (21% oxygen) in the bottle was not replaced to form an aerobic environment. Twenty milliliters of headspace gas was collected using a 50 mL gas-tight syringe through the rubber stopper every 12 h to detect N_2O. The samples were collected in triplicate.

Impact of temperature, C source and C/N To determine the influence of temperature, 2 mL of the bacterial suspension were collected at the exponential phase, centrifuged, washed by sterilized DD H_2O, re-suspended to 2 mL and inoculated (2% inoculum) into 100 mL DTM with an initial NO_3^--N concentration of 50 mg L^{-1}. The suspension was then cultured in rotary shaker at temperatures of 15°C, 20°C, 25°C, 30°C, 35°C and 40°C. Additional conditions were as follows: the carbon source was sodium succinate and the C/N was 60. For carbon sources,

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