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Aerobic Denitrifying Bacteria That Produce Low Levels of Nitrous Oxide

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Most denitrifiers produce nitrous oxide (N_2O) instead of dinitrogen (N_2) under aerobic conditions. We isolated and characterized novel aerobic denitrifiers that produce low levels of N_2O under aerobic conditions. We monitored the denitrification activities of two of the isolates, strains TR2 and K50, in batch and continuous cultures. Both strains reduced nitrate (NO_3^-) to N_2 at rates of 0.9 and 0.03 μmol min^{-1} unit of optical density at 540 nm^{-1} at dissolved oxygen (O_2) (DO) concentrations of 39 and 38 μmol $liter^{-1}$, respectively. At the same DO level, the typical denitrifier *Pseudomonas stutzeri* and the previously described aerobic denitrifier *Paracoccus denitrificans* did not produce N_2 but evolved more than 10-fold more N_2O than strains TR2 and K50 evolved. The isolates denitrified NO_3^- with concomitant consumption of O_2. These results indicated that strains TR2 and K50 are aerobic denitrifiers. These two isolates were taxonomically placed in the β subclass of the class *Proteobacteria* and were identified as *P. stutzeri* TR2 and *Pseudomonas* sp. strain K50. These strains should be useful for future investigations of the mechanisms of denitrifying bacteria that regulate N_2O emission, the single-stage process for nitrogen removal, and microbial N_2O emission into the ecosystem.

Nitrous oxide (N_2O) is a gaseous nitrogen oxide that is present at a concentration of about 350 ppb in the atmosphere. The concentration of this compound was maintained below 300 ppb in the global nitrogen cycle before the 20th century. However, recent reports suggest that the atmospheric concentration of N_2O is now increasing at a rate as high as 0.3% per year (1). N_2O has a 200- to 300-fold-stronger greenhouse effect than carbon dioxide (CO_2) and has the potential to destroy the ozone layer (17). Therefore, the N_2O balance is critical to the natural environment. The proposed sources of N_2O are chemical industries, burning fossil fuels, and biomass, as well as soil denitrification of nitrogenous compounds resulting from excess agricultural fertilizer (3, 6, 25). Another critical source of N_2O is wastewater treatment plants, in which considerable amounts of nitrogen pollutants removed from treated water are released into the atmosphere as N_2O, as well as dinitrogen (N_2).

Currently, nitrogen removal in wastewater treatment plants is essentially based on the activity of nitrifying and denitrifying microorganisms, both of which are inhabitants of activated sludge. Nitrifying bacteria aerobically oxidize ammonium contaminants to nitrite (NO_2^-) and nitrate (NO_3^-), which are then reduced by denitrifying bacteria to gaseous nitrogen forms such as N_2O and N_2. Efficient wastewater treatment relies on successively exposing water to aerobic and anaerobic conditions, since nitrification and denitrification are aerobic and anaerobic processes, respectively (4, 18). These properties represent a shortcoming of current systems since both denitrification and nitrification produce N_2O as a by-product in the absence of correctly regulated oxygen (O_2) concentrations (2, 20). For example, the denitrifying activity of most denitrifying bacteria is suppressed when anaerobiosis is insufficient and they cannot catalyze the final step of denitrification (reduction of N_2O to N_2) and produce N_2O (2, 8, 13, 19). Because complete removal of dissolved O_2 is difficult before the anaerobic denitrification that follows aerobic nitrification, current systems release considerable amounts of N_2O during denitrification.

To overcome this problem, novel aerobic denitrifying bacteria are required that could be used for constructing aerobic denitrifying processes. *Paracoccus denitrificans* (formerly *Thiosphaera pantotropha*) (2, 19, 20) is an aerobic denitrifier that has been isolated from activated sludge and it is the best-characterized such organism. This species reduces NO_3^- even in the presence of a saturating concentration of O_2. More recent surveys of aerobic denitrifiers have revealed some novel species, such as *Microvirgula aerodenitrificans* (16) and *Thaurea mechernichensis* (22); the former organism denitrifies as efficiently as *P. denitrificans* under aerobic conditions (15). Previously published results show that these denitrifiers remove NO_3^- quite efficiently from treated water or from culture medium. However, the previous reports did not address the effect of O_2 on the reduction of N_2O to N_2.

In the present paper we describe a novel method for screening and characterizing natural aerobic denitrifiers that produce N_2 gas by reducing NO_3^- under oxic conditions. The strains described here produce less N_2O under aerobic conditions than the previously described aerobic denitrifiers produce, indicating that they potentially could be used to construct an aerobic denitrifying system that emits low levels of N_2O.

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MATERIALS AND METHODS

Strains and media. *P. denitrificans* (*T. pantotropha*) ATCC 35512 originated from the American Type Culture Collection. *Pseudomonas stutzeri* ZoBell (=

ATCC 14405) was provided by W. G. Zumft. Other strains were isolated in this study. The following media were used: bromothymol blue (BTB) medium (0.1% L-asparagine, 0.1% KNO_3, 0.1% KH_2PO_4, 0.005% $FeCl_2 \cdot 6H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 1 ml of BTB liter^{-1} [1% in ethanol], 2% agar; pH 7.0 to 7.3), screening medium (SM) [0.284% sodium succinate, 10 mM $NaNO_3$, 0.136% KH_2PO_4, 0.027% $(NH_4)_2SO_4$, 0.1% yeast extract (Difco), 0.019% $MgSO_4 \cdot 7H_2O$, 1 ml of a trace element solution (14) liter^{-1}; pH 7.2], denitrification medium (DM) (0.472% sodium succinate, 10 mM $Na^{15}NO_3$, 0.15% KH_2PO_4, 0.042% Na_2HPO_4, 0.06% NH_4Cl, 0.5% Casamino Acids [Difco], 0.1% $MgSO_4 \cdot 7H_2O$, 2 ml of a trace element solution [14] liter^{-1}; pH 7.2), artificial wastewater (AWW) (0.085% $NaNO_3$, 0.06% peptone, 0.04% bouillon extract, 0.01% urea, 0.003% NaCl, 0.01% KH_2PO_4, 0.0014% KCl, 0.002% $MgSO_4 \cdot 7H_2O$, 0.00185% $CaCl_2 \cdot 2H_2O$), and Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl).

Screening of denitrifiers. Samples from rice ponds, domestic wastewater, and soil were transferred to 200 ml of SM in 500-ml Erlenmeyer flasks with cotton plugs and incubated at 30°C for 3 days. Fresh SM was inoculated with 5 ml of culture and incubated under the same conditions. These procedures were repeated three times. The resultant bacterial suspension was streaked on BTB medium plates with 8.5 g of sodium succinate liter^{-1} and incubated at 30°C for 1 to 3 days. Soil samples were sometimes suspended in 0.9% NaCl and plated directly on BTB medium plates. Regardless, soil samples were incubated in DM or AWW instead of SM. Resulting blue colonies were isolated and screened as follows (second screening). The bacteria were transferred to 200 ml of Luria-Bertani medium with 10 mM $NaNO_3$ in a 500-ml Erlenmeyer flask. The flask was sealed with a butyl rubber stopper and rotary shaken at 120 rpm at 30°C (preculture). The atmospheric air in the headspace was not replaced, so the initial conditions were aerobic. A portion (50 ml) of the preculture was collected by centrifugation, washed twice with 0.9% NaCl, transferred to 100 ml of DM containing ^{15}N ($Na^{15}NO_3$) in a 500-ml Erlenmeyer flask, and incubated as described above. Aerobic denitrification by the bacteria was measured by determining time-dependent production of $^{15}N_2$ and the amount of residual O_2 in the headspace gas.

Batch culture. Batch cultures in flasks were incubated essentially under the conditions described above for the second screening. To investigate their effects on denitrification, various carbon sources were added to DM instead of succinate at a C/N ratio of 24.

Continuous culture. Precultures (100 ml) were transferred to 1-liter fermentation jars (BMJ-01; Able, Tokyo, Japan) containing 500 ml of DM or AWW with formate in which $Na^{15}NO_3$ was substituted for $Na^{14}NO_3$. Each culture was magnetically stirred at 300 rpm and 30°C (pH 7.4). Initially, 20% O_2 balanced with argon was added to the cultures at a constant flow rate of 20 ml/min. Exhaust gas that flowed into gas sampling tubes was collected after passing through a vapor condenser. The culture broth was aseptically withdrawn through sampling tubes, and then NO_3^-, NO_2^-, and biomass were analyzed. A continuous nutrient flow was started when cultures reached the logarithmic growth phase and was continued at a dilution rate of 0.14 h^{-1}. After 48 h, exhaust gas was analyzed in duplicate to confirm the steady state. The O_2 supply conditions were manipulated by changing the feed gas composition and/or the stirring speed. The cultures were constantly supplied with nutrients for over 48 h, which was equivalent to 6.7 replacements of the working volume to ensure a steady state.

Determination of the 16S rRNA gene sequences. Total bacterial DNA was purified as described previously (14). The genes encoding 16S rRNA were amplified by PCR by using total DNA (0.1 μg) as the template and primers AGA GTTTGATCCTGGCTCAG and GGTTACCTTGTTACGACTT (26). Genes were amplified by 30 cycles of 94°C for 20 s, 50°C for 1 min, and 72°C for 1.5 min, followed by extension at 72°C for 10 min.

Analytical methods. We analyzed gaseous O_2 and N_2O by gas chromatography (GC) (23). Isotope-labeled N_2 gas was measured by isotope mass spectroscopy (Delta plus, Finnigan MAT) as described previously (24), and NO_3^- and NO_2^- were measured colorimetrically (12).

Enzyme assays. Nitrate reductase (Nar) activity was assayed as described previously (7) by using methylviologen-dithionite as the electron donor. Nitrite reductase (Nir) activity was assayed by using NADH-phenazine methosulfate as the electron donor by determining the amount of NO produced by the P450nor-trap method (7). Nitric oxide reductase (Nor) activity was determined by determining NADH-dependent N_2O formation by GC as described previously (11). Nitrous oxide reductase activity was assayed as described by Kshirsagar et al. (9).

Nucleotide sequence accession numbers. The nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB096261 (strain TR2) and AB096260 (strain K50).

RESULTS

Isolation of novel aerobic denitrifiers. We developed plate assays to isolate bacterial strains that denitrify under aerobic conditions,. The method is based on the changes in the pH of a medium due to NO_3^- depletion by denitrification. The plates contained KNO_3 and the pH indicator BTB. The pH of the medium was initially adjusted to between 7.0 and 7.3. Plates inoculated with a bacterial suspension were incubated for several days at the appropriate temperature, and then bacterial NO_3^- consumption was monitored by examining blue colonies and/or halos due to the increasing pH of the medium. Positive strains obtained from this initial screening analysis were screened further by culturing them in flasks containing DM under initially aerobic conditions. Denitrification was periodically monitored by measuring N_2O and $^{15}N_2$ in the gas phase by GC and GC-mass spectrometry, respectively. Most strains completely consumed O_2 before they produced N_2. However, some strains produced significant amounts of N_2 even in the presence of 3% O_2 in the gas phase of the flasks. Among these, strains that produced the least N_2O (aerobic denitrifiers emitting low N_2O levels) were selected.

In a typical experiment we screened 97 bacterial colonies, and we found 29 strains which were positive in BTB plate assays and 9 strains that produced N_2 even in the presence of 3% O_2. Six of the nine strains evolved less than 1 μmol of N_2O flask^{-1} under the same culture conditions. The most prominent aerobic (O_2-tolerant) N_2 producers (strains TR2 and K50) from several screening analyses were analyzed as follows.

Properties of denitrifiers in batch culture. The time-dependent denitrification by strain TR2 or K50 was compared with that of the typical denitrifiers *P. stutzeri* ZoBell and *P. denitrificans* ATCC 35512 during batch culture in flasks with a headspace that was initially filled with air to create aerobic conditions. After the incubation was started, the O_2 concentration in the gas phase was gradually decreased, and N_2 evolved (Fig. 1). The rates of N_2 production were significantly different for the different cultures. Initiation of N_2 production by *P. stutzeri* ZoBell required a lag period of 2.5 h, whereas the other three strains evolved N_2 without a lag phase, indicating that N_2 production by *P. stutzeri* ZoBell is more sensitive to O_2 than N_2 production by the other strains is (Fig. 1A). The aerobic denitrifier *P. denitrificans* produced N_2 without a lag but concomitantly produced a considerable amount of N_2O (Fig. 1B). Strains TR2 and K50 produced N_2 without a lag period, and little N_2O was detected during the entire incubation period. These results demonstrated that strains TR2 and K50 should be aerobic denitrifiers that produce low levels of N_2O even under aerobic conditions. The NO_3^- initially added (1 mmol) was completely consumed by these cultures (data not shown). The amount of consumed N atoms recovered in N_2 was greatest with strain K50, in which the 0.27 mmol of N_2 obtained corresponded to 54% recovery (Fig. 1C). The final level of recovery with strain TR2 was 28% (Fig. 1D).

Denitrification in continuous culture. We further investigated the effects of aeration on N_2 and N_2O production by the isolated bacteria. Table 1 shows the steady-state characteristics observed with continuous cultures of the strains isolated and of the typical denitrifiers. Changing the composition of the aerating gas and agitation speed allowed us to establish anoxic

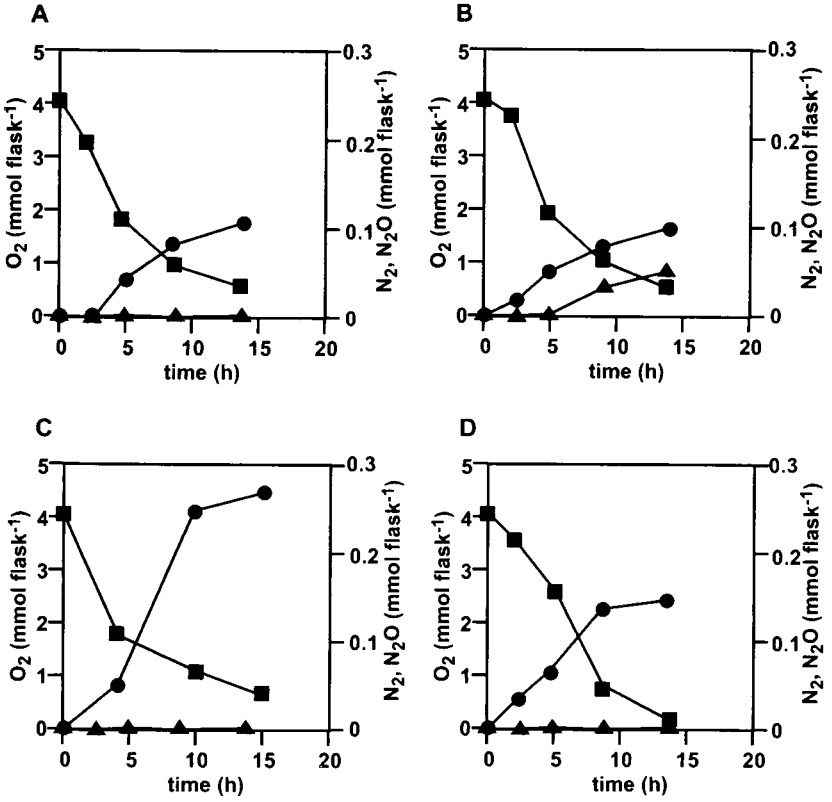

FIG. 1. Denitrification by new isolates and control denitrifiers in initially aerobic batch cultures. The strains were cultured in flasks containing DM, and the gas phase was monitored periodically as described in Materials and Methods. (A) *P. stutzeri* ZoBell; (B) *P. denitrificans* ATCC 35512; (C) strain K50; (D) strain TR2. Symbols: ●, N_2; ▲, N_2O; ■, O_2. Typical results from more than three independent experiments are shown.

(dissolved oxygen [DO] concentration, 0 μmol liter^{-1}), hypoxic (DO concentration, 5.3 to 9.4 μmol liter^{-1}), and oxic (DO concentration, 28 to 39 μmol liter^{-1}) aeration conditions. Most of the cultures produced N_2 under anoxic and hypoxic conditions; the only exception was a *P. denitrificans* culture in which there was too little growth to detect N_2 produced under anoxic conditions. All of the strains produced N_2 at a higher rate under hypoxic conditions than under anoxic conditions (data not shown). However, under anoxic conditions the rate of N_2 production was higher than that under hypoxic conditions, as for other bacterial denitrifiers (2, 4), when the rates were expressed on the basis of cell mass (optical density at 540 nm [OD_{540}]). This is because the cell mass was significantly larger under hypoxic conditions than under anoxic conditions.

TABLE 1. Steady-state characteristics of continuous cultures of bacterial strains[a]

Strain	Conditions[b]	DO concn (μmol liter^{-1})	OD_{540}	NO_3^- consumption (μmol min^{-1} unit of OD_{540}^{-1})	N_2O production (μmol min^{-1} unit of OD_{540}^{-1})	N_2 production (μmol min^{-1} unit of OD_{540}^{-1})
TR2	A	0	0.5	24	9.9×10^{-5}	6.8
	H	5.3	1.3	7.3	9.7×10^{-4}	2.8
	O	39	2.4	3.5	1.6×10^{-4}	0.9
K50	A	0	0.4	30	9.5×10^{-3}	6.5
	H	6.3	1.7	6.8	1.6×10^{-3}	1.8
	O	38	3.7	0	6.2×10^{-4}	0.03
P. stutzeri ZoBell	A	0	0.5	24	1.5×10^{-2}	2.9
	H	7.8	1.3	8.8	6.7×10^{-3}	1.5
	O	38	1.0	8.4	6.2×10^{-3}	0.0
P. denitrificans ATCC 35512	A	0	0.2	60	9.5×10^{-3}	0.0
	H	9.4	3.2	2.7	4.1×10^{-3}	1.4
	O	28	6.7	0.4	2.8×10^{-2}	0.0

[a] DM was used for the cultures. The dilution rate was 0.14 h^{-1}. The data are averages of two independent experiments.
[b] A, anoxic (the influent gas was 100% Ar, and the agitation rate was 300 rpm); H, hypoxic (the influent gas was 20% O_2–80% Ar, and the agitation rate was 300 rpm); O, oxic (the influent gas was 20% O_2–80% Ar, and the agitation rate was 430 rpm).

TABLE 2. Carbon source utilization of strains TR2 and K50[a]

Carbon source	Reaction rate (μmol h^{-1})					
	Strain TR2			Strain K50		
	N_2	N_2O	O_2	N_2	N_2O	O_2
Glycerol	8.8	1.3	230	4.8	0.05	140
Glucose	8.8	6.5	230	15.0	ND[b]	390
Succinate	36.3	ND	380	42.5	ND	410
Citrate	17.5	2.1	310	16.3	0.23	310
Acetate	13.8	2.6	250	28.8	ND	410
Ethanol	2.5	8.3	263	28.8	ND	450
Methanol	0.50	1.6	230	0.04	0.73	150
Formate	0.16	12.0	200	ND	1.0	380

[a] The values are the initial reaction rates for N_2 and N_2O production and O_2 consumption during the first 8 h of flask culture. N_2, N_2O, and O_2 concentrations in the gas phase were determined by GC. The initial amounts of NO_3^- and O_2 were 1 and 4 mmol, respectively. The data are averages of two independent experiments.
[b] ND, not detected.

The value per cell for strain TR2 (2.8 μmol of N_2 min^{-1} unit of $OD_{540}{}^{-1}$) was double that for *P. denitrificans* (1.4 μmol of N_2 min^{-1} unit of $OD_{540}{}^{-1}$) under hypoxic conditions. When challenged with oxic conditions, *P. stutzeri* ZoBell and *P. denitrificans* produced no N_2 and considerable amounts of N_2O, indicating that O_2 inhibited the reduction of N_2O to N_2 under these conditions. By contrast, strains TR2 and K50 could still produce N_2 under oxic conditions (Table 1). The activity of strain TR2 (0.9 μmol of N_2 min^{-1} unit of $OD_{540}{}^{-1}$) was as much as 32% of the activity under hypoxic conditions. Both TR2 and K50 produced less N_2O than the other strains produced under the corresponding aeration conditions. TR2 produced less N_2O than K50 produced, indicating that strain TR2 reduces N_2O to N_2 more efficiently than K50 reduces N_2O to N_2. Our results showed that *P. denitrificans*, a typical aerobic denitrifier, produced N_2O at a much higher rate than the other strains produced N_2O, indicating that this organism produces N_2O instead of N_2 under oxic conditions. These findings indicate that the strains isolated in the present study are aerobic denitrifiers that produce low levels of N_2O.

Electron donor specificity. We investigated the electron donor specificity of denitrification by strains TR2 and K50 in flask cultures under initially aerobic conditions (Fig. 1). Table 2 shows that both strains consumed O_2 and produced N_2 and/or N_2O with all carbon sources tested, indicating that they can use a variety of carbon sources as electron donors for O_2 respiration and denitrification. Of the carbon sources tested, succinate supported O_2 respiration and denitrification most efficiently in TR2. Strain K50 utilized O_2 efficiently in the presence of ethanol and acetate in addition to succinate. Although the N_2 production rates were lower and more N_2O evolved than the N_2O that evolved with the other carbon sources, C_1 compounds, such as methanol and formate, could support denitrification by both bacteria. Strain K50 evolved little N_2O irrespective of the electron donor.

Identification of the strains. Strains TR2 and K50 were both gram-negative rods with catalase and oxidase activities. They also metabolized glucose to organic acid, indicating that they are pseudomonads. The nucleotide sequences of their 16S rRNA genes and a phylogenetic analysis revealed that these strains are members of the genus *Pseudomonas* in the β subclass the class *Proteobacteria*. The levels of identity of the sequences of strains TR2 and K50 were greatest with the sequences of *P. stutzeri* (99%) and *Pseudomonas mendocina* NCIB 10541 (99%), respectively. The phenotype of strain TR2 was characteristic of *P. stutzeri*; namely, there were no fluorescent pigments, organic growth factors were not required, and the organism was gelatinase negative and amylase positive. The ability to grow at 41°C is also a phenotypic criterion that distinguishes *P. stutzeri* strains from other *Pseudomonas* spp. Thus, we identified strain TR2 as *P. stutzeri* and designated it *P. stutzeri* TR2. Strain K50 accumulated poly β-hydroxybutyric acid, which *P. mendocina* does not do. Therefore, we could not identify this strain and designated it *Pseudomonas* sp. strain K50.

Performance in AWW system. We estimated the denitrification by continuous cultures of *P. stutzeri* TR2 in organic wastewater. We added formate to conventional wastewater as an additional electron donor assuming that it should provide selective pressure for survival that should allow *P. stutzeri* TR2 to become the dominant species in the culture. Table 3 shows that neither *P. stutzeri* TR2 nor the control strain *P. stutzeri* ZoBell evolved N_2O at any DO level. TR2 more efficiently reduced NO_3^- and produced more N_2 than *P. stutzeri* ZoBell. When the DO concentration was increased to 113 μmol $liter^{-1}$, *P. stutzeri* ZoBell produced neither N_2 nor N_2O. This is in contrast to *P. stutzeri* TR2, which produced a significant amount of N_2 even under highly aerobic conditions (DO concentration, 141 μmol $liter^{-1}$). The level of recovery of N atoms in N_2 in an aerobic culture of *P. stutzeri* TR2 was 14% of the consumed NO_3^-. Other N atoms of the consumed NO_3^- should have been incorporated into the biomass. These results indicate that the denitrifying system of *P. stutzeri* TR2 is more resistant to

TABLE 3. Steady-state characteristics of strains in AWW supplemented with formate[a]

Strain	DO concn (μmol $liter^{-1}$)	OD_{540}	NO_3^- consumption (μmol min^{-1} unit of $OD_{540}{}^{-1}$)	NO_2^- production (μmol min^{-1} unit of $OD_{540}{}^{-1}$)	N_2 production (μmol min^{-1} unit of $OD_{540}{}^{-1}$)
P. stutzeri TR2	3	0.22	51.4	7.7	5.5
	72	0.50	23.0	0.18	5.6
	141	0.62	18.2	0.10	1.3
P. stutzeri ZoBell	3	0.45	23.1	0.89	1.3
	113	0.50	22.4	0.14	ND
	160	0.49	22.7	0.08	ND

[a] The dilution rate was 0.14 h^{-1}. The data are typical results of three independent experiments. No N_2O production was detected for any strain.

O_2 than the denitrifying system of *P. stutzeri* ZoBell is and that the TR2 strain produced N_2 under aerobic conditions in AWW.

Enzyme activities. The activities of respiratory NO_3 reductase, NO_2^- reductase, NO reductase, and N_2O reductase in crude extracts prepared from denitrifying cells of *P. stutzeri* TR2 were determined, and the results obtained with 3-day cultures are shown in Fig. 1. The specific activities of these enzymes were 4,400, 63, 6.8, and 64 nmol of product min^{-1} mg of $protein^{-1}$, respectively, indicating that the activity observed should have been responsible for denitrification by *P. stutzeri* TR2.

DISCUSSION

Although N_2O production by denitrifying bacteria under insufficient anaerobic conditions is an established phenomenon that causes serious global warming, in most studies of aerobic denitrifiers the workers have described only NO_3^- and NO_2^- consumption under aerobic conditions, and studies in which the workers focused on denitrified gas (N_2O and N_2) have been limited. This is probably due to difficulties in detecting denitrified N_2 under aerobic conditions; under such conditions contamination with atmospheric N_2 interferes with precise quantitation of denitrified N_2. Here, we used heavy-isotope-labeled NO_3^- ($^{15}NO_3^-$) and highly sensitive mass spectrometry to screen and isolate denitrifying bacteria emitting low levels of N_2O under aerobic conditions. To our knowledge, we are the first researchers to isolate and characterize denitrifiers that produce less N_2O than other bacteria produce. Our screening method should be applicable to isolation of aerobic denitrifying bacteria that exhibit such properties.

Our results indicated that both of the denitrifiers isolated, *P. stutzeri* TR2 and *Pseudomonas* sp. strain K50, produced N_2 even under oxic conditions (DO concentration, 38 to 39 μmol $liter^{-1}$) (Table 1), conditions under which other typical aerobic denitrifiers could not produce N_2, indicating that the novel strains are O_2 resistant, aerobic denitrifiers. Physiologically, O_2 is the best electron acceptor for supporting growth, and it receives electrons through the respiratory chain. Most denitrifying microorganisms perform O_2 respiration and repress the denitrification mechanism when O_2 is available. Therefore, the physiological significance of denitrification under oxic conditions (aerobic denitrification) is quite intriguing. Aerobic denitrification has also been identified in *P. denitrificans* (19, 20), as well as in the novel species *M. aerodenitrificans* (15) and *T. mechernichensis* (22). The strains identified here, together with these organisms, should be useful for future investigations of the mechanisms of aerobic denitrification by bacteria and the role of aerobic denitrification in the ecosystem.

We found that all of the bacteria tested emitted considerably more N_2O under continuously aerobic conditions than under anaerobic conditions (Table 1). This is consistent with the observation that in most denitrifiers, the activity of N_2O reductase is inactivated by O_2, which also represses expression of the encoding gene and N_2 production (4, 8). We also found that both *P. stutzeri* TR2 and *Pseudomonas* sp. strain K50 produced less N_2O and more N_2 under our conditions than the other denitrifiers produced (Fig. 1 and Table 1). Furthermore, our results demonstrated that the N_2- and N_2O- producing activities of the strains were dependent on the culture. For example, carbon sources seemed to affect denitrification to various degrees depending on the strain (Table 2). Factors other than carbon sources can also affect aerobic denitrification. In the presence of formate as an electron donor, *P. stutzeri* TR2 produced N_2O in batch culture (Table 2), whereas little N_2O was detected in continuous culture (Table 3). This may have been due to differences in conditions between the batch and continuous cultures or to the compositions of the media. The mechanism with which the isolates suppressed N_2O production remains to be studied. However, the results suggest that aerobic denitrification that produces less N_2O should be a complicated system that is regulated by various factors, including the O_2 supply and the composition of the medium. It is interesting that *P. stutzeri* TR2 and ZoBell have different denitrifying properties, especially for N_2O production under oxic conditions (Table 1) and for N_2 production in response to sudden exposure to O_2 (Fig. 1), despite their close phylogenetic relationship. A comparison of the genes of these strains should reveal the critical gene(s) required for aerobic denitrification and for N_2O suppression.

The novel bacteria isolated in the present study denitrified under conditions that mimicked those in wastewater treatment plants (Table 3). This finding suggests that the isolates could be maintained in the mixed population of microorganisms found in sludge as is observed with *P. denitrificans*, which reduces NO_3^- more efficiently than a control reactor without the strain when it is mixed with activated sludge (9). Sometimes, a C_1 carbon source, such as methanol or formate, has been used to manipulate the dominant bacterial species in sludge (5, 21). Our novel strains should be useful for constructing new aerobic denitrification processes that do not produce N_2O, since these strains could be selected as the dominant organisms by using C_1 compounds as the electron donors.

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