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Heterotrophic Nitrification by *Alcaligenes faecalis*: NO_2^-, NO_3^-, N_2O, and NO Production in Exponentially Growing Cultures

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Heterotrophic nitrification by *Alcaligenes faecalis* DSM 30030 was not restricted to media containing organic forms of nitrogen. In both peptone-meat extract and defined media with ammonium and citrate as the sole nitrogen and carbon sources, respectively, NO_2^-, NO_3^-, NO, and N_2O were produced under aerobic growth conditions. Heterotrophic nitrification was not attributable to old or dying cell populations. Production of NO_2^-, NO_3^-, NO, and N_2O was detectable shortly after cultures started growth and proceeded exponentially during the logarithmic growth phase. NO_2^- and NO_3^- production rates were higher for cultures inoculated in media with pH values below 7 than for those in media at alkaline pH. Neither assimilatory nor dissimilatory nitrate or nitrite reductase activities were detectable in aerobic cultures.

Besides the broad spectrum of denitrifiers, autotrophic nitrifiers have attracted special scientific interest, since it was found that these microorganisms are able to produce NO and N_2O during nitrification and, therefore, may contribute significantly to the biogenic emissions of these trace gases into the atmosphere (2, 6, 8, 14, 18, 19, 30). Heterotrophic nitrification, first described in 1894 for a fungus (24), is thought to be restricted to old or stationary-phase bacterial cultures (for examples, see references 1 and 22) and to be a minor component of the biogeochemical cycle of nitrogen (3, 4). However, heterotrophic nitrification may be an important process in heath and conifer forest soils (15, 17, 21, 25). In these studies however, it was not determined whether N_2O or NO is produced during heterotrophic nitrification in addition to nitrite or nitrate. The present experiments were conducted to identify microorganisms able to nitrify under aerobic heterotrophic growth conditions; characterize the growth phase, during which heterotrophic nitrification activities occur; and quantify the potential products of heterotrophic nitrification including N_2O, NO, and NO_2.

MATERIALS AND METHODS

Alcaligenes faecalis DSM 30030, *Alcaligenes eutrophus* DSM 428, *Pseudomonas aeruginosa* DSM 50071, *Arthrobacter simplex* DSM 20130, *Arthrobacter* sp. DSM 312, and *Arthrobacter* sp. 917 were obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany. Stock cultures of the bacteria were maintained under aerobic conditions at 28°C on peptone-meat extract (PM) agar plates (5 g of peptone from meat [no. 7224; Merck, Darmstadt, Federal Republic of Germany], 3 g of extract of meat [no. 3979; Merck] per liter of aqua bidest., 2% [wt/vol] agar). The pH of the PM medium was adjusted to 7 with 0.1 N NaOH prior to autoclaving. For experiments on heterotrophic nitrification from organically bound nitrogen, PM medium was used but agar was omitted. In addition, a defined ammonium citrate (AC) medium was used. The basal medium contained the following components (molar): sodium citrate, 9.5×10^{-3}; NH_4Cl, 9.35×10^{-3}; KH_2PO_4, 1.47×10^{-3}; $MgSO_4 \cdot 7H_2O$, 1.62×10^{-4}; $CaCl_2 \cdot 2H_2O$, 1.36×10^{-7}; and $FeSO_4 \cdot 7H_2O$ (as EDTA complex), 3.6×10^{-5}. The pH of the medium was adjusted to 6.5 with 5% Na_2CO_3 or 1 N NaOH prior to autoclaving.

For testing NO_2^-, NO_3^-, N_2O, and NO production during heterotrophic nitrification, mid-log cells of *A. faecalis* grown in PM or AC medium (28°C; 120 rpm on a rotary shaker) were centrifuged (4°C, 20,000 × *g*) and washed twice in PM or AC medium, respectively. Cells were suspended in the original volume of the respective medium, and 10 μl of the suspension (about 10 to 35 μg of protein) was inoculated into Fernbach flasks (total volume, 7.0 ml) containing 2.0 ml of medium. Flasks were stoppered with sterile serum rubber stoppers and incubated at 28°C on a rotary shaker at 120 rpm. The gas phase was analyzed for N_2O, NO, and O_2 every 60 min. Thereafter, portions (1 ml) of suspension were transferred to Eppendorf tubes and centrifuged at 13,000 × *g* (Biofuge A; Heraeus-Christ GmbH). Supernatants and cell pellets were stored frozen (−30°C). After thawing, the supernatants were analyzed immediately for nitrite and nitrate concentrations; the pellets were analyzed for protein after suspension to the original volume in double distilled water. In addition, portions of the cultures were diluted in physiological sodium chloride solution and were analyzed for colony-forming ability after plating on PM agar. Colonies were counted after 24 and 48 h of incubation at 28°C. The measurement of protein increase and increase in viable cell numbers paralleled each other and, therefore, could equally be used to characterize growth.

NO_2^- was determined by the method of Snell and Snell (23). NO_3^- was quantified enzymatically by the nitrate reductase assay according to the instructions of the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany). In order to ensure the performance of these methods, NO_2^- and NO_3^- in the supernatants were also quantified by isocratic reversed-phase high-pressure liquid chromatography (column: ODS Hypersil RP 18, 4 mm by 250 mm; particle size: 5 μM; flow rate: 1 ml of 0.005 M LOW-UV-PIC-A-Reagent [Waters Associates, Inc., Milford, Mass.] per min). Both methods gave identical results. In all experiments, uninoculated PM medium and AC medium served as controls. NO_2^- and NO_3^- contents of uninoculated media were below the limits of detection of the methods employed.

N_2O and O_2 in cultures were determined by injecting

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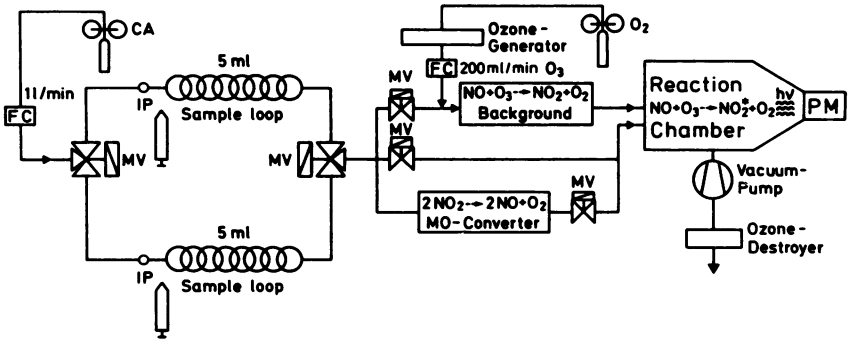

FIG. 1. Schematic drawing of the instrumental design for the determination of NO and NO_2 by the chemoluminescence detector including the two injection ports and the two sample loops. The gas samples were injected by gastight syringes through the septa of the injection ports into the sample loops (volume, 5 ml). By switching the magnetic valves, the sample could be eluted by the gas stream (compressed air) without any pressure buildup. Direct injection of samples into the continuous gas stream would result in a sudden pressure increase, the peak of which would simulate the presence of NO or NO_2. Abbreviations: CA, compressed air; FC, flow controller; IP, injection port with septum; MV, magnetic valve; PM, photomultiplier; MO-converter, molybdenum converter.

100-μl portions of the gas phase with gastight syringes into a Perkin-Elmer Sigma 3B gas chromatograph equipped with a ^{63}Ni electron capture detector and a PoraPLOT Q megabore column (GS-Q column, 30 m by 0.53 mm; ict Handels GmbH, Frankfurt, Federal Republic of Germany). Chromatographic conditions were as follows: carrier gas, 4 ml of He per min; makeup gas, 30 ml of 95% Ar plus 5% CH_4 per min; injector temperature, 100°C; and detector temperature, 350°C. The column temperature was kept at 50°C for 4 min; then the temperature was increased to 100°C at a rate of 30°C per min. The final temperature (100°C) was held for 6 min before the oven was cooled down to 50°C. N_2O, CO_2, and O_2 could be separated and determined in the same run under these conditions. For standardization, 100-μl portions of appropriate dilutions of N_2O (99%; Messer Griesheim, Düsseldorf, Federal Republic of Germany) in ambient air were injected into the gas chromatograph. Integration of peaks and data analysis were performed with a Sigma 15 Chromatography Data Station (Perkin-Elmer).

The amount of NO in the gas phases of cultures was determined after its conversion to NO_2 by O_3 with a NO-NO_2-NO_x analyzer (chemoluminescence detector model 14B/E; Thermo Electron Corp., Waltham, Mass.). Portions (5 ml) were taken with gastight syringes and injected into sample loops (Fig. 1), from which the sample could be transferred without pressure buildup into the gas stream (flow rate of compressed air, 1 liter per min). NO_2 signals (from NO) were registered by a chart recorder. Gas phases of uninoculated media served as controls. NO concentrations were calculated on a peak area basis from a standard curve obtained by injection of 5-ml volumes of appropriate dilutions of standard NO gas (21 ppm [21 μl/liter] in nitrogen; Messer Griesheim) into the instrument. NO_2 in the gas phases of cultures was either detected with a LUMINOX-NO_2 instrument (Scintrex Unisearch LMA-3, Ontario, Canada) or the same chemoluminescence detector used for the determination of NO with the molybdenum converter being connected to the gas stream.

For tests on the presence of nitrate reductase and nitrite reductase (NR and NiR, respectively) activities in aerobic cultures, which produced NO_2^-, NO_3^-, N_2O, and NO in PM or AC medium during the logarithmic growth phase, cells were harvested by centrifugation (20,000 × *g*, 4°C), washed twice with the respective medium, and suspended in medium to an optical density at 680 nm of 0.140 (3×10^7 viable cells per ml; 0.15 mg of protein per ml). In vivo tests were performed either in glass tubes (for aerobic incubations) or in 7-ml Fernbach flasks (for anaerobic incubations) in a shaking water bath at 28°C. The assay mixtures contained the following in a total volume of 2 ml: 0.9 ml of medium, 1 ml of cell suspension, and 0.1 ml of either 60 mM NO_3^- for NR assay (final concentration, 3 mM) or 0.3 mM NO_2^- for NiR (final concentration, 0.015 mM) assay. The reactions were started after 3 min of equilibration by addition of the NO_3^- or NO_2^- solution. For anaerobic incubations, the Fernbach flasks containing medium and cells were closed until gastight with serum rubber stoppers, evacuated, and flushed several times with pure nitrogen gas (99.999% N_2; Messer Griesheim). Anaerobic solutions of NO_3^- and NO_2^- were injected through the serum rubber stoppers in order to start the reactions. After 0, 20, 40, and 60 min of incubation, 0.3-ml samples were withdrawn and centrifuged, and the supernatant was analyzed for NO_2^- (23) produced from NO_3^- (NR) or NO_2^- consumed (NiR), respectively. Incubations without NO_3^- or NO_2^- were used as a measure of the values for NO_3^- and NO_2^- heterotrophically produced during the incubations to be subtracted from the values obtained in the assays for NR-NiR.

Protein content of whole cells was determined as described by Herbert et al. (9). Data shown in Table 1 and Fig. 2 represent the means of three independent experiments with at least five replicates each.

RESULTS AND DISCUSSION

Among the six bacterial strains tested, four were found to heterotrophically produce NO_2^- in significant amounts during growth on PM medium: *A. faecalis* DSM 30030, *A. eutrophus* DSM 428, *P. aeruginosa* DSM 50071, and *Arthrobacter* sp. DSM 312. *Arthrobacter* sp. DSM 917 and *Arthrobacter simplex* DSM 20130 did not produce NO_2^- under the same conditions. The strains of *A. faecalis*, *A. eutrophus*, and *P. aeruginosa* were found to accumulate NO_2^- during exponential growth, whereas *Arthrobacter* sp. DSM 312 produced NO_2^- heterotrophically only after exponential growth. The amount of NO_2^- produced by *A. faecalis* DSM 30030 was six to ten times higher than the amount produced by the other strains tested regardless of whether heterotrophic nitrification took place during the exponential or stationary phase of growth. Therefore, heterotrophic nitrification was characterized with this microorganism.

TABLE 1. Media and substrates for heterotrophic nitrification (nitrite, nitrate, and N_2O production) by *A. faecalis* DSM 30030 under aerobic growth conditions[a]

Medium and substrate	Concn	Production of[b]:			Growth[b]
		Nitrite	Nitrate	N_2O	
Complex medium					
PM	5 g, 3 g/liter	+	+	+	+
Peptone	5 g/liter	+	−	+	+
Meat extract	3 g/liter	+	−	+	+
Substrate on defined medium[c]					
NH_4Cl + citrate	9.35, 9.5 mM	+	+	+	+
Citrate	9.5 mM	−	−	−	−
NH_4Cl	9.35 mM	−	−	−	−
$(NH_4)_2HPO_4$	3.8 mM	+	ND[d]	+	+
$(NH_4)_2SO_4$	3.8 mM	+	ND	+	+
Urea	8.3 mM	+	ND	+	+
Arginine	9.5 mM	−	ND	−	+
Kreatine	2.7 mM	−	ND	−	−
Arginine	9.5 mM	−	ND	−	−
Glutamic acid	3.3 mM	−	ND	−	+
Glutamine	9.9 mM	−	ND	−	−

[a] *A. faecalis* was grown under aerobic conditions in the media listed above at an initial pH of 6.5 and at 28°C on a rotary shaker (120 rpm). Mid-log cultures were analyzed for NO_2^-, NO_3^-, and N_2O production and growth.

[b] Results are indicated as positive (+) or negative (−).

[c] AC medium was used as defined medium. $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, urea, and arginine were used as substrates in place of NH_4Cl on defined medium. Kreatine, arginine, glutamic acid, and glutamine were used as substrates in place of NH_4Cl and citrate on defined medium.

[d] ND, Not determined.

Analysis of the dependence of NO_2^- production on the initial pH in PM medium showed that specific activities were highest at pH 6.5 with 5.34 μmol of NO_2^- produced per h per mg of protein and that they rapidly declined at more acidic (pH 5.6) or more alkaline (pH 7.1, 8.5) levels. Therefore, most of the further experiments were conducted with an initial pH of 6.5 in the media.

Heterotrophic production of NO_2^- and other possible products of heterotrophic nitrification by *A. faecalis* was first analyzed in a qualitative way testing different media with different substrates (Table 1). Under aerobic conditions, *A. faecalis* was found to produce NO_2^-, NO_3^-, and N_2O not only on PM medium but also on a defined medium (AC medium) containing citrate as the sole source of carbon and NH_4Cl as the sole source of nitrogen. Apparently, heterotrophic nitrification was not restricted to media containing nitrogen in organic form but could also have been achieved with ammonium.

When NH_4Cl or citrate was omitted from the AC medium, growth or heterotrophic nitrification activity was not observed (Table 1). Attempts to grow *A. faecalis* like a chemolithotrophic nitrifier in the defined medium (without citrate but with carbonate as the carbon source) failed but were successful with a *Nitrosomonas europaea* strain as a control (data not shown). When cells of *A. faecalis* were incubated for up to 10 weeks in this medium, no NO_2^- or NO_3^- could be detected. Therefore, contamination of *A. faecalis* stock cultures with autotrophic nitrifiers could be excluded. From these experiments, it is also clear that EDTA present in AC medium could not be used as a nitrogen or carbon source by *A. faecalis*.

Heterotrophic nitrification activity was strictly dependent on the presence of a carbon source (other than CO_2) and a nitrogen source in the medium (Table 1). NH_4Cl could be replaced by other inorganic nitrogen sources such as $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ or by urea as an organic compound. With arginine, growth was obtained but NO_2^- and N_2O production were undetected. Replacing citrate plus ammonium chloride with substrates, which theoretically could serve as carbon and nitrogen sources for heterotrophic nitrification activity (kreatine, arginine, glutamic acid, and glutamine) did not result in growth of cells, with the exception of glutamic acid. NO_2^- or N_2O production was not observed under these conditions. On the other hand, with PM medium, NO_3^- was produced by the cultures in addition to NO_2^- and N_2O. Cultures grown on peptone or meat extract medium alone produced NO_2^- and N_2O but failed to produce any nitrate under the conditions employed (Table 1).

In order to identify possible correlations among growth, growth phase, and the release of products of heterotrophic nitrification under aerobic conditions, cultures were grown on PM medium (Fig. 2) and analyzed every hour for protein, NO_2^-, and NO_3^- content in the media and N_2O and NO content in the gas phases of the cultures. Both NO_2^- and NO_3^- could be detected in experiments with PM medium (Fig. 2) approximately 2 h after the cells had started to grow (approximately 12 h after inoculation). The production of NO_2^- and NO_3^- proceeded continuously during the exponential growth phase in a logarithmic way. The gaseous nitrogen oxides N_2O and NO were also produced exponentially during the growth of the cultures, although obviously aerobic conditions prevailed (Fig. 2). These results clearly show that heterotrophic NO_2^- and NO_3^- production by *A. faecalis* took place during active growth of the cultures and cannot be attributed to stationary-phase cultures or dying cell populations. The main product of heterotrophic nitrifi-

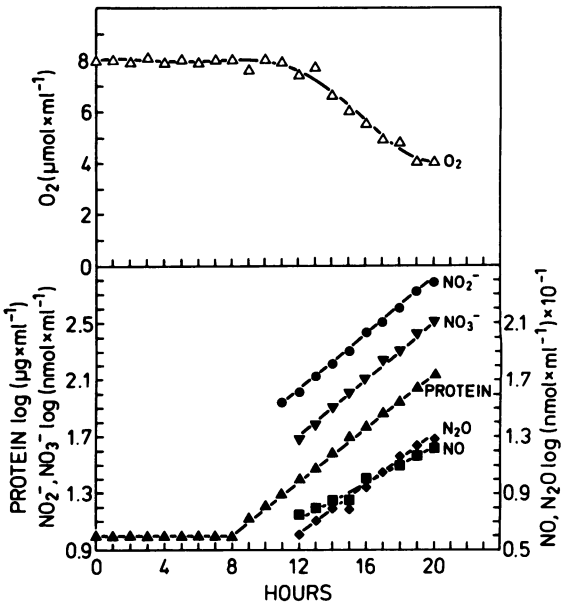

FIG. 2. Heterotrophic nitrification by *A. faecalis* DSM 30030 under aerobic conditions in PM medium. Semilogarithmic plots of NO_2^-, NO_3^-, NO, and N_2O produced during growth (protein) in PM medium. In addition, the oxygen concentration in the flasks is shown.

cation by *A. faecalis* after 20 h of incubation in PM medium was NO_2^- (1,200 nmol per culture), followed by NO_3^- (630 nmol per culture) and about equal amounts of N_2O (9.3 nmol per culture) and NO (8.3 nmol per culture). Mean specific activities (in micromoles per hour per milligram of protein) of NO_2^-, NO_3^-, N_2O, and NO production were, respectively, the following: 5.34, 2.60, 0.029, and 0.023. These activities correspond to a ratio of NO_2^-:NO_3^-:N_2O:NO of 1:0.49:0.0054:0.0043. In experiments on PM medium, trace amounts of NO_2 could also be detected in the gas phases during active growth of the bacteria (data not shown).

When cultures of *A. faecalis* were grown in the defined AC medium, similar results to those with PM medium were obtained: N_2O and NO were produced by heterotrophic nitrifying cultures under aerobic conditions during the whole phase of exponential growth. After 19 h, the cultures had accumulated (per culture) 900 nmol of NO_2^-, 560 nmol of NO_3^-, 109 nmol of N_2O, and 0.7 nmol of NO. These values are 1.1 (NO_2^-), 1.3 (NO_3^-), and 12 (NO) times lower than those obtained in the PM medium with the exception of N_2O, which was produced in amounts 12 times higher in AC medium than those in PM medium. Mean specific activities (in micromoles per hour per milligram of protein) of NO_2^-, NO_3^-, N_2O, and NO productions were the following: 2.79, 1.11, 0.59, and 0.014. These activities correspond to a ratio of nitrite:nitrate:N_2O:NO production of 1:0.39:0.21:0.005.

In order to show whether denitrification enzymes were present, cultures actively producing NO_2^-, NO_3^-, N_2O, and NO in PM and AC media were analyzed aerobically and anaerobically for NR and NiR activities. All attempts to find any dissimilatory (and assimilatory) NR and NiR activities within 40 min of aerobic and anaerobic incubations were unsuccessful (data not shown). Anaerobic incubations were performed for up to 40 min, as incubation times of 60 min and more caused the induction of both NiR and NR in *A. faecalis*. This induction is connected with the ability of *A. faecalis* to grow as a denitrifier under anaerobic conditions in the presence of NO_2^- or NO_3^- as respiratory electron acceptors (for examples, see references 2 and 11).

The results presented here show that NO_2^- was produced heterotrophically from organic substrates present in PM medium during the growth of *A. faecalis* DSM 30030, *A. eutrophus* DSM 428, and *P. aeruginosa* DSM 50071. The bacterial strains differed markedly in their heterotrophic NO_2^--producing capabilities during exponential growth in PM medium, with *A. faecalis* showing the highest activities of NO_2^- production (5.34 μmol/mg of protein per h). However, this may be the consequence of the medium applied and may differ with other media and substrates. *Arthrobacter* sp. DSM 312 was also able to heterotrophically produce NO_2^- in PM medium but only when active growth had already ceased. These observations suggest that heterotrophic nitrification can occur during the stationary phase of growth (1, 15, 22, 26) but also takes place in exponentially growing cultures, depending on the microorganism.

Heterotrophic NO_2^- production during active growth has been described for *Arthrobacter* sp. 9006 (29) but only occurred in a mineral medium when acetate was added along with tryptone and yeast extract. Furthermore, in contrast to these results with strains of *A. faecalis*, *A. eutrophus*, *P. aeruginosa*, and *Arthrobacter* sp., there was no heterotrophic NO_2^- production in a complex medium containing peptone or meat extract alone. On the other hand, the presence of organically bound nitrogen was not essential for heterotrophic nitrification activity by *A. faecalis* DSM 30030 but also occurred in a defined AC medium with ammonium chloride as the sole source of inorganic nitrogen (Table 1).

With the data presented in Fig. 2, it was calculated that in PM medium (pH 6.5) *A. faecalis* accumulated NO_2^- to about 10 mg of N per liter. Similar results were obtained with *Arthrobacter* sp. 9006 accumulating NO_2^- in the presence of acetate (pH 7.4) up to 15 mg of N per liter (29). *A. faecalis* produced NO_2^- more efficiently at acidic pHs than at neutral or alkaline pHs, while other nitrifying heterotrophs are thought to produce hydroxylamine, NO_2^-, NO_3^-, or other oxidized nitrogen compounds only when grown on neutral or alkaline media (10, 26–28). Recent experiments with several fungi and two *Bacillus* spp., however, isolated from a podzolic brown earth (pH 3.5) showed significant rates of heterotrophic nitrification (17). These findings as well as the data presented here support the view that nitrification can be performed by certain heterotrophs under acidic conditions and may, therefore, be of greater significance in nature, e.g., in acidic soils than hitherto presumed (17).

In PM (Fig. 2) as well as in AC medium, *A. faecalis* DSM 30030 produced NO_3^- in addition to NO_2^- as a product of heterotrophic nitrification during exponential growth. NO_3^- production was about 50% of NO_2^- production. Apparently, heterotrophic nitrification by *A. faecalis* differs basically from chemolithotrophic nitrifiers, which during active growth either produce NO_2^- (ammonia oxidizers) or NO_3^- (nitrite oxidizers). It is believed that NO_3^- is produced by fewer heterotrophically nitrifying microorganisms than NO_2^- (7). To our knowledge, the presence of both NO_2^- and NO_3^- in media as products of heterotrophic nitrification by the same microorganism has been described only for an *Arthrobacter* sp. (27) and *Aspergillus flavus* CMC 5 (10); however, in these studies heterotrophic nitrification was observed under conditions when active growth had already ceased.

Investigations on the identification of products of heterotrophic nitrification concentrated on hydroxylamine, NO_2^-, and NO_3^-, but N_2O and NO as products of heterotrophic nitrification have not been reported (16, 27). The present experiments with *A. faecalis* DSM 30030 show that, during aerobic heterotrophic nitrification, N_2O and NO are also produced. The production of these gases paralleled those of NO_2^- and NO_3^- during logarithmic growth of the cultures in both PM (Fig. 2) and AC media. Since enzymes of denitrification, aerobic denitrification (2, 20), and assimilatory NO_3^- and NO_2^- reduction could not be detected, we carefully conclude that these enzymes were not responsible for the production of N_2O or NO in this study. These observations are consistent with the finding of a strain of *A. faecalis* capable of heterotrophic nitrification lacking dissimilatory NR and NiR activities when incubated under aerobic conditions with carbon sources other than pyruvicoxime (5). Further research will clarify whether other enzyme systems may catalyze the production of N_2O and NO under conditions of aerobic heterotrophic nitrification by *A. faecalis* DSM 30030. Whether the NO_2 observed in the gas phase of cultures was caused by heterotrophic nitrification activity of the bacteria or by chemodenitrification could not be decided from the experiments performed.

These experiments suggest that, besides denitrification and autotrophic nitrification (2, 6, 8, 14, 18, 19, 30), heterotrophic nitrification has to be considered as a potential source for gaseous atmospheric nitrogen oxides of biogenic origin. In comparison to chemolithotrophic nitrifiers (12–14), nitrification rates of heterotrophs are described to be 3 orders of magnitude lower (16). Obviously, this is not the

case with *A. faecalis*, the specific NO_2^- production activity of which (5.34 μmol/mg of protein per h) was found in this study to be comparable to that of *N. europaea* (about 3 to 6 μmol/mg of protein per h, calculated from data presented earlier [14]). On the other hand, one has to realize that in view of the greater numbers of different fungal and bacterial genera capable of heterotrophic nitrification (17, 28), the total nitrification activity caused by heterotrophs may even exceed that caused by chemolithotrophic nitrifiers in certain environments. This seems to be the case especially with respect to nitrous oxide production activity: e.g., for *N. europaea* grown in air on 11.6 mM NH_4^+, it was calculated that the total N_2O N formed as a percentage of the NO_2^- N formed was 0.09% (14). With *A. faecalis* DSM 30030 under aerobic conditions in AC medium and with comparable ammonium concentrations (9.5 mM NH_4Cl), N_2O N was 12.1% of the NO_2^- N produced; in PM medium, it was 0.65%. Therefore, the amount of N_2O produced during heterotrophic nitrification may be 2 orders of magnitude higher than with autotrophic ammonia oxidizers under comparable conditions. Further experiments will have to show whether heterotrophic nitrification is also an important factor in the biogenic emission of gaseous nitrogen oxides under field conditions.

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