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Codenitrification and Denitrification Are Dual Metabolic Pathways through Which Dinitrogen Evolves from Nitrate in *Streptomyces antibioticus*

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We screened actinomycete strains for dinitrogen (N_2)-producing activity and discovered that *Streptomyces antibioticus* B-546 evolves N_2 and some nitrous oxide (N_2O) from nitrate (NO_3^-). Most of the N_2 that evolved from the heavy isotope ($[^{15}N]NO_3^-$) was $^{15}N^{14}N$, indicating that this nitrogen species consists of two atoms, one arising from NO_3^- and the other from different sources. This phenomenon is similar to codenitrification in fungi. The strain also evolved less, but significant, amounts of $^{15}N^{15}N$ from $[^{15}N]NO_3^-$ in addition to $^{15}N^{15}NO$ with concomitant cell growth. Prior to the production of N_2 and N_2O, NO_3^- was rapidly reduced to nitrite (NO_2^-) accompanied by distinct cell growth, showing that the actinomycete strain is a facultative anaerobe that depends on denitrification and nitrate respiration for anoxic growth. The cell-free activities of denitrifying enzymes could be reconstituted, supporting the notion that the $^{15}N^{15}N$ and $^{15}N^{15}NO$ species are produced by denitrification from NO_3^- via NO_2^-. We therefore demonstrated a unique system in an actinomycete that produces gaseous nitrogen (N_2 and N_2O) through both denitrification and codenitrification. The predominance of codenitrification over denitrification along with oxygen tolerance is the key feature of nitrate metabolism in this actinomycete.

The biological process of dinitrogen (N_2) gas formation from fixed nitrogen compounds such as nitrate (NO_3^-) plays an important role in maintaining homeostasis of the global environment. Bacterial denitrification was long considered the sole biological reaction responsible for this condition, and its systems have been characterized in detail (3, 8, 20). Denitrification physiologically functions as anaerobic respiration in which NO_3^- is used as the terminal electron acceptor when oxygen (O_2) is unavailable. Known bacterial denitrifying systems consist of four steps that successively reduce NO_3^- to N_2 and involve nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) as intermediates. Production of the enzymes catalyzing each step is induced by nitrogen oxides and suppressed by O_2 (3, 20). During the past decade, denitrifiers have been discovered in a variety of taxa including filamentous fungi (9, 10), yeasts (15), and actinomycetes (1, 11). All of these novel denitrifiers produce N_2O as the major denitrification product. Both nitrogen atoms in the N_2O product are derived from nitrate (or nitrite) (1, 9–11, 15). The phenomenon is therefore defined as denitrification, since the process should include the formation of an N—N bond (20). This system in several fungi is localized at respiring mitochondria, where it acts as anaerobic respiration, as it does in bacterial systems (5, 13, 16). Another unique feature of the fungal system is the involvement of cytochrome P450 (P450nor) as NO reductase (Nor) (6).

Biological processes other than bacterial denitrification evolve N_2 from fixed nitrogen compounds, but their molecular mechanisms and physiological significance remain to be elucidated. We identified simultaneous fungal codenitrification and denitrification, in which a hybrid N_2 species is formed by combining two nitrogen atoms derived from NO_2^- and from other nitrogen compounds (10, 14). The denitrifying fungi *Fusarium solani* and *Cylindrocarpon tonkinense* evolve hybrid N_2 species (10). The fact that the denitrifying fungus *Fusarium oxysporum* evolves N_2O instead of N_2 by codenitrification only when a nitrogen compound in addition to NO_2^- such as azide, salicylhydroxamic acid, or ammonium (NH_4^+) is available (14) suggests that the mechanisms of this process differ among fungal species. Anammox is a third N_2-generating metabolic process that has been identified in the strictly anaerobic chemolithotrophic *Planctomycetales* (12), in which NH_4^+ is combined with NO_2^- to form N_2.

The actinomycetes form a unique taxon among gram-positive bacteria. Although actinomycetes naturally proliferate in soil and in aqueous environments, little is known about how they accomplish denitrification. Denitrifiers also occur among actinomycetes (1, 11), and the system of *Streptomyces thioluteus* has been characterized previously (11). All of the actinomycete denitrifiers found in these studies evolve N_2O from NO_3^- or NO_2^-, and thus, no known actinomycete strains contain a complete denitrifying system that can thoroughly reduce NO_3^- to N_2.

The present study continues screening for denitrifying actinomycetes (11) by using a highly sensitive N_2 detection system equipped with an isotope mass spectrometer and NO_3^- labeled with a stable isotope ($[^{15}N]NO_3^-$). The results showed that *Streptomyces antibioticus* B-546 has N_2-producing activity

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and that most of the N_2 molecules are formed via intracellular codenitrification that is induced simultaneously with denitrification.

MATERIALS AND METHODS

Strains and media. The strains used in this study originated from the JCM (Japan Collection of Microorganisms, RIKEN) or IFO (Institute for Fermentation, Osaka, Japan) type culture collections, except for strains A, B, C, and N. We isolated the latter four strains from soil, and *S. antibioticus* B-546 originated from a different culture collection in the Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo. All cells were seeded, precultured, or cultured in glycerol-peptone (GP) medium (3% glycerol, 0.2% peptone, 10 mM KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and inorganic salts) (11) unless otherwise stated.

Batch cultures in flasks. Actinomycetes were batch cultured in flasks as follows. Seed cultures (10 ml) in 50-ml tubes were inoculated into 300 ml of GP medium in 500-ml Erlenmeyer flasks and rotary shaken at 30°C for 4 days at 120 rpm (preculture). Finally, portions of the preculture (100 ml) were transferred to 500-ml flasks containing 100 ml of GP medium supplemented with 10 mM $NaNO_3$ and incubated as described above (for preculture). Denitrifying actinomycetes were screened under initially aerobic conditions that were attained by sealing the flask after inoculation with a rubber stopper without replacing headspace air. $NaNO_3$ was labeled with 10% heavy isotope nitrogen (^{15}N). Time-dependent changes in nitrogen oxides were investigated under two aerating conditions. Initially aerobic cultures were incubated in the same manner as for screening. Anaerobic cultures were incubated in the same manner, but the headspace air was replaced with argon gas before the flask was sealed. Both cultures used $[^{15}N]NaNO_3$ instead of $[^{14}N]NaNO_3$. When necessary, acetylene gas was added to a ratio of 10% of the headspace volume.

Quantification of nitrogenous oxides, dinitrogen, and cell weight. We determined NO_3^- and NO_2^- with an ion-pair, high-performance liquid chromatograph equipped with a TSK gel IC-Anion PW column (Tosoh, Tokyo, Japan) as described previously (9). Oxygen, N_2O, and N_2 concentrations in the gas phase were determined by gas chromatography (GC) as described previously (9). ^{15}N-labeled N_2 gas was analyzed by isotope mass spectrometry (MS) with a Finnigan DELTA Plus isotope mass spectrometer as described previously (15). Collected cells were dried at 80°C for 2 h and then weighed (cell dry weight).

Preparation of crude extract and subcellular fractionation. Denitrifying cells of *S. antibioticus* B-546 were harvested by centrifugation, resuspended in buffer (100 mM potassium phosphate [pH 7.2], 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 0.25 μM *N*-tosyl-L-phenylalanyl chloromethyl ketone, and 0.25 μM phenylmethanesulfonyl fluoride), and sonicated (Sonifier 250; Branson) for 60 min with occasional cooling. The sonicate was separated by centrifugation at 10,000 × *g* for 20 min to obtain the supernatant (crude extract), which was further sedimented by centrifugation at 100,000 × *g* for 60 min. The resulting supernatant (soluble fraction) and pellet (membranes) were analyzed as follows.

Enzyme assay. Nitrate reductase (Nar) was assayed as described previously (5) with methyl viologen-dithionite as the electron donor. Nitrite reductase (Nir) was assayed with NADH-phenazine methosulfate as the electron donor by determining the amount of NO produced by the P450nor-trap method (5). Nor activity was determined by measuring NADH-dependent N_2O formation by GC as described previously (6). Nitrous oxide reductase (N_2Or) was assayed as described previously (4) with the following modification. The reaction mixture (0.85 ml of 10 mM potassium phosphate [pH 7.2] and 0.05 ml of 10 mM methyl viologen) in 1-ml Zumberg-type cuvettes was flushed with argon and an appropriate volume of 1 mM sodium dithionite to give an A_{600} of 1.0 ± 0.2. An aliquot of the enzyme was injected, and the A_{600} was monitored for 1 min to measure the background oxidation rate before starting the reaction by injecting 0.08 ml of N_2O gas. The reaction was measured by monitoring the N_2O-dependent oxidation of reduced methyl viologen at 600 nm. The protein concentration was determined with a protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, Calif.).

RESULTS

Screening actinomycetes that produce N_2. We examined denitrification in the following strains: *Saccharopolyspora gregorii* IFO15045T, *Microbispora grisealba* IFO14840T, *Nocardia asteroides* IFO3384, *Nocardia brasiliensis* IFO14402T, *Streptomyces prunicolor* IFO13075T, *Streptomyces roseolus* IFO12816T, *Streptomyces acrimycini* IFO12736T, *Streptomyces badius* IFO12745T, *Nocardia carnea* IFO14403T, *Streptomyces daghestanicus* IFO12762T, *Streptomyces umbrinus* IFO13091T, *Streptomyces variabilis* IFO12825T, *Micromonospora citrae* IFO14025T, *Streptomyces coelicolor* JCM4375, *Streptomyces lividans* JCM4783, *Nocardia salmonicida* JCM4826, *Streptomyces rubescens* IAM0074, *S. antibioticus* B-546, strain B, strain C, strain E, and strain N. Each strain was initially cultured under aerobic conditions (see Materials and Methods) in medium containing NO_3^-, 10% of which was labeled with a heavy isotope ($[^{15}N]NO_3^-$). The headspace gas of the culture flask was analyzed after the incubation by GC-MS for isotopically labeled N_2 gas. Among the strains tested, only *S. antibioticus* B-546 produced a significant amount (9.5×10^1 μmol) of N_2 species ($^{14}N^{15}N$) after a 4-day incubation, indicating N_2 production from NO_3^-. We then screened four other *S. antibioticus* strains (JCM3117, JCM4690, IFO12652, and IFO13271) and found that strain JCM3117 evolved up to 1.9×10^1 μmol of N_2 species ($^{14}N^{15}N$) after a 4-day incubation. These results showed that strains of this actinomycete species evolve more N_2 than do the other species tested.

Codenitrification and denitrification by *S. antibioticus*. We investigated N_2 production by *S. antibioticus* B-546 in more detail. The strain was initially incubated under the aerobic conditions described above but with the stable isotope species of NO_3^-, in which 99% of N atoms are labeled with ^{15}N. We changed the content of ^{15}N with the expectation that the $^{14}N^{15}N$-N_2 species observed in the previous experiment would be replaced by $^{15}N^{15}N$-N_2 species. However, the major N_2 species produced remained $^{14}N^{15}N$ (Fig. 1) with a very small but distinct amount of $^{15}N^{15}N$. Furthermore, the N_2O that was also formed was in fact a $^{15}N^{15}NO$ species (data not shown). Neither $^{15}N^{15}N$ nor $^{15}N^{15}NO$ was observed in the control experiment without cells (data not shown), indicating that $^{15}N^{15}N$ and $^{15}N^{15}NO$ were formed because of denitrification. Since almost all of the added NO_3^- was of the heavy isotope species, the $^{14}N^{15}N$-N_2 species should be generated by a combination of two nitrogen atoms, one derived from NO_3^- and the other from other nitrogen sources (codenitrification). The hybrid N_2 species was not detected in the control experiment without the cells. The medium contained 10 mM phosphate buffer (pH 7.2), and the final pH of the medium after 5 days of incubation was above 6.0. These results rule out the possibility that the hybrid N_2 species was formed by chemical means (2) but indicate that N_2 arises from a physiological reaction like that in fungal denitrifiers (10, 14).

Time-dependent changes in each component during the initial aerobic culture show that the culture was divided into two phases (Fig. 1). In the first phase, NO_3^- and O_2 were rapidly consumed with the concomitant production of NO_2^- (Fig. 1A) and $^{15}N^{15}N$-N_2 species (Fig. 1B), as well as cell growth (Fig. 1C). Most (70%) nitrogen atoms of the consumed NO_3^- seemed to be recovered into NO_2^-. However, these activities almost stopped or significantly declined within 24 h. During the second phase after the 1-day incubation, N_2O began to be evolved whereas the emission of $^{15}N^{15}N$-N_2 species declined. By contrast, the hybrid $^{14}N^{15}N$-N_2 species was emitted during the first and the second phases throughout the incubation. Some portion of the nitrogen atoms of consumed NO_3^- were recovered into $^{14}N^{15}N$-N_2 (6%), $^{15}N^{15}N$-N_2 (0.0001%), and N_2O (0.3%). In contrast, NO was not detected throughout the

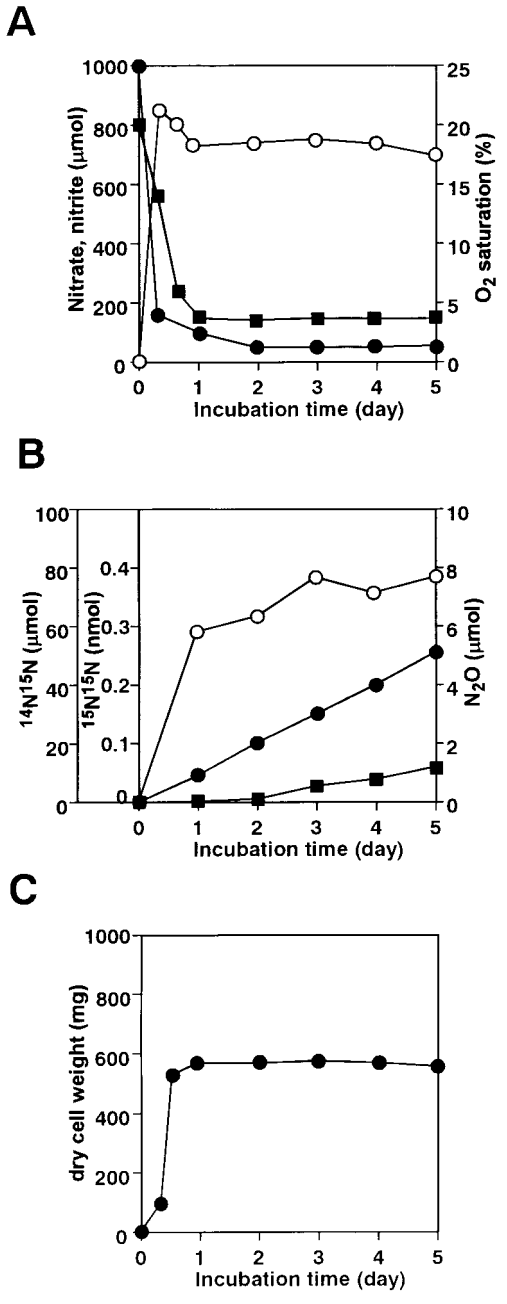

FIG. 1. N_2 production by *S. antibioticus* B-546 under aerobic conditions. Flask headspace was filled with air to initially maintain aerobic conditions. (A) Amounts of NO_3^- (solid circles) and NO_2^- (open circles) in medium and O_2 in the gas phase (solid squares) were determined at indicated times. (B) Amounts of $^{14}N^{15}N$ (solid circles), $^{15}N^{15}N$ (open circles), and N_2O (solid squares) in the gas phase were determined at indicated times. (C) Cell growth. Results are representative values from three experiments.

incubation. The consumption of O_2 stopped in the second phase, although O_2 still accounted for 2% of the headspace gas. These results showed that the strain can reduce $^{15}NO_3^-$ to produce $^{15}N^{14}N$, $^{15}N^{15}N$, N_2O, and NO_2^- species under aerobic conditions, which is in sharp contrast to other bacterial denitrifiers.

Nitrate metabolism by *S. antibioticus* under anoxic conditions. The aerating (initial aerobic) conditions above (Fig. 1) mimicked those required for fungal denitrification (18). Next we examined similar cultures under anoxic conditions. Figure 2 shows that NO_3^- was first converted to NO_2^- as in the aerobic culture (Fig. 1), that the conversion was almost stoichiometric, and that $^{15}N^{14}N$, $^{15}N^{15}N$, and N_2O gas species evolved. Cell growth was distinct even under anoxic conditions (Fig. 2C) although cell yield was much lower than that under aerobic conditions (Fig. 1C). Figure 3 shows the time-dependent accumulation of each gas species per milligram of cells (dry

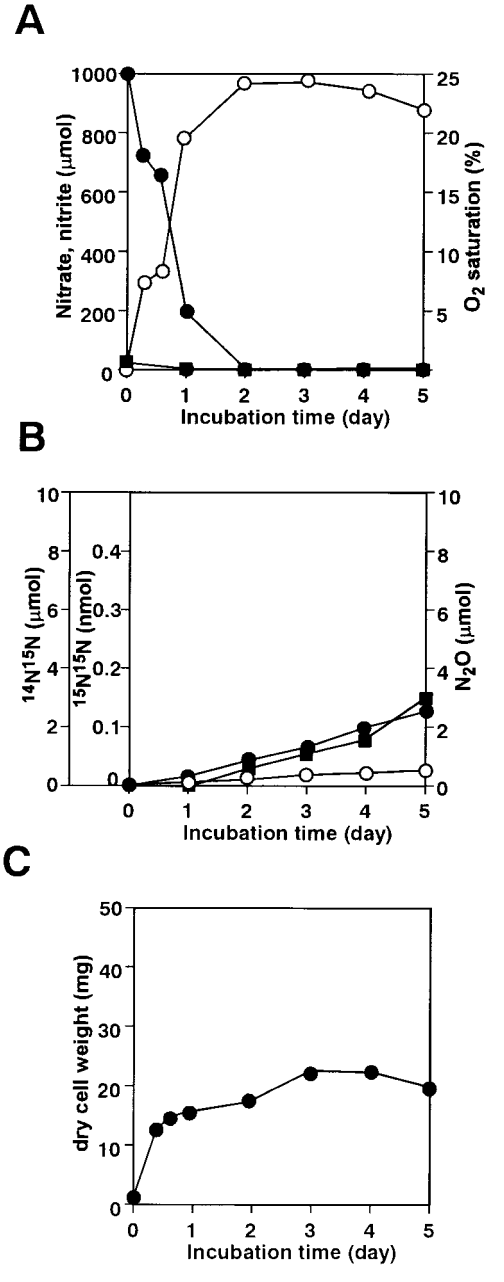

FIG. 2. N_2 production by *S. antibioticus* B-546 under anaerobic conditions. Flask headspace was filled with argon to maintain anaerobic conditions. Symbols are as described for Fig. 1. Results are representative values from three experiments.

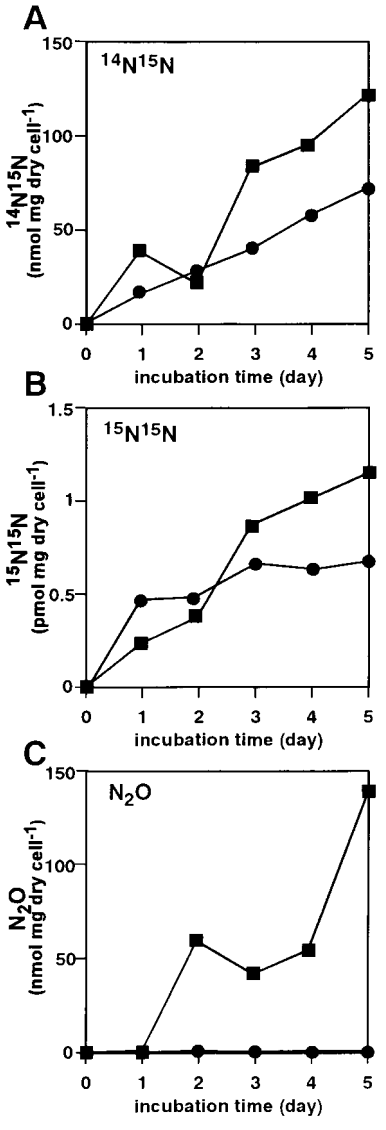

FIG. 3. Denitrification by *S. antibioticus* B-546. Time-dependent accumulation of each gas species per milligram of dry cells was calculated and plotted. Circles, aerobic culture (Fig. 1); rectangles, anoxic culture (Fig. 2).

weight) during aerobic (Fig. 1) and anoxic (Fig. 2) cultures. More of all of the gas species accumulated (per milligram of cells) under anoxic conditions. In particular, the amounts of N_2O evolved were increased by 500- to 1,000-fold under anoxic, compared with aerobic, conditions.

Denitrifying enzyme activities. The formation of $^{15}N^{15}N$, $^{15}N^{15}NO$, and NO_2^- from $^{15}NO_3^-$ suggests that *S. antibioticus* B-546 contains a conventional denitrifying system common to bacteria. We therefore examined the reconstitution of cell-free activities of enzymes that should be involved in the system. Table 1 shows that nitrate reductase (Nar), nitrite reductase (Nir), and nitric oxide reductase (Nor) activities were detected in crude extract or subcellular fractions prepared from the denitrifying cells (on day 3 in Fig. 1). Significant Nar, Nir, and Nor activities were recovered in the membranes as well as in the soluble fraction. In contrast, we could not reconstitute nitrous oxide reductase (N_2Or) activity.

TABLE 1. Denitrifying enzyme activities of *S. antibioticus* B-546[a]

Denitrifying enzyme	Total activity (nmol min^{-1})		
	Crude extract	Soluble fraction	Membranes
NO^-_3 reductase	12.5	6.7	2.7
NO^-_2 reductase	396	260	49
NO reductase	56	13	6.0

[a] S. antibioticus B-546 was initially cultured under aerobic conditions for 3 days, homogenized, and then fractionated as described in Materials and Methods.

Effect of acetylene on denitrification of *S. antibioticus*. Since cell-free N_2Or activity could not be reconstituted, we further examined this step with respect to the in vivo activity of the actinomycete strain. N_2Or activities both in vivo and in vitro are generally inhibited by acetylene; thus, in vivo incubation of the denitrifying system usually results in N_2O accumulation (17). When *S. antibioticus* B-546 was initially cultured under aerobic conditions, acetylene did not affect the in vivo production of $^{14}N^{15}N$, $^{15}N^{15}N$, and N_2O from $^{15}NO_3^-$ (data not shown), showing that the production of N_2 by strain B-546 is insensitive to acetylene.

Dissimilar nitrate reduction by *S. antibioticus*. The above results indicate that nitrate metabolism by *S. antibioticus* supports anoxic cell growth (Fig. 2C). This notion was further supported by the following findings. The cell-free specific activity of Nar was higher in the anoxic cells than in those grown under aerobic conditions (Fig. 4), which is consistent with the complete conversion of NO_3^- to NO_2^- (Fig. 2A). The addition of tungstate, an inhibitor of molybdenum enzymes, to the culture medium considerably inhibited the cell-free enzyme activity in both the aerobic and anoxic cells, indicating that Nar activity is dependent on a molybdenum enzyme (Fig. 4). These properties are similar to those of bacterial Nar, which is involved in nitrate respiration (20).

NO_3^- reduction during the first phase accompanied cell growth even in the absence of O_2 (Fig. 2). When NO_3^- was omitted from medium that still contained another nitrogen source (peptone), little growth was attained by the anoxic cul-

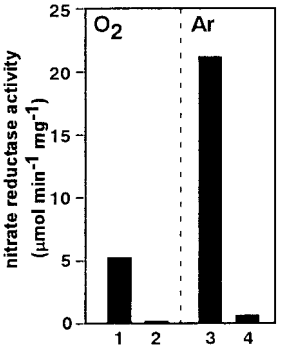

FIG. 4. Nitrate reductase activity of *S. antibioticus* B-546 under anaerobic conditions. Flask headspace was filled with air (bars 1 and 2) or argon (bars 3 and 4) and incubated for 24 h (the first phase) in medium with (bars 2 and 4) or without (bars 1 and 3) 10 mM sodium tungstate. Results are typical of over three experiments.

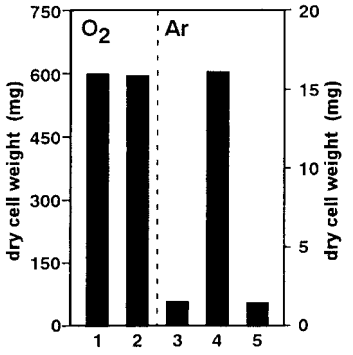

FIG. 5. Nitrate-dependent cell growth of *S. antibioticus* B-546 under anaerobic conditions. Flask headspace was filled with air (bars 1 and 2) or argon (bars 3 to 5) and incubated for 24 h (the first phase in Fig. 1 and 2). GP medium was supplemented with (bars 2, 4, and 5) or without (bars 1 and 3) 10 mM $NaNO_3$. Sodium tungstate (10 mM) was added (bar 5). Typical results of more than three experiments are shown.

ture in sharp contrast to the normal growth attained under (initial) aerobic conditions in the absence of NO_3^- (Fig. 5). The addition of tungstate to the culture inhibited the NO_3^--dependent cell growth under anoxic conditions (Fig. 5, bar 5), again contrasted with the absence of a tungstate effect on the aerobic growth (data not shown). These results unequivocally demonstrated that anoxic cell growth depended on the Nar-dependent, dissimilatory reduction of NO_3^-, whereas the high cell yield by the aerobic culture was mostly supported by O_2 respiration.

The anoxic cell growth (Fig. 2C) consisted of two phases, consistent with the initial reduction of NO_3^- to NO_2^- and the following initiation of N_2O evolution (Fig. 2A and B). These results support the notion that this actinomycete strain prefers, as the anoxic energy-yielding process, the reduction step from NO_3^- to NO_2^- by Nar to the subsequent reduction steps. When NO_3^- became unavailable due to consumption, the strain began to utilize the accumulated NO_2^- for further reduction, which resulted in denitrification. The strain was selective about the denitrification product, preferring N_2O to N_2 (detected above as $^{15}N^{15}N$). The Nir and Nor activity detected in the cell extracts (Table 1) should correspond to the further reduction of NO_2^- to N_2O.

DISCUSSION

We are the first to demonstrate that denitrifiers produce N_2 as the denitrification product in actinomycetes. We also showed that the denitrifying system of *S. antibioticus* B-546 is distinct from other known systems of bacteria in that most N_2 production depends on codenitrification, where N_2 is formed by combining two nitrogen atoms, one from NO_3^- and one from other nitrogen sources. The hybrid N_2 species appears to be formed from NO_2^- (or NO) as it is in fungal codenitrification (10) but not directly from NO_3^-, since the formation continued long after NO_3^- was consumed (Fig. 1 and 2). Another N_2 species ($^{15}N^{15}N$ from $^{15}NO_3^-$) should be formed by reducing N_2O since both nitrogen atoms of N_2O were derived from NO_3^- ($^{15}N^{15}NO$ when $^{15}NO_3^-$ was applied), showing that both products (N_2O and homo-N_2 species) are formed by normal denitrification. Thus, N_2 is formed in *S. antibioticus* B-546 via dual pathways of nitrate metabolism, namely, denitrification and codenitrification. Less hybrid N_2 species than denitrification product (N_2 O) is produced by known fungal codenitrification processes (10). Therefore, the unique feature of nitrate metabolism by the actinomycete strain is that codenitrification predominates over, or at least is comparable to, denitrification.

These results are also the first to show that an actinomycete strain can attain growth even when the O_2 supply is completely lost (Fig. 2) and that anoxic growth depends on denitrification (nitrate respiration) (Fig. 2 and 4). Another unique feature of this type of nitrate metabolism is its aerobic or O_2-resistant nature, which is in sharp contrast to the nitrate metabolic processes of other bacteria that are suppressed by atmospheric O_2 (3). NO_3^- was converted to NO_2^- and all the gas species were formed under both aerobic and anoxic conditions (Fig. 1 and 2), although the formation rate for each metabolite differed between the conditions. The formation rate per milligram of cell (Fig. 3) for the denitrification products (N_2O and $^{15}N^{15}N$) was much higher under anoxic than under aerobic conditions, supporting the importance of denitrification for anoxic growth. By contrast, the rates for conversion of NO_3^- to NO_2^- or the formation of the hybrid N_2 gas species ($^{14}N^{15}N$) were comparable between the conditions. The aerobic nitrate metabolism does not apparently contribute to cell growth (Fig. 1C and 5). However, this does not rule out the possibility that nitrate metabolism is an energy-yielding process, since NO_3^- was converted to NO_2^- (possibly supported by Nar) simultaneously with O_2 uptake, and O_2 respiration should be much more effective than nitrate respiration for energy production. This means that, under conditions such as the first stage shown in Fig. 1, suppressing nitrate respiration would be energetically even more favorable because nitrate should compete for electrons with O_2. In the second stage of the aerobic culture, the cell mass remained almost constant (Fig. 1C). The number of cells grown under aerobic conditions should have been too large for the weak denitrifying activity to support more growth (compare Fig. 1C and 2C).

Codenitrification was expressed in *S. antibioticus* irrespective of the extent of aeration. Thus, codenitrification appears to be associated with the formation of NO_2^- from NO_3^-. However, the present results cannot confirm whether codenitrification contributes to cell growth. Another nitrogen source for codenitrification could be the peptone that was added to the medium as well as nitrate. The O_2-resistant nature of Nar along with codenitrification in the actinomycete strain has provided new insight into the dissimilar metabolism of nitrate by microorganisms, whereas their physiological significance or mechanism(s) remains to be elucidated.

We recently showed that many soil fungi are not obligatory aerobes but facultative anaerobes and that facultative anaerobiosis is supported not only by denitrification but also by ammonia fermentation, which is a novel anoxic metabolism of nitrate by fungi (19). Most known actinomycetes reside in soils, and like fungi, they have been considered obligatory aerobes. Therefore, the present study has revealed the possibility that many soil actinomycetes are in fact facultative anaerobes.

Our characterization indicates that the denitrifying system of the actinomycetes is more similar to that of fungal systems than

bacterial systems. Codenitrification (10, 14), aerobic qualities (9, 18), formation of N_2O as the main product of denitrification (9, 10, 15), and rapid excretion of NO_2^- into the medium after conversion from NO_3^- (18) are all features of fungal denitrification. On the other hand, the P450nor involved in the fungal systems (Nor) seems not to occur among actinomycetes, although P450nor is classified into the same phylogenetic branch as other actinomycete P450s within the P450 superfamily (7). The phylogenetic relationship among the denitrifying systems of fungi, actinomycetes, and other bacteria requires further clarification. The contributions of the novel denitrifying systems of actinomycetes and of fungal systems to the nitrogen cycle in nature remain to be assessed.

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