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Nitrous Oxide Reduction by an Obligate Aerobic Bacterium, *Gemmatimonas aurantiaca* Strain T-27

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ABSTRACT N_2O-reducing organisms with nitrous oxide reductases (NosZ) are known as the only biological sink of N_2O in the environment. Among the most abundant *nosZ* genes found in the environment are *nosZ* genes affiliated with the understudied *Gemmatimonadetes* phylum. In this study, a unique regulatory mechanism of N_2O reduction in *Gemmatimonas aurantiaca* strain T-27, an isolate affiliated with the *Gemmatimonadetes* phylum, was examined. Strain T-27 was incubated with N_2O and/or O_2 as the electron acceptor. Significant N_2O reduction was observed only when O_2 was initially present. When batch cultures of strain T-27 were amended with O_2 and N_2O, N_2O reduction commenced after O_2 was depleted. In a long-term incubation with the addition of N_2O upon depletion, the N_2O reduction rate decreased over time and came to an eventual stop. Spiking of the culture with O_2 resulted in the resuscitation of N_2O reduction activity, supporting the hypothesis that N_2O reduction by strain T-27 required the transient presence of O_2. The highest level of *nosZ* transcription (8.97 *nosZ* transcripts/*recA* transcript) was observed immediately after O_2 depletion, and transcription decreased ~25-fold within 85 h, supporting the observed phenotype. The observed difference between responses of strain T-27 cultures amended with and without N_2O to O_2 starvation suggested that N_2O helped sustain the viability of strain T-27 during temporary anoxia, although N_2O reduction was not coupled to growth. The findings in this study suggest that obligate aerobic microorganisms with *nosZ* genes may utilize N_2O as a temporary surrogate for O_2 to survive periodic anoxia.

IMPORTANCE Emission of N_2O, a potent greenhouse gas and ozone depletion agent, from the soil environment is largely determined by microbial sources and sinks. N_2O reduction by organisms with N_2O reductases (NosZ) is the only known biological sink of N_2O at environmentally relevant concentrations (up to ~1,000 parts per million by volume [ppmv]). Although a large fraction of *nosZ* genes recovered from soil is affiliated with *nosZ* found in the genomes of the obligate aerobic phylum *Gemmatimonadetes*, N_2O reduction has not yet been confirmed in any of these organisms. This study demonstrates that N_2O is reduced by an obligate aerobic bacterium, *Gemmatimonas aurantiaca* strain T-27, and suggests a novel regulation mechanism for N_2O reduction in this organism, which may also be applicable to other obligate aerobic organisms possessing *nosZ* genes. We expect that these findings will significantly advance the understanding of N_2O dynamics in environments with frequent transitions between oxic and anoxic conditions.

KEYWORDS *Gemmatimonadetes*, nitrous oxide, nitrous oxide reduction, RT-qPCR

The recent meteorological data from the first half of the year 2016 witnessed the highest worldwide average temperature ever recorded for the same period of the year, supporting the concern of the scientific world about ongoing climate change

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(GISS Surface Temperature Analysis [http://www.ncdc.noaa.gov/sotc/global/201606]). The increase in greenhouse gas emission due to various anthropogenic activities has been regarded as the major culprit of the general upward trend in the global temperature and climate anomalies occurring with alarming frequency across the globe (1). Nitrous oxide (N_2O) is a greenhouse gas with a global warming potential ~300 times that of CO_2 and is the third most important contributor to global warming (6.2%) after CO_2 (78%) and CH_4 (16%) (1–4). Further, the abolition of chlorofluorocarbons has left N_2O as the largest contributor to the destruction of the ozone layer in the stratosphere (5, 6). Therefore, control of N_2O emissions is indispensable in the efforts to curb global warming and climate change.

Both natural and anthropogenic sources of N_2O have a predominantly biological origin, with biological transformation of N fertilizer applied to agricultural soils being the single largest source (1, 7, 8). Nitrification produces N_2O as a by-product of ammonia oxidation, and denitrification emits N_2O as a stable intermediate or an end product (8–11). Other relatively minor sources of N_2O include dissimilatory reduction to ammonium (DNRA) and chemodenitrification (8, 12–14). In contrast to the diverse pathways leading to the production of N_2O, the sole biological sink process of N_2O in the environment is its reduction by the organisms expressing nitrous oxide reductases (NosZ) (8, 15–17). N_2O reduction was originally regarded merely as a part of the denitrification cascade. N_2O reduction as an independent respiratory reaction had not garnered the deserved interest until recent discoveries unveiled the unexpectedly broad diversity of *nosZ* (15, 18). *nosZ* genes of the novel clade II are often found in organisms lacking the genes encoding the key denitrification enzymes, namely *nirK* or *nirS*, indicating that these organisms utilize N_2O reduction as a respiratory reaction independent from denitrification (19). Thus, these nondenitrifying N_2O reducers function as *de facto* sinks of N_2O, as confirmed by physiological observations in experiments with isolates possessing NosZ (20, 21). Indeed, a recent study on the kinetics of N_2O reductions revealed that the clade II *nosZ*-containing organisms have significantly higher affinity to N_2O than the clade I *nosZ*-containing organisms, supporting the hypothesis that the organisms with clade II *nosZ* may contribute to the mitigation of N_2O emissions from nonpoint sources (17).

Metagenomic analyses of environmental DNA have revealed that clade II *nosZ* genes are, in fact, abundant in diverse environments, ranging from tropical forest and hot desert to Arctic tundra and polar desert (18, 22). Among the most abundant phylogenetic groups of *nosZ* in the environment are clade II *nosZ* genes affiliated with the *Gemmatimonadetes* phylum (16, 18). *Gemmatimonadetes* have been identified as one of the most abundant phyla of bacteria in soil environments and often constitute >2% of the total bacterial population (23, 24); however, only three representative strains of this phylum (*Gemmatimonas aurantiaca* strain T-27, *Gemmatirosa kalamazoonesis* strain KBS708, and *Gemmatimonas phototrophica* strain AP64) have been isolated to date, and the physiological characteristics of these organisms are virtually unknown (25–28). Although clade II *nosZ* genes were found in the genomes of *G. aurantiaca* strain T-27 and *G. kalamazoonesis* strain KBS708, these strains were both characterized as obligate aerobes, and respiration on any other electron acceptors, e.g., N_2O, has yet to be explored (25–27). Therefore, questions remain unanswered regarding the functionality of this *Gemmatimonadetes* NosZ and its potential physiological role as a respiratory enzyme. As this particular group of *nosZ* genes constituted up to 33% of the entire *nosZ* gene pools identified in soil metagenomes, an understanding of the N_2O reduction phenotype of the *Gemmatimonadetes* phylum is crucial for predicting N_2O sink capabilities of subsurface soil environments. In this study, N_2O reduction by *G. aurantiaca* strain T-27 was observed in both the absence and presence of oxygen. The inability of this organism to consume N_2O in the complete absence of oxygen and the unexpected transcription pattern of *nosZ*, i.e., upregulation in the presence of oxygen and downregulation in the absence of oxygen, suggest a novel regulatory mechanism for N_2O respiration by obligate aerobic microorganisms.

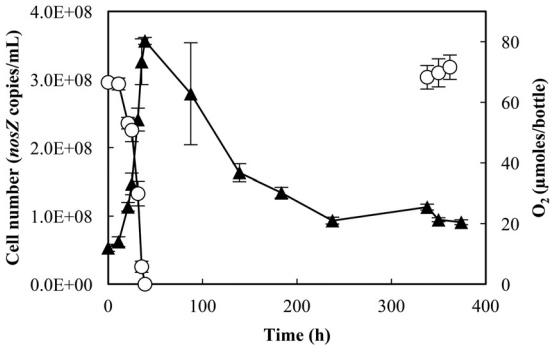

FIG 1 Aerobic growth of *G. aurantiaca* strain T-27 cultures in partially oxic conditions. The changes in amounts of O_2 (○) and *nosZ* copy numbers (▲) in the reaction bottles were monitored. Additional O_2 was added at $t = 339$ h. The data points are averages of duplicate experiments, with the error bars representing the standard deviations.

RESULTS

Aerobic growth of *G. aurantiaca* strain T-27. *Gemmatimonas aurantiaca* strain T-27 was incubated with 66.6 ± 1.1 μmol O_2 as the electron acceptor to obtain its growth curve under aerobic conditions (Fig. 1). The cell number, quantified by determining the *nosZ* gene copy number, increased from $5.5 \times 10^7 \pm 0.5 \times 10^7$ cells · ml^{-1} to $3.6 \times 10^8 \pm 0.5 \times 10^8$ cells · ml^{-1} at an exponential-growth rate of 0.068 ± 0.004 · h^{-1} (see Fig. S1 in the supplemental material). The exponential-growth phase lasted until O_2 was depleted. Cell decay began immediately after O_2 depletion; there was no stationary phase. The cell number decreased by more than 3-fold, from $3.6 \times 10^8 \pm 0.5 \times 10^8$ cells · ml^{-1} to $1.1 \times 10^8 \pm 0.4 \times 10^8$ cells · ml^{-1}, over 299 h of anoxic incubation following O_2 depletion. As 66.6 ± 1.1 μmol O_2 was consumed, the cellular yield on O_2 respiration was calculated to be $1.14 \times 10^8 \pm 0.03 \times 10^8$ cells (μmol e^- eq)$^{-1}$. The addition of O_2 at $t = 338$ h failed to revitalize the inactivated *G. aurantiaca* strain T-27 cells, as three consecutive measurements at 12 h-intervals confirmed no significant increase in cell number or decrease in the amount of O_2. Unexpectedly, no significant decrease in glucose concentration was observed throughout the experiment (data not shown), indicating that other organic compounds in the complex medium NM-1 (polypeptone, monosodium glutamate, and/or yeast extract) also served as electron donors and sources of carbon for *G. aurantiaca* strain T-27.

N_2O consumption under anoxic and partially oxic conditions. Initially, *G. aurantiaca* strain T-27 was examined for N_2O reduction activity under completely anoxic conditions (Fig. 2A). The amount of N_2O in the culture bottle after 312 h of incubation (105.1 ± 6.4 μmol/bottle) was not significantly different ($P > 0.05$) from the initial amount of N_2O (103.5 ± 1.7 μmol/bottle). When *G. aurantiaca* strain T-27 cells were incubated with O_2 added to the headspace (55.8 ± 3.9 μmol/bottle) along with N_2O (125.8 ± 9.9 μmol/bottle), active N_2O reduction was observed, but only after O_2 was depleted (Fig. 2B). The O_2 concentration dropped below the detection limit of the oxygen meter (0.02% in the headspace) within 25 h of inoculation, and the consumption of N_2O started upon the depletion of O_2. N_2O was depleted after 138.5 h of anoxic incubation. The observations from these anoxic and partially oxic incubations of strain T-27 with N_2O suggested that N_2O reduction activity in strain T-27 is dependent on O_2. No decrease in N_2O concentration was observed in killed-cell controls over 336 h of incubation (Fig. S2), confirming that the N_2O consumption was entirely biological.

Long-term N_2O consumption in the absence of oxygen. In a subsequent experiment under identical experimental conditions, N_2O consumption by *G. aurantiaca* strain T-27 was monitored over the long term (392 h) to investigate whether N_2O reduction activity is sustained in the absence of O_2 (Fig. 3A). Neither the cell yield ($1.27 \times 10^8 \pm 0.18 \times 10^8$ cells [μmol e^- eq]$^{-1}$) nor the exponential-growth rate (0.081 ± 0.009 h^{-1}) during the initial aerobic growth was significantly different from

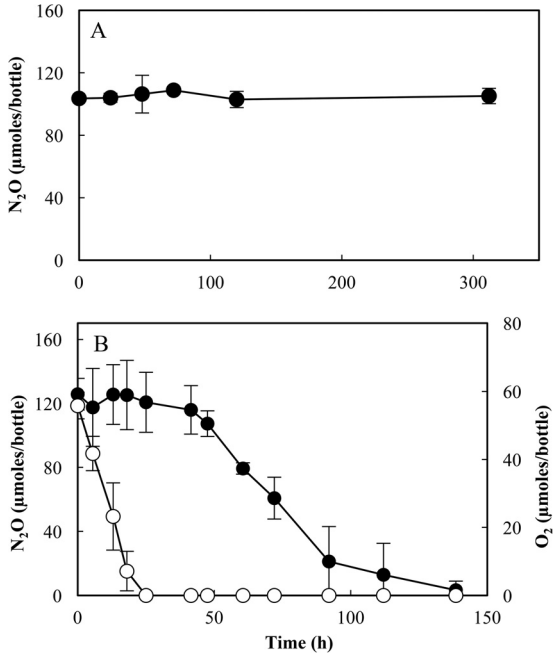

FIG 2 N_2O (●) and O_2 (○) consumption by *G. aurantiaca* strain T-27 cultures in the absence of O_2 (A) and initially amended with 55.8 ± 3.9 μmol O_2 (B). The data points are averages of triplicate experiments, with the error bars representing the standard deviations of the triplicate measurements.

the value determined from the aerobic incubation without N_2O (Fig. S1). The initial batch of N_2O (125.8 ± 0.9 μmol) was rapidly consumed within 81.5 h after O_2 depletion. After an injection of an additional 133.1 ± 2.7 μmol N_2O, strain T-27 continued to consume N_2O until the reaction slowed down significantly after $t = 237.5$ h and came to a near-complete stop at $t = 338$ h, after a total of 159.9 μmol N_2O was consumed. N_2O respiration did not appear to be coupled to growth in strain T-27, as no significant increase in cell number ($P > 0.05$) was observed after the depletion of O_2. Unlike when strain T-27 was incubated without N_2O, O_2 depletion did not lead to an immediate decrease in cell number. The cell number was sustained for 144.5 h after the depletion of O_2, although a statistically insignificant ($P > 0.05$) drop from $3.6 \times 10^8 \pm 0.3 \times 10^8$

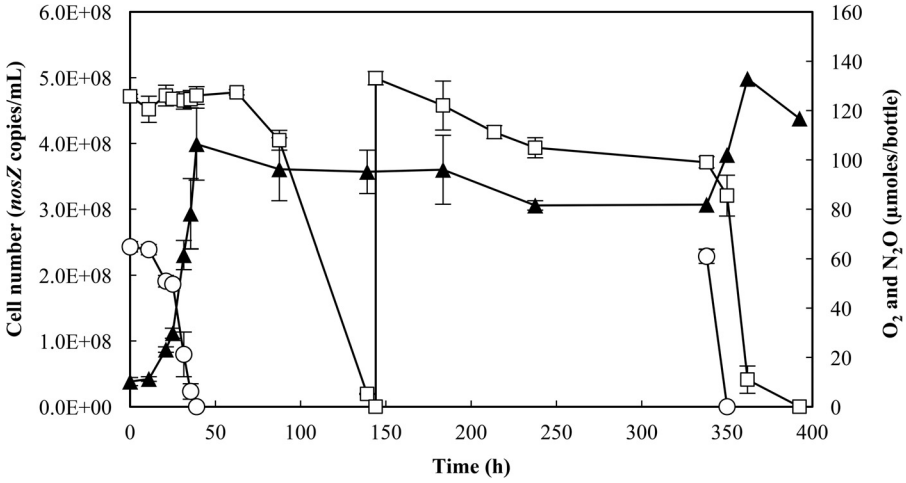

FIG 3 Long-term incubation of *G. aurantiaca* strain T-27 with N_2O after initial growth on O_2. The amounts of N_2O (□) and O_2 (○) in the reaction bottles were monitored, and the copy numbers of *nosZ* genes (▲) were measured at selected time points using qPCR for cell counts. N_2O was added upon N_2O depletion at $t = 144$ h, and O_2 was added at $t = 338$ h. The data points are averages of triplicate experiments, with the error bars representing the standard deviations of the triplicate measurements.

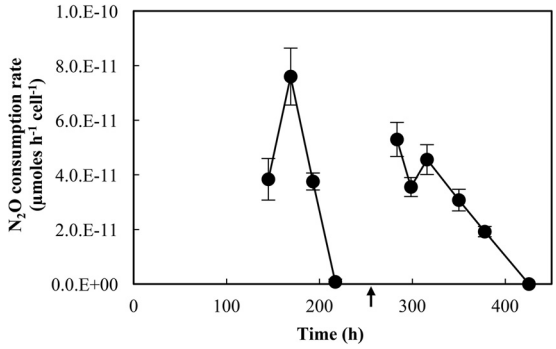

FIG 4 Monitoring of the change in rates of N_2O consumption by *G. aurantiaca* strain T-27. Each data point represents the N_2O consumption rate determined from linear regression of time versus N_2O amount (the average of triplicate samples) data. The error bars represent the standard errors of linear regression analyses. The arrow mark indicates the time of O_2 addition.

copies · ml^{-1} to $3.1 \times 10^8 \pm 0.8 \times 10^8$ copies · ml^{-1} was observed after that time point. The addition of O_2 at $t = 338$ h resulted in immediate consumption of O_2 accompanied by an immediate increase in the *nosZ* gene copy number to $5.0 \times 10^8 \pm 0.7 \times 10^8$ copies · ml^{-1}, followed by resuscitation of N_2O consumption. Residual N_2O (99.0 ± 2.3 mol) at the time of O_2 injection was rapidly reduced within 42.5 h of O_2 depletion. The different responses of the cultures amended with and without N_2O to O_2 depletion suggested that N_2O reduction helped sustain cell integrity and, in part, metabolic activities during transient anoxia.

In another set of experiments, the rates of N_2O consumption were measured at different time points during anoxic incubation following initial growth on O_2 (Fig. 4). The N_2O consumption rate peaked with $7.6 \times 10^{-11} \pm 1.0 \times 10^{-11}$ μmol · h^{-1} · $cell^{-1}$ at $t = 169$ h (132 h after O_2 depletion) and decreased to zero at $t = 217$ h. N_2O reduction resumed after consumption of added O_2, and the maximum N_2O consumption rate, $5.3 \times 10^{-11} \pm 0.6 \times 10^{-11}$ μmol · h^{-1} · $cell^{-1}$, was measured immediately after O_2 depletion. The maximum N_2O consumption rate was 69.7% of the highest value measured before resuscitation; however, N_2O consumption was sustained for a longer period of time after (144 h) than before (95 h) resuscitation. These kinetics measurements confirmed that N_2O consumption is not sustainable in the prolonged absence of O_2.

***nosZ* transcription analyses.** The transcription of *nosZ* was monitored with the samples withdrawn from a replicate of the batch culture used for monitoring of N_2O consumption and cell growth (Fig. 5, Fig. S3). The transcription of *nosZ* increased significantly, from 0.21 ± 0.21 *nosZ* transcripts/*recA* transcript upon inoculation to 8.97 ± 0.90 *nosZ* transcripts/*recA* transcript, immediately after the consumption of 63.1 μmol O_2 ($P < 0.05$). The transcription of *nosZ* decreased to 0.15 ± 0.23 *nosZ* transcripts/*rec* transcript 419 h after O_2 depletion, and the downregulation of *nosZ* transcription explained the disappearance of N_2O reductase activity. As expected from the resumption of N_2O reduction activity after injection of O_2, O_2 addition resuscitated *nosZ* transcription, and the maximum transcription level (1.86 ± 0.16 *nosZ* transcripts/*rec* transcript) was observed 91 h after O_2 depletion. This elevated transcription activity was sustained for >300 h after the oxygen depletion, even though a notable decreasing trend was observed after the peak, suggesting a less stringent regulation of *nosZ* transcription than that observed after the first O_2 depletion event. This observation was consistent with a less precipitous decrease in N_2O reduction rate after resuscitation than before O_2 addition. The highest peaks in *nosZ* transcription were observed immediately after O_2 was depleted, indicating that *nosZ* transcription occurred in the presence of O_2. These observations suggested that *G. aurantiaca* strain T-27 may require N_2O reductases expressed in the presence of O_2 to utilize N_2O as a surrogate electron acceptor in the anoxia that follows.

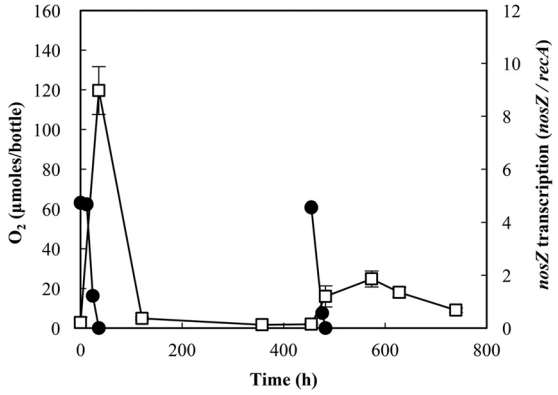

FIG 5 *nosZ* transcription in the *G. aurantiaca* strain T-27 culture during the long-term anoxic incubation with N_2O following initial partial-oxic incubation. The samples for the RT-qPCR analysis were collected at 9 crucial time points from the same culture vessel examined in Fig. S3. *nosZ* transcription level (□) was presented as the *nosZ* transcript copy numbers normalized with *recA* transcript copy numbers. O_2 (●) concentration is shown to indicate the time points of O_2 addition and depletion. The *nosZ* transcription data points are averages of triplicate samples treated independently through RNA extraction, purification, and reverse transcription procedures, and the error bars represent their standard deviations.

DISCUSSION

The *Gemmatimonadetes* phylum is one of the most abundant phylogenetic groups of microorganisms that constitute the soil bacterial communities (29, 30). Apart from the synthesis of a novel carotenoid by *G. aurantiaca* strain T-27 (31), not much has been reported regarding their physiology until recently, mainly due to the paucity of available isolates. The recent discovery of a full operon encoding type 2 photosynthetic reaction centers in the *G. phototrophica* strain AP-64 genome suggested that the *Gemmatimonadetes* phylum of bacteria may have adopted an oxygen-independent energy-generating metabolism to supplement an oxygen-dependent heterotrophic lifestyle in cases of transient hypoxia or anoxia (32). The utilization of N_2O by *G. aurantiaca* strain T-27 observed in this study may be interpreted in a similar context. Strain T-27 was previously characterized as an obligate aerobic bacteria unable to grow with any nonoxygen electron acceptors (26), and our experiments have confirmed that the presence of N_2O alone could not initiate N_2O reduction under conditions of complete anoxia or support growth; however, in the short term, N_2O utilization as the surrogate electron acceptor helped sustain cell viability in the absence of O_2. Similar transient enhancement of reduction of nonoxygen electron acceptors, i.e., NO_2^- and NO, was previously observed in obligate aerobic nitrifiers under hypoxic or semioxic conditions (33, 34). Geets et al. (34) proposed the hypothesis that one of the possible physiological roles of this nitrifier denitrification may be related to survival during anoxic periods, but this hypothesis has not yet been supported by experimental evidence. In recent research with *Nitrosomonas eutropha* strain C91, NO_2 gas was found to have a similar role of sustaining cell viability in oxygen-depleted cultures (35). The transcriptomics and proteomics data in the study suggested that *N. eutropha* strain C91 tuned down the assimilatory metabolism while retaining or stimulating dissimilatory metabolism upon anoxic incubation with NO_2 as the surrogate oxidant. A Gram-positive obligate aerobic bacterium, *Streptomyces coelicolor* A3(2), was also found to utilize its Nar-type respiratory nitrate reductase to survive extended periods of anoxia, although no growth was observed in the absence of O_2 (36, 37). The utilization of N_2O as a temporary substitute for O_2 in *G. aurantiaca* strain T-27 may be another such lifestyle that strictly aerobic microorganisms have adopted to generate cell maintenance energy to survive through periods of anoxia. In fact, in support of this hypothesis, several other organisms that have been characterized as obligate aerobes harbor clade II *nosZ* genes, including *Leptospira* spp., *Runella slithyformis*, *Haliscomenobacter hydrossis*, and *Marivirga tractuosa* (38–41).

The physiology of N_2O reduction by *G. aurantiaca* strain T-27 observed in this study

was distinguishable from that of previously studied denitrifiers or nondenitrifying N_2O reducers in that (i) N_2O reduction was decoupled from cell growth and (ii) N_2O reduction activity was dependent on the transient presence of O_2 and dissipated under prolonged anoxia. Although N_2O reductase is a soluble periplasmic enzyme, earlier studies have demonstrated that the electron transfer chain to N_2O reductase involves cytochromes and thus is coupled to proton translocation across the membrane and energy conservation (42, 43). In fact, diverse groups of denitrifiers and nondenitrifying N_2O reducers were able to grow with N_2O as the sole electron acceptor (17, 43, 44). Albeit with different efficiencies and concentration thresholds, organisms with active clade I and clade II N_2O reductases examined in a recent kinetics study invariably coupled N_2O reduction to growth, and N_2O reduction entirely decoupled from cell growth has not been previously observed.

A plausible explanation for nongrowth in the absence of O_2 may be that O_2 is required for synthesis of metabolites necessary for strain T-27 to reproduce while N_2O can be used only as a temporary substitute for oxygen, as it can provide energy but not these necessary metabolites. A similar phenomenon was previously observed with *Campylobacter jejuni* strain 11168, a gastrointestinal pathogen capable of utilizing NO_3^-, NO_2^-, fumarate, and trimethylamine-*N*-oxide as electron acceptors for energy conservation under microaerophilic conditions, but not in the complete absence of oxygen (45). The oxygen dependence of *C. jejuni* strain 11168 was attributed to the requirement for oxygen-dependent ribonucleotide reductase (RNR). Another plausible explanation for the oxygen requirement of strictly aerobic bacteria is the use of HemF, the oxygen-dependent coproporphyrinogen III oxidase, for coproporphyrinogen III decarboxylation in heme synthesis (46). Neither case applies to *G. aurantiaca* strain T-27, as it possesses the genes encoding class II RNR and HemN, the oxygen-independent counterparts for class I RNR and HemF, respectively, in its genome; however, it is still possible that other essential metabolic functions for biosynthesis in *G. aurantiaca* strain T-27 may be carried out by oxygen-dependent enzymes.

Nitrous oxide reductase, like other enzymes in the denitrification cascade, has long been known to be sensitive to O_2 concentration (47, 48). That *G. aurantiaca* required the transient presence of O_2 for expression and activation of nitrous oxide reductases is nothing new. *Ensifer meliloti* strain 1021 was found to require partial oxygenation before activation of anaerobic denitrification and N_2O reduction, and *Paracoccus denitrificans* exhibited the highest level of *nosZ* expression immediately following O_2 depletion (49, 50). Nevertheless, the eventual termination of N_2O reduction in prolonged anoxia has not been observed in these organisms. In *E. meliloti* strain 1021, the expression levels of denitrification-related genes *napA*, *nirS*, *nosC*, and *nosZ* were sustained at an order-of-magnitude-higher level under completely anoxic conditions than under partially oxic conditions, suggesting continued expression and activity of denitrification and N_2O reduction (51). In *P. denitrificans*, the high level of *nosZ* transcription was sustained in the anoxic phase until all nitrogen oxides were depleted, indicating that NosZ enzymes synthesized during anoxic incubation were utilized for N_2O reduction (52). Further, in four strains of bacteria examined for kinetic properties, N_2O served as the sole source of electron acceptor for exponential growth in the complete absence of O_2 (17). As nitrous oxide reductase is active only in the absence of O_2 and thus is supposedly more beneficial for its owners under anoxic conditions (52), the disappearance of *nosZ* transcription activity in oxygen-depleted cultures of *G. aurantiaca* strain T-27 is rather unexpected and is unprecedented in any studies on denitrifiers or nondenitrifying N_2O reducers alike. These novel findings substantially expand the knowledge of microbial processes that contribute to N_2O emission mitigation, as this study demonstrated a novel regulatory mechanism that may be more generally applicable to *nosZ*-harboring obligate aerobic bacteria, which constitute an appreciable portion of the *nosZ*-harboring population in the environment (15, 18).

Subsurface soil environments alternate between oxic and anoxic conditions due to both natural and anthropogenic events. Precipitation events often lead to flooding of the vadose zone, hindering mass transfer of atmospheric O_2 into the subsurface soil

environment (53). In fertilized grassland, soil compaction from frequent mowing may also result in temporarily diminished O_2 availability (54). Elevated N_2O emissions have been observed during such transitions from oxic to anoxic conditions (55–57). The obligate aerobic organisms harboring *nosZ* genes, including *G. aurantiaca* strain T-27, may benefit from the elevated local N_2O concentrations, as they are capable of utilizing N_2O as a temporary surrogate for O_2 to survive temporary anoxia. These aerobic N_2O reducers may be undertaking an unexpectedly important role in reducing the amounts of N_2O emitted to the atmosphere in events of oxic-to-anoxic transitions. Future research is warranted for the development of novel experimental methods for an investigation of the true contribution of these obligate aerobic organisms to N_2O emission reduction, as enrichment-based investigations would not be able to capture these important N_2O sinks.

MATERIALS AND METHODS

Bacterial culture and growth conditions. *Gemmatimonas aurantiaca* strain T-27 was acquired from the Japan Collection of Microorganisms (JCM 11422). The culture medium used in this study was developed from NM-1 medium, a semidefined medium previously developed for isolation and culturing of *G. aurantiaca* strain T-27 (26). The medium contained, per liter, 0.5 g of glucose, 0.5 g of polypeptone, 0.5 g of monosodium glutamate, 0.5 g of yeast extract, 0.44 g of K_2HPO_4, 0.1 g of $(NH_4)_2SO_4$, and 0.1 g of $MgSO_4$. As nitrous oxide reductase is a copper-dependent enzyme and a lack of copper may hinder the activity of the enzyme (58), $CuCl_2$ was added to a concentration of 5 μM. The pH was adjusted to 7.0 with 5 N NaOH solution. For preparation of anoxic or partially oxic cultures, 100-ml aliquots of the medium were distributed into 160-ml serum bottles (Wheaton, Millville, NJ) and flushed with >99.999% N_2 for ~20 min to remove O_2. This degassing step was omitted for oxic precultures, prepared with 50 ml of medium in 160-ml serum bottles. The serum bottles were then sealed with butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK). After autoclaving, 10% of headspace (6 ml of 60-ml headspaces) was aseptically replaced with air to prepare partially oxic cultures (~2.1% O_2 concentration in the headspace). No air was added to anoxic controls. Half a milliliter of 200× Wolin's vitamin solution (59) was added, and 2.0 ml of *G. aurantiaca* strain T-27 preculture was inoculated. Three milliliters of >99.999% N_2O (Deokyang Co., Ulsan, South Korea) was aseptically added using a disposable syringe connected to 0.2-μm-pore-size syringe filters (Advantec, Inc., Tokyo, Japan) after the same volume of headspace gas was removed. All culture vessels were incubated at 30°C with shaking at 140 rpm. Glucose was added in excess (0.5 g/liter), as a theoretical stoichiometric calculation estimated that only 15.0% (42.5 μmol) of added glucose would be consumed to reduce 125.8 μmol O_2 and 258.9 μmol N_2O (the maximum amounts of electron acceptors used in this research), even if glucose is used as the sole electron donor. Glucose concentrations were measured after the reactions were completed to confirm that the amount of electron donor added was not a limiting factor for the growth of *G. aurantiaca* strain T-27.

Analytical procedure. The amounts of N_2O in the serum bottles were quantified using an HP6890 series gas chromatograph equipped with an HP-PLOT/Q column and an electron capture detector (Agilent Technologies, Santa Clara, CA). The injector, oven, and detector temperatures were set to 200, 85, and 250°C, respectively (17). For each measurement, 200 μl of headspace gas was removed using a 1700 series gas-tight syringe (Hamilton Company, Reno, NV), and 100 μl of the withdrawn sample was manually injected into the gas chromatograph. The syringe was flushed at least three times with pressurized N_2 gas to remove O_2 before use, and 200 μl of N_2 was added upon each sampling event to prevent a pressure drop in the culture bottles. The change in the amounts of N_2O due to gas sampling was accounted for in the subsequent calculations (60). Oxygen concentrations were monitored with fiber-optic oxygen sensor spots and a FireStingO2 oxygen meter (Pyroscience, Aachen, Germany). The total amounts of N_2O and O_2 in the reaction vessels were calculated from the headspace concentrations as described previously (17). The dimensionless Henry's constants (calculated as moles in the headspace/moles in the aqueous phase) of N_2O and O_2 at 30°C were calculated to be 1.92 and 33.3, respectively (61). These dimensionless Henry's constants were used to calculate the aqueous concentrations of N_2O and O_2. The amounts of the gases in the headspace and the aqueous phase were summed to determine the total amounts of N_2O and O_2 in the vessels. Glucose concentrations were measured colorimetrically using a glucose (HK) assay kit (Sigma-Aldrich, St. Louis, MO).

Monitoring of N_2O consumption. A series of incubation experiments was performed to investigate the consumption of N_2O by *G. aurantiaca* strain T-27. Oxic precultures for the experiments were prepared from glycerol stocks of strain T-27. For anoxic control experiments, 50 ml of the oxic precultures was harvested at the late-exponential phase (optical density at 600 nm [OD_{600}], ~0.043) and centrifuged at 4,800 × *g* for 10 min at 4°C. In an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) filled with 95% N_2 and 5% H_2, the pellets were equilibrated for 1 h and resuspended into 2 ml of medium taken from the anoxic culture bottles prepared as described above. The concentrated precultures were reinjected into the anoxic serum bottle, and the N_2O concentration was monitored for 312 h. After confirmation of the absence of N_2O consumption activity in the anoxic cultures, the N_2O consumption experiments were performed in partially oxic culture bottles prepared with a low concentration (2.1%) of O_2 in the headspace. One milliliter of frozen stock of strain T-27 was grown in 50 ml of oxic medium in a 160-ml serum bottle until the late-exponential phase. Each partially oxic culture bottle (with 100 ml

of medium) was inoculated with 2 ml of these late-exponential-phase cultures. O_2 concentrations were monitored until the concentrations dropped below the detection limit of the oxygen meter, and N_2O concentrations were measured at 5- to 26.5-h intervals until no remaining N_2O was detected. The killed-cell negative controls were inoculated with autoclaved aerobic precultures harvested at the late-exponential phase and concentrated by centrifugation. The concentrations of O_2 and N_2O were monitored for 336 h at 30°C.

Long-term incubation of *G. aurantiaca* strain T-27 on N_2O. In order to examine whether O_2 is required for sustenance of N_2O reduction activity, *G. aurantiaca* strain T-27 culture was initiated with 51.5 μmol O_2 and 122.6 μmol N_2O. Each time N_2O was depleted, 3 ml (nominally 121.6 μmol) of N_2O was aseptically injected into the culture bottle. After N_2O consumption came close to a halt, 6 ml of headspace was aseptically replaced with air, providing an additional 51.5 μmol O_2, to examine whether O_2 addition resuscitates N_2O reduction activity. O_2 and N_2O concentrations were monitored throughout the experiment at appropriate time intervals (3.5 to 100.5 h), and aqueous-phase samples were collected for quantification of *nosZ* genes and/or transcripts. As strain T-27 contains a single *nosZ* copy in its published genome (accession no. NC_012489.1), the copy number of the *nosZ* gene was used as a surrogate for cell counts. In the no-N_2O controls amended only with 51.5 μmol O_2, O_2 concentrations and cell numbers were monitored until 323 h after O_2 depletion. After O_2 replenishment, three measurements of O_2 concentrations and *nosZ* copy numbers were made at 12-h intervals.

In an independent set of experiments, the decrease in N_2O consumption rate was monitored in anoxia following initial O_2 consumption (51.5 μmol). Three independent cultures of *G. aurantiaca* strain T-27 were prepared identically to the above-described experiments. After O_2 depletion and onset of N_2O consumption, N_2O consumption rates were measured by taking at least three consecutive measurements of N_2O concentrations at a constant time interval (intervals were varied depending on the rates of N_2O consumption) and performing linear regression to calculate the slope of the time-versus-amount curves for the N_2O consumption rates. The average of the time points was taken as the representative time for each rate measurement. After each rate determination, N_2O in the culture bottles was replenished to the initial amount (~121.6 μmol N_2O/bottle) by adding >99.999% N_2O aseptically through 0.2-μm-pore-size filters. One milliliter of the aqueous phase was extracted immediately after N_2O replenishment for cell counting by quantitative PCR (qPCR), and the volumetric loss was replaced with fresh anoxic NM-1 medium. The N_2O consumption data were normalized, with the numbers of *nosZ* genes measured by qPCR. Upon cessation of N_2O reduction, 51.5 μmol O_2 was added, and N_2O reduction rates were monitored using the same protocol.

Cell culture sampling for DNA/RNA extraction. A qPCR technique was used for quantification of cell numbers, and reverse transcription-quantitative PCR (RT-qPCR) analyses were performed to examine the effect of O_2 on transcription of *nosZ*. A cell suspension was sampled at the time points determined to be crucial for observation of N_2O reduction by *G. aurantiaca* strain T-27. Each sampling was performed by withdrawing 1.0 ml (samples subjected to either qPCR or RT-qPCR) or 1.6 ml (samples subjected to both qPCR and RT-qPCR) of the aqueous phase using a sterile disposable syringe flushed with N_2 gas. To avoid pressure loss in the vessel, the same volume of N_2 gas was added upon each sampling event. Triplicate 0.2-ml aliquots were transferred to 1.5-ml DNase- and RNase-free tubes (Eppendorf, Hamburg, Germany) for DNA extraction. After centrifugation at 15,000 $\times$ *g* for 1 min, the supernatant was removed. The cell pellet was stored in a −20°C freezer until DNA extraction. For RNA extraction, triplicate 0.3-ml aliquots were transferred to 1.5-ml DNase- and RNase-free tubes, and each aliquot was mixed vigorously with 0.6 ml of RNAprotect Bacteria reagent (Qiagen, Hilden, Germany). After centrifugation at 5,000 $\times$ *g* for 10 min, the supernatant was carefully removed and the pellet was stored at −80°C until further treatment.

DNA/RNA extraction and purification procedure. DNA extraction from the pelleted cell culture samples were performed with the DNeasy blood and tissue kit (Qiagen) according to the protocol provided by the manufacturer. The DNA samples were stored at −20°C until qPCR analyses. A two-step RT-qPCR approach was taken for analyses of *nosZ* transcription according to the established protocol (62). Before extraction, 1 μl of luciferase control mRNA (Promega, Madison, WI) diluted to 10^{10} copies · μl^{-1} was added to each sample as an internal standard to account for RNA loss during extraction, purification, and reverse transcription processes (63). The recovery of the control RNA was used to check for the validity of transcription analyses. A mixture of 350 μl of RLT buffer from an RNeasy minikit (Qiagen) and 7 μl of β-mercaptoethanol was added to the tubes containing cell pellets, and the tubes were vortexed for 10 s. Each suspension was transferred to a 2.0-ml reinforced tube containing 0.1-mm-diameter glass beads (Omni International, Kennesaw, GA). Cells were disrupted for 5 min at 5 m/s in a Bead Ruptor 12 homogenizer (Omni International). Total RNA was extracted using an RNeasy minikit according to the protocol provided by the manufacturer. The eluents were then treated with RNase-free DNase I (Qiagen) to remove residual DNA (64). The DNase-treated samples were purified using the RNeasy MinElute cleanup kit (Qiagen), and 20-μl eluent volumes were collected after purification. To 10 μl of the eluents, 1 μl of a 10 mM dinucleoside triphosphate (dNTP) mixture (Invitrogen, Waltham, MA) and 2 μl of random hexamers (Invitrogen) were added. The remaining eluents were stored at −20°C and later employed to check for DNA contamination by using qPCR assays targeting the *nosZ* gene. The mixture was incubated at 65°C for 5 min. After cooling in ice for 1 min, 4 μl of 5× First-Strand buffer, 1 μl of 0.1 M dithiothreitol (DTT; Invitrogen), and 1 μl of RNaseOUT solution (Invitrogen) were added, and the reaction mixture was incubated at room temperature for 2 min. After 1 μl of SuperScript III reverse transcriptase (Invitrogen) was added, the reaction mixture was incubated with the following temperature cycle: 10 min at room temperature, 3 h at 42°C, and 15 min at 70°C. The resulting cDNA was chilled on ice before 1 μl of RNase H (Invitrogen) was added to digest RNA strands of RNA-cDNA hybrids and residual RNA in the solution.

TABLE 1 Primers used for qPCR and RT-qPCR analyses and their qPCR calibration curve parameters

Primer	Primer sequence (5′ to 3′)	Target gene (locus tag)	Amplicon length (bp)	Slope	*y* intercept	Amplification efficiency	R^2	Reference or source
GenosZf1000	TCGATCTACTTCCTGCCGAC	*nosZ* (GAU_1385)	151	−3.523	36.21	92.2	0.993	This study
GenosZr1150	CGAACGCCTGATCCTTGATG							
GerecAf780	CGACATCATGTACGCGGAAG	*recA* (GAU_1917)	183	−3.449	35.99	95.0	0.999	This study
GerecAr972	CTTCACCTTGTCCTCGACCT							
lucf	TACAACACCCCAACATCTTCGA	Luciferase control mRNA	67	−3.32	34.69	100.3	1	62
lucr	GGAAGTTCACCGGCGTCAT							

The reaction mixture was incubated at 37°C for 20 min, and the resulting cDNA solution was diluted 5-fold with the addition of 84 μl of nuclease-free water to reduce inhibitory effects of the reagents on qPCR. The triplicate samples collected at each time point were treated independently through extraction, purification, and reverse transcription procedures and yielded three separate cDNA samples later subjected to qPCR.

Quantitative PCR/RT-qPCR assays for cell number determination and analyses of *nosZ* transcription. Quantification of *nosZ* genes in the genomic DNA and the cDNA samples were performed with quantitative PCR using established protocols, with modifications (62) (Fig. 5). *recA*, the housekeeping gene encoding recombinase A, was quantified in the cDNA samples for normalization of the RT-qPCR data, and the *nosZ* gene expression levels were presented as the *nosZ* transcript copy number per *recA* transcript. The new primers used in this study were designed using the Primer3 software, and amplicon sizes were limited to <200 bp for accurate quantification (Table 1) (65). SYBR green detection chemistry was used for qPCR assays performed with the QuantStudio 3 real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA). The reaction mixture was prepared with 2× Power SYBR green PCR master mix solution (Applied Biosystems, Waltham, MA). The calibration curves were constructed with a serial dilution series of the target fragments inserted into PCR2.1 vectors using the TOPO TA cloning kit (Thermo Fisher Scientific). The qPCR assay for all three target genes yielded consistent results for DNA copy numbers as low as 10^1 copies/μl. Amplification of the no-template controls and DNase-treated RNA samples yielded negative results. Consistent melting curves indicated the target specificity of the qPCRs. The RNA recovery rates, as determined from the recovery of the *luc* control, ranged from 19.61% to 41.28%. For each DNA or cDNA sample analyzed, qPCRs were performed in triplicate, and the average threshold cycle (C_T) values of these technical replicates were obtained for calculation of the copy number.

Statistical analyses. Statistical analyses (*t* tests) were performed with SPSS 24 software (IBM Corp., NY, USA). Unless otherwise mentioned, the presented data are the averages and standard deviations of the results from triplicate experiments. *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00502-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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