Quorum Quenching of *Nitrobacter winogradskyi* Suggests that Quorum Sensing Regulates Fluxes of Nitrogen Oxide(s) during Nitrification

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**ABSTRACT** Quorum sensing (QS) is a widespread process that bacteria use to coordinate gene expression with cell density, diffusion dynamics, and spatial distribution through the production of diffusible chemical signals. To date, most studies on QS have focused on model bacteria that are amenable to genetic manipulation and capable of high growth rates, but many environmentally important bacteria have been overlooked. For example, representatives of proteobacteria that participate in nitrification, the aerobic oxidation of ammonia to nitrate via nitrite, produce QS signals called acyl-homoserine lactones (AHLs). Nitrification emits nitrogen oxide gases (NO, NO₂, and N₂O), which are potentially hazardous compounds that contribute to global warming. Despite considerable interest in nitrification, the purpose of QS in the physiology/ecology of nitrifying bacteria is poorly understood. Through a quorum quenching approach, we investigated the role of QS in a well-studied AHL-producing nitrite oxidizer, *Nitrobacter winogradskyi*. We added a recombinant AiiA lactonase to *N. winogradskyi* cultures to degrade AHLs to prevent their accumulation and to induce a QS-negative phenotype and then used mRNA sequencing (mRNA-Seq) to identify putative QS-controlled genes. Our transcriptome analysis showed that expression of *nirK* and *nirK* cluster genes (*ncgABC*) increased up to 19.9-fold under QS-proficient conditions (minus active lactonase). These data led us to query if QS influenced nitrogen oxide gas fluxes in *N. winogradskyi*. Production and consumption of NO, increased and production of N₂O decreased under QS-proficient conditions. Quorum quenching transcriptome approaches have broad potential to identify QS-controlled genes and phenotypes in organisms that are not genetically tractable.

**IMPORTANCE** Bacterial cell-cell signaling, or quorum sensing (QS), is a method of bacterial communication and gene regulation that is well studied in bacteria. However, little is known about the purpose of QS in many environmentally important bacteria. Here, we demonstrate quorum quenching coupled with mRNA-Seq to identify QS-controlled genes and phenotypes in *Nitrobacter winogradskyi*, a nitrite-oxidizing bacterium. Nitrite oxidizers play an important role in the nitrogen cycle though their participation in nitrification, the aerobic oxidation of ammonia to nitrate via nitrite. Our quorum quenching approach revealed that QS influences production and consumption of environmentally important nitrogen oxide gases (NO, NO₂, and N₂O) in *N. winogradskyi*. This study demonstrated a novel technique for studying QS in difficult-to-work-with microorganisms and showed that nitrite oxidizers might also contribute to nitrification-dependent production of nitrogen oxide gases that contribute to global warming.
tified in representatives of proteobacteria that participate in the process of nitrification (5–10).

During nitrification, diverse genera of chemolithotrophic bacteria and/or archaea oxidize ammonia (NH$_3$) to nitrite (NO$_2^-$) and then to nitrate (NO$_3^-$) (11–14). Generally, NH$_3$ is oxidized to NO$_2^-$ by ammonia oxidizers, including bacteria (AOB) and archaea (AOA), while NO$_2^-$ is oxidized to NO$_3^-$ by nitrite-oxidizing bacteria (NOB). Recently, complete oxidation of NH$_3$ to NO$_3^-$ was identified in representatives of the genus *Nitrospira*, a group previously characterized as NOB (15, 16). Nitrification is a key part of the nitrogen cycle in natural, agricultural, and industrial systems and is a contributor to gas emissions of nitric oxide (NO), nitrogen oxides (NO$_x$), and nitrous oxide (N$_2$O), which are hazardous gases that contribute to global warming (11, 17).

In many proteobacteria, QS is accomplished through the production of acyl-homoserine lactone (AHL) signaling compounds or autoinducers (1). AHLs represent the best-studied class of autoinducers (1). AHLs are generally located adjacent to each other in the genome and are generally positively autoregulated (1).

One method to study AHL QS is by specifically inactivating all AHL autoinducers through the use of recombinant lactonase to promote a QS-deficient phenotype (18, 19). AiiA, an AHL lactonase identified from *Bacillus* spp., is a well-characterized enzyme that specifically hydrolyzes the homoserine lactone (HSL) ring of AHLs, regardless of the chain length of the acyl group or other moiety (20, 21). So-called “quorum quenching” approaches have been implemented through both heterologous expression in a host of interest and addition of purified AHL lactonase (18, 20, 22–24). In this study, we used purified AiiA lactonase to identify QS-controlled gene expression and phenotypes in *Nitrobacter winogradskyi*, a well-characterized NOB that is currently not genetically tractable.

The genus *Nitrobacter* consists of a ubiquitous group of NOB in the family Bradyrhizobiaceae isolated from soil, water, and wastewater treatment systems (13, 14). *N. winogradskyi* is a well-studied example of NOB due to its superior growth rate and wastewater treatment systems (13, 14). In addition, the genome of *N. winogradskyi* has been sequenced and this bacterium has been the subject of recent global transcriptome studies (6, 25, 26). Expression of *N. winogradskyi* genes *nwiI* and *nwiR*, encoding an autoinducer synthase, and *nwiR*, encoding a receptor-transcriptional regulator, was shown to be cell density dependent and to correlate with the AHL concentration in culture (10). The structure of the predominant AHL was identified as that of an unsaturated AHL with a 10 carbon acyl chain, C$_{10:1}$-HSL (10). Nuclear magnetic resonance (NMR) spectroscopy analysis of AHL extracts produced via heterologous expression in *Escherichia coli* identified the isomeric form of C$_{10:1}$-HSL (27) but suggested a location for the double bond that is different from that previously described (10). However, heterologous expression of autoinducer synthases in *E. coli* often produces AHLs that are different from those in the native strain (27, 28).

Previous attempts have been made to identify QS-controlled phenotypes in *N. winogradskyi* (10, 27). Mellbye et al. showed that the growth rate decreased as transcription of *nwiI* and *nwiR* increased and AHLs began to accumulate (10). Shen et al. observed up to a 2-fold increase or 5-fold decrease in the expression of select genes of the nitrite oxidoreductase (NXR) gene cluster after the addition of purified C$_{10:1}$-HSL to cultures at saturating concentrations but did not observe any statistically significant phenotypic changes (27).

Here, we utilized a quorum quenching approach to identify both primary and secondary regulatory effects of AHL QS in *N. winogradskyi*. Using purified AiiA lactonase, AHLs were depleted from *N. winogradskyi* cultures and QS-controlled genes were identified through comprehensive mRNA sequencing (mRNA-Seq) analysis. Our transcriptome analysis showed that depletion of AHLs affected the expression of a significant percentage (52%) of the genetic inventory in *N. winogradskyi* and also suggested a link between QS and nitrogen oxide fluxes in this bacterium. Our experiments confirm a previous report that *N. winogradskyi* can produce N$_2$O (29) and present new evidence that QS affects NO$_x$ fluxes. Our work demonstrates that AiiA-mediated quorum quenching coupled with mRNA-Seq is a useful technique to identify QS-controlled genes and phenotypes in difficult-to-study organisms.

**RESULTS**

AiiA lactonase treatment of *N. winogradskyi* cultures depletes AHLs. To determine the effect of QS inhibition in *N. winogradskyi*, we initiated and monitored three batch culture treatments: (i) AiiA lactonase treatment (QS-deficient), (ii) heat-denatured AiiA lactonase treatment (QS-proficient) (to determine if protein addition had an effect), and (iii) no-added-lactonase treatment (QS-proficient). Depending on cell density, approximately 0.28 or 0.71 µg protein ml$^{-1}$ was added to both lactonase and heat-denatured lactonase treatments daily (see Text SI in the supplemental material). Although the treatments showed no significant differences in nitrite oxidation rate, growth rate, or growth yield (Fig. 1A), the addition of AiiA lactonase prevented the accumulation of bioassay-detectable AHL (Fig. 1B). Lactonase-treated and heat-denatured lactonase-treated cultures were harvested on day 3 during peak signal production as observed in our previous work (10) to collect RNA for mRNA-Seq (Fig. 1).

**Transcriptome responses to QS inhibition.** The transcriptome of *N. winogradskyi* under QS-deficient (AiiA-treated) conditions was compared to that present under QS-proficient (heat-denatured AiiA-treated) conditions. All changes in gene expression are expressed as the ratio of the number of transcripts seen under the QS-proficient treatment conditions to the number seen under QS-deficient treatment conditions. First, we validated our quorum quenching approach by noting an increase in the transcript abundance of the signal synthase *nwiI* gene and the signal receptor *nwiR* gene under QS-proficient conditions (Table 1). As previously noted, many bacterial QS genes, particularly the signal synthase gene, are autoregulated, creating a positive-feedback loop (1, 10). In addition, levels of methionine biosynthesis transcripts increased up to 7.7-fold, possibly due to increased use of S-adenosyl methionine for AHL biosynthesis (Table 1). The transcriptome analysis revealed 1,631 genes showing statistically significant changes in expression in QS-proficient cells, but many changes were <3-fold (see Table S1 and Dataset S1 in the supplemental material). In total, expression of 1,346 genes changed marginally and expression of 237 genes changed >3-fold between QS-deficient and QS-proficient conditions (see Dataset S1). Grouping
Nitrite metabolism and signal transduction genes are induced under QS-proficient conditions. An in-depth scan of the QS transcriptome of *N. winogradskyi* showed that the largest changes in expression involved genes encoding proteins associated with biosynthetic metabolism, nitrogen metabolism, and signal transduction, particularly those associated with nitrite metabolism (Table 1). Under QS-proficient conditions, assimilatory nitrite reductase gene *nirBD* decreased in expression up to 9.3-fold, suggesting imminent growth arrest and induction of the stringent response, as nitrite was the sole nitrogen source in the medium (Table 1). In addition, expression of nitrite reductase gene *nirK* increased 2.2-fold whereas expression of *nirK* cluster genes, including *nirGABC*, increased up to 19.9-fold under QS-proficient conditions (Table 1). Furthermore, expression of Nwi0557, a homolog of *nirS*, a putative NO-responsive membrane protein gene, increased 8.1-fold (Table 1). These changes suggest a possible link between QS effects on N biosynthesis metabolism and NO production.

QS-proficient conditions also changed the expression levels of several genes involved in signal transduction and flagellum biosynthesis. Guanine nucleotide secondary messenger (e.g., *ci-dGMP*, ppGpp) biosynthesis and response genes, including genes encoding a diguanylate cyclase/phosphodiesterase homolog (*Nwi0500*), a Crp domain regulator (*Nwi2061*), a RelA/SpoT homolog (*Nwi1922*), and Ppx/GppA phosphatase (*Nwi2151*), increased in expression by 2.3- to 8.9-fold (Table 1). Fourteen genes associated with flagellum biosynthesis and assembly decreased in expression up to 2.6-fold (Table 1). That said, no obvious phenotype differences, such as changes in motility, biofilm, or aggregate formation, were observed under QS-proficient or -deficient conditions.

**Quorum sensing in *N. winogradskyi* influences NO fluxes.** Following the transcriptome analysis prediction that QS affects nitrite metabolism through expression of *nirK* cluster genes, production and consumption of NO gases by *N. winogradskyi* were measured. In order to observe the biggest difference in gas fluxes, cells were incubated at high cell density in sealed vials conducive to AHLL accumulation. Preliminary tests detected abiotic NOX accumulation in the headspace above sterile nitrite-containing growth medium, most likely due to the aqueous chemical decomposition of protonated NOX (nitrous acid [HNO2 also known as HONO]) to NO and NO2, collectively referred to as NOX gases (31). Therefore, we included both heat-killed cells and sterile medium controls along with our QS-proficient and -deficient treatments in the experiments. NOX gas measurements were made from such suspensions of concentrated QS-proficient or -deficient *N. winogradskyi* cells during 24 h of NOX oxidation. We predicted that an increase in expression of the *nirK* cluster genes under QS-proficient conditions would either increase NOX production as earlier studies have suggested (32–34) or decrease NOX production by consuming NO as previously reported (34–36).

A statistically significant (*P < 2 × 10^-16*) accumulation of NOX (measured as parts per billion [ppb] by volume) was registered for both QS-proficient and -deficient treatments (approximately 1,699 and 1,240 ppb, respectively) after 2 h of incubation, compared to the accumulations seen with medium alone and with heat-killed cell controls (approximately 87 and 213 ppb, respectively) (Fig. 3A). The peak NOX accumulation in both the QS-proficient and -deficient treatments was transient and was followed by the disappearance of NOX as NO2 was consumed by...
N. winogradskyi (Fig. 3). In contrast, heat-killed cells and medium-alone controls slowly accumulated NOx in the headspace over time and NO2/HN02 concentrations did not change significantly (Fig. 3). The pH of N. winogradskyi cultures and controls did not change significantly during the experiments (data not shown). Although these data suggest that some abiotic NOx accumulation occurred in the controls without N. winogradskyi cells, transient NOx production occurring during active NO2/HN02 oxidation by N. winogradskyi was significantly greater than that seen with the controls. QS-proficient cells both produced and consumed NOx at significantly greater rates ($P < 0.003$) than AiiA-treated, QS-deficient cells (Fig. 3A). Addition of heat-denatured AiiA to either growth medium or heat-killed cell controls did not affect the rate of NOx accumulation or consumption (data not shown). QS-proficient cells produced approximately 756 ppb NOx h−1 during the initial 2 h of the experiment, while QS-deficient cells produced 514 ppb NOx h−1 (Fig. 3A). Between h 4 and h 8, the net levels of consumption of NOx by QS-proficient and QS-deficient cells were 262 ppb h−1 and 191 ppb h−1, respectively (Fig. 3A).

**QS inhibition increases N2O production by N. winogradskyi.** Following a previous unsubstantiated report of N2O production by Nitrobacter species (29), we measured N2O accumulation by both QS-proficient and -deficient concentrated N. winogradskyi cells after 24 h of NO2/HN02 oxidation. Despite the absence of a known nitric oxide reductase gene (nor) in the N. winogradskyi genome, N. winogradskyi cells accumulated significantly ($P < 0.002$) more N2O than either growth medium alone or heat-killed cell controls (Fig. 4). AiiA-treated, QS-deficient cells accumulated approxi-

| Gene category or number(s) | Gene name(s) | Description or role | Fold change$^a$
|---------------------------|--------------|---------------------|------------------|
| Quorum sensing            |              |                     |                  |
| Nwi0283, Nwi0284, Nwi0403, Nwi0586, Nwi2890 | metH, metW | Methionine biosynthesis | 1.5 to 7.7       |
| Nwi0626                   | nwil         | Autoinducer synthase | 2.5              |
| Nwi0827                   | nwiR         | AHL-binding LuxR    | 1.3              |
| Biosynthetic metabolism   |              |                     |                  |
| Nwi0719, Nwi0720          | nirBD        | Assimilatory nitrite reductase | −2.5 to −9.3 |
| Nitrogen metabolism       |              |                     |                  |
| Nwi2648                   | nirK         | Putative nitrite reductase, NO production/consumption | 2.2 |
| Nwi2653–Nwi2649           | ncgABC       | nirK cluster genes, NO production/consumption | 2.7 to 19.9 |
| NO and/or guanine nucleotide signaling |          |                     |                  |
| Nwi0500                   |              |                     |                  |
| Nwi0529, Nwi0597–Nwi0599, Nwi1111, Nwi1121–Nwi1124, Nwi1130, Nwi1132–Nwi1134 | fltA, fltH, fltG, fltE, fltM, fltP, fltB, fltC, fltE | Diguanylate cyclase/phosphodiesterase | 3.7 |
| Nwi0557                   | nrrS         | NO-related gene product | 8.1 |
| Nwi1922                   |              | RelA/SpoT homolog | 2.3 |
| Nwi2061                   |              | Crp domain regulator | 8.9 |
| Nwi2151                   |              | Ppx/GppA phosphatase | 3.2 |

$^a$ Fold change data correspond to the difference in mRNA transcript levels between AiiA-treated QS-deficient cells and QS-proficient cells ($P \leq 0.05, n = 4$).

**FIG 2** Clusters of orthologous group (COG) assignments of gene expression under QS-proficient conditions. Bars indicate the number of genes with increased expression (black) or the number of genes with decreased expression (gray) under QS-proficient conditions for each functional group. A quantity of 100 genes corresponds to 3.2% of COG assignments in the genome. In total, 56.3% of the COG assignments changed in expression level. Expression changes correspond to the difference in mRNA transcript levels between AiiA-treated QS-deficient cells and QS-proficient cells ($P \leq 0.05$).
Quorum Sensing Regulates NOx Fluxes in *N. winogradskyi*

**DISCUSSION**

Quorum quenching transcriptomics is a novel technique to identify QS-controlled genes and phenotypes. We used a quorum quenching transcriptomic technique to investigate the role of QS in *N. winogradskyi*, a nitrite-oxidizing bacterium that participates in the nitrogen cycle. Our experiments identified a QS-controlled phenotype and revealed a link between QS and NOx metabolism in a nitrifying microorganism. While mutational analysis is the preferred method to study QS, there are many bacteria, including those of environmental importance, with no established genetic system and new techniques are needed to determine the purpose of QS in these organisms. Quorum quenching also represents a different methodology to study animal pathogens and social evolution (20, 22–24). Mutant construction in most bacteria takes several generations of selection that may introduce unintended changes, particularly during social-evolution experiments. As previously suggested, quorum quenching through addition of a lactonase or chemical inhibitor is a quicker method to induce a QS-deficient phenotype without the need for several generations of selection and the possibility of pleiotropic effects (23, 24, 37).

**NOx metabolism in *N. winogradskyi***. NO production and consumption in *N. winogradskyi* have generated considerable interest and confusion for almost 30 years. Earlier work by Freitag et al. and Freitag and Bock suggested that *Nitrobacter* strains consume NO to generate NADH and produce N2O under various conditions (29, 35). Starkenburg et al. published the sequenced genome of *N. winogradskyi* and noted that it lacked the gene(s) known to produce N2O but possessed a putative NO-producing gene, *nirK*, and *ncgABC*, closely related to homologs in *N. europaea* (6). However, later work by Starkenburg et al. could not confirm production of NO by *N. winogradskyi* when *nirK* was expressed but did show that *N. winogradskyi* was able to consume NO (36). Coculture studies of the AOB *Nitrosomonas europaea* and *N. winogradskyi* suggest that *N. winogradskyi* can consume NO as less NO accumulated during coculture but expression of *nirK* in *N. winogradskyi* increased (25, 38). Finally, the studies described above and work on other NOB and AOB were incorporated into a multispecies metabolic model to assess sources and sinks of NO in relation to N2O production during wastewater treatment (34). The model predicts that NOB likely oxidize NO to NO3− but do not substantially contribute to N2O production (34).

Our quorum quenching transcriptomics approach led us to investigate production and consumption of NOx gases in *N. winogradskyi* and showed that *N. winogradskyi* may function as a source and/or a sink of NOx and N2O. We hypothesized that if increased expression of *nirK* and associated genes under QS-proficient conditions increased the concentration of NOx gases, ultimately 525 ppb N2O, 1.68-fold more N2O than QS-proficient cells (313 ppb N2O) (*P* < 0.0001) (Fig. 4). In addition, the molar ratio of NO2-N at 2 h to normalized N2O-N accumulated at 24 h (after all NOx was consumed) was considerably higher (6.90 NO-N/N2O-N ratio) than that seen under QS-deficient conditions (2.78 NO-N/N2O-N ratio). These data suggest that QS also affects N2O production by *N. winogradskyi*, likely through changes in NOx fluxes.

![FIG 3](image1.png) **FIG 3** QS-dependent production and consumption of NOx gases in *N. winogradskyi*. Closed circles (QS−) indicate AiiA lactonase-treated (QS-deficient) cells, closed triangles (QS+) indicate untreated cells (QS-proficient), open squares (killed) indicate heat-inactivated cell controls, and open diamonds (medium) indicate sterile medium controls. All measurements were made over 24 h (*x* axis). (A) Values correspond to NOx gases accumulated in the headspace measured as parts per billion above atmospheric N2O (ppb; *y* axis). (B) Values show NOx− concentrations (millimolar; *y* axis) in solution. Values are the means of the results of four independent biological replicates. Error bars indicate the standard deviations of the means.

![FIG 4](image2.png) **FIG 4** QS-dependent production of N2O by *N. winogradskyi*. N2O accumulation in headspace is shown as parts per billion above atmospheric N2O (ppb; *y* axis). The dark line (medium) indicates medium controls, the light gray bar (killed) indicates heat-inactivated cell controls, the black bar (QS+) indicates untreated QS-proficient cells, and the white bar (QS−) indicates AiiA lactonase-treated QS-deficient cells. Values are the means of the results of four independent biological replicates. Error bars indicate the standard deviations of the means. Different letters represent significant differences between treatments determined by a one-way analysis of variance (*P* < 0.0001, *n* = 4).
then more NO would be available for abiological or nonspecific mechanism-based reduction to N2. Contrary to our prediction, QS-deficient cells produced significantly more N2 than QS-proficient cells. A closer inspection of the molar ratio of peak NOx-N and N2O-N produced shows that QS-proficient cells not only produced more NOx than QS-deficient cells but also directed considerably less of the NOx to the N2O pool than QS-deficient cells. These data possibly suggest consumption of NOx via oxidation back to nitrite and, subsequently, nitrate, as previously suggested (36), or that there are alternate fates for NO such as unspecified signaling roles.

Note that N. winogradskyi produced considerably less N2O than NOx, as previously predicted (34). N. winogradskyi does not contain a known nitric oxide reductase (nor), and, while our data show that some of the N2O formation is dependent on live cells, there may be abiotic reactions of NO with cellular components as has been recently suggested during ammonia oxidation by Thau- nurea (39). This hypothesis may partially explain why QS-deficient cells produce more N2O, since their consumption of NOx is slower than that by QS-proficient cells.

Considering the close homologies between nirK and nrgABC of N. winogradskyi and N. europaea, the two gene clusters may serve similar purposes. In N. europaea, both nirK and nrgABC were shown to confer tolerance to NOx−; however, nirK had a negative fitness effect in nrgABC mutants (40, 41). Our observation of an up to 19.9-fold increase in nrgABC transcripts (Table 1; see also Table S1 and Dataset S1 in the supplemental material) under QS-proficient conditions suggests that these genes may play a role in NOx consumption or detoxification in N. winogradskyi, but future work is needed to investigate this prediction.

One interesting observation arising from our work on NOx flux is the abiotic generation of NOx from NO2−, likely via chemical decomposition of aqueous HNO2 or of its gaseous analog, HONO. Although this phenomenon was studied extensively in the past (31), reviewed in the nitrification and engineering fields (33), and recently appreciated in soils (42, 43), most studies in the nitrification and environmental engineering fields have still largely ignored it. Many studies on nitrifying microorganisms, particularly NH3 oxidizers, routinely measure NO but do not account for NOx generated abiotically from the NO2− end product of NH3 oxidation. In addition, many metabolic modeling studies, including an important study cited here modeling NO and N2O turnover (34), have not included abiotic formation of NOx as a significant source. Clearly, future models and studies need to consider the contribution of abiotic reactions to NOx production.

Why does QS regulate NOx metabolism? The immediate rapid generation of NO by concentrated N. winogradskyi cells is an initially puzzling response for a NO2− oxidizer, since reductant would be required to reduce NO2− to NO. However, metabolic modeling of electron flow in Nitrobacter has suggested that generation and consumption of NO would help explain previous experimental data (32). Some support for QS regulation of this metabolic response recently emerged, as Nitrobacter accumulated fewer AHLs under mixotrophic than under autotrophic growth conditions, perhaps also suggesting less generation of NO when organic carbon is available (27). Another possible explanation is the model proposed by Starkenburg et al. that suggests that NO production is a strategy to reversibly block the terminal cytochrome oxidase and redirect electrons toward generation of reductant (36). This hypothesis would make sense if one role for QS is to promote redirection of electrons away from reductive cellular biosynthesis and toward generation of electron-rich storage compounds such as poly-β-hydroxybutyrate as previously reported (6).

In other bacteria, QS generally controls production of a public good that can be used by the entire population (1). According to previous reports that Nitrobacter generates NADH from NO oxidation (35), an increase in NO generation at higher cell densities (QS-proficient conditions) may function as a public good for energy generation. NO generated in a large population of Nitrobacter is more likely to be utilized by nearby Nitrobacter cells and may benefit the population.

Another possible function for QS regulation of the nirK cluster and other genes could be preparation for stationary phase, partially through NO signaling. Signal integration of QS and stress responses has been previously demonstrated in Pseudomonas aeruginosa and other bacterial species (3). Transcriptome data suggest that QS-proficient conditions both prepare for growth arrest via repression of assimilatory nitrate reductase nirBD and induction of the stringent response and promote transition from a motile to sessile state through inhibition of flagellar expression and possible guanine secondary messenger signaling (Table 1). Many of these and other changes in transcription could be indirect effects of NO signaling. According to transcriptome data, QS activation triggers transcription of a putative nrrS homolog (Table 1). While the role of nrrS in N. winogradskyi is unknown, this gene was previously shown to be transcriptionally induced during exposure to NO and to regulate chemotaxis in Rhodobacter sphaeroides (44, 45). NrrS has recently been proposed to function as an NO sensor (46), and we speculate that it may serve a similar role in N. winogradskyi. NO signaling would also be a convenient way to detect nearby AOB, since some NO is produced via NH3 oxidation.

The identification of QS regulation of NOx metabolism in N. winogradskyi raises questions about NOx metabolism in other NOB. A cursory search of genomic databases shows that all NOB and comammox bacteria contain nirK homologs and that all except Nitroccus and Nitrolancea contain nrrS homologs but that only Nitrobacter species contain both nrgABC and correctly annotated autoinducer synthase and receptor genes associated with QS. Since Nitrobacter species are r-strategists with the ability to exploit higher substrate concentrations and sporadically grow to higher densities, they may make better use of cell-density-dependent QS genetic regulation (10, 47). In addition, as r-strategists, Nitrobacter species might use QS-controlled preparation for starvation as an important strategy to recognize transitions to an unfavorable energy-limited situation (48). Future research into nirK function in NOB is needed to confirm the role of nirK and nrrS in NOx fluxes and to determine if NO signaling occurs in these microorganisms.

MATERIALS AND METHODS

Chemicals. N-Decanoyl-m-homoserine lactone (C10-HSL) was purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid and high-performance-liquid-chromatography (HPLC)-grade ethyl acetate were purchased from VWR International (Radnor, PA) and EMD Chemicals (Darmstadt, Germany), respectively.

Bacterial strains, plasmids, and growth medium. Bacterial strains and plasmids used in this study are outlined in Table S3 in the supplemental material. N. winogradskyi was routinely cultivated in 60 mM NaNO3-supplemented mineral salts medium as described previously (26), with minor modifications for NO3 and N2O measurements (see Text S1 in the
supplemental material and the descriptions of AiiA QS inhibition and culturing experiments below). *N. winogradskyi* cultures were routinely screened for heterotrophic contamination by plating 200-μl aliquots of culture on Luria-Bertani (LB) agar plates. *Agrobacterium tumefaciens* was prepared and cultivated as described elsewhere (49, 50). *Escherichia coli* strains were grown in LB medium on a rotary shaker at 200 rpm and 37°C.

AiiA lactonase production and activity measurement. Plasmid pDSK519 carrying aiiA was kindly provided by Max Teplitzki and Mengsheng Gao of the University of Florida. The aiiA gene was cloned, and AiiA was expressed and purified as outlined in Text S1 in the supplemental material. AiiA-specific activity units were determined by measuring reductions of AHL concentrations after 4 hours as outlined in Text S1 in the supplemental material.

AHL bioassay. AHLS were extracted from *N. winogradskyi* cultures and quantified in Miller units by broad-range *Agrobacterium tumefaciens* bioassay as described previously (10, 49, 50). AHL concentrations (nanomolar) were estimated using standard concentrations of C10-HSL (see Fig. S2 in the supplemental material). For determining AiiA activity, 200 μl of the assay solution was directly added to *A. tumefaciens* culture as described previously (49, 50).

AiiA QS inhibition and culturing experiments. Batch cultures of *N. winogradskyi* were prepared in 100 ml 60 mM NO₂⁻-supplemented medium at pH 7.5 as outlined above, inoculated to an optical density at 600 nm (OD₆₀₀) of 0.001 from mid-exponential-phase cultures, and cultured experiments below).

Analytical methods. NO₃⁻ levels were determined by chemical assay as described previously (51). NOₓ levels were measured using a portable NOₓ detector (LMA-3D and LNC-3D; Unisearch Associates Ltd., Rockford, IL).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01753-16/-/DCSupplemental.

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**REFERENCES**

1. Schuster M, Sexton DJ, Diggle SP, Greenberg EP. 2013. Acyl-homoserine lactone quorum sensing: from evolution to application. Annu Rev Microbiol 67:3–63. [http://dx.doi.org/10.1146/annurev-micro-092412-155635](http://dx.doi.org/10.1146/annurev-micro-092412-155635).

2. Hense BA, Schuster M. 2015. Core principles of bacterial autoinducer systems. Microbiol Mol Biol Rev 79:153–169. [http://dx.doi.org/10.1128/MMBR.00024-14](http://dx.doi.org/10.1128/MMBR.00024-14).

3. Mellbye B, Schuster M. 2011. More than just a quorum: integration of stress and other environmental cues in acyl-homoserine lactone signaling.
networks. In Storz G, Hengge R (ed), Bacterial stress responses, 2nd ed. ASM Press, Washington, DC.

4. Mellbye B, Schuster M. 2014. Physiological framework for the regulation of quorum sensing-dependent public goods in *Pseudomonas aeruginosa*. J Bacteriol 196:1155–1164. http://dx.doi.org/10.1128/JB.01223-13.

5. Gutierrez A, Pezzotti M, Pellitteri MG, Hickey WJ. 2005. Identification of acyl-homoserine lactone signal molecules produced by *Nitrosomonas europaea* strain Schmidt. Appl Environ Microbiol 71:4906–4909. http://dx.doi.org/10.1128/AEM.71.8.4906-4909.2005.

6. Starkenburg SR, Chain PS, Sayavedra-Soto LA, Hauser L, Land ML, Larimer FW, Malfatti SA, Klotz MG, Bottomley PJ, Arb D, Ward B, Bottomley PJ. 2008. Complete genome sequence of *Nitrobacter hamburgensis* X14 and comparative genomic analysis of species within the genus *Nitrobacter*. Appl Environ Microbiol 74:2852–2863. http://dx.doi.org/10.1128/AEM.01311-07.

7. Nasuno E, Kimura N, Fujita MJ, Nakatsu CH, Kamagata Y, Hanada S. 2012. Phylogenetically novel LuxI/LuxR-type quorum sensing systems isolated using a metagenomic approach. Appl Environ Microbiol 78: 8067–8074. http://dx.doi.org/10.1128/AEM.01442-12.

8. Gao J, Ma A, Zhuang X, Zhuang G. 2014. An N-acyl homoserine lactone synthase in the ammonia-oxidizing bacterium *Nitrosospira multiformis*. Appl Environ Microbiol 80:951–958. http://dx.doi.org/10.1128/AEM.03636-13.

9. Mellbye BL, Bottomley PJ, Sayavedra-Soto LA. 2015. Nitrite-oxidizing bacterium *Nitrobacter winogradskyi* produces N-acyl-homoserine lactone autoinducers. Appl Environ Microbiol 81:5917–5926. http://dx.doi.org/10.1128/AEM.01103-15.

10. Ward BB. 2011. Nitrite in the environment: an introduction and overview of the state of the field, p 3–8. In Ward BB, Arb D, Klotz MG (ed), Nitrification. ASM Press, Washington, DC.

11. Sayavedra-Soto LA, Arb D. 2011. Ammonia-oxidizing Bacteria: their biochemistry and molecular biology, p 11–37. In Ward BB, Arb D, Klotz MG (ed), Nitrification. ASM Press, Washington, DC.

12. Starkenburg SR, Speieck E, Bottomley PJ. 2011. Metabolism and genomics of nitrite-oxidizing *Bacteria*; emphasis on studies of pure cultures and of *Nitrobacter* species, p 267–293. In Ward BB, Arb D, Klotz MG (ed), Nitrification. ASM Press, Washington, DC.

13. Daims H, Lederle EV, Pjevac P, Han P, Herbold C, Albertsen M, Jehmlich N, Palatinszky M, Vierheilig I, Bulaev A, Kirkegaard RH, von Bergen M, Ratti E, Bendinger B, Nielsen PH, Wagner M. 2015. Complete nitrification by *Nitrobacter* bacteria. Nature 528:504–509. http://dx.doi.org/10.1038/nature16461.

14. Van Kessel MA, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJ, de Vries BM, Rattei T, Bendinger B, Nielsen PH, Wagner M. 2015. Identification of quorum-sensing mutants. *Infect Immun* 83:5036–5042. http://dx.doi.org/10.1128/IAI.00671-15.

15. Freitag A, Rudert M, Bock E. 1987. Growth of *Nitrobacter* by dissipatoric nitrate reduction. FEMS Microbiol Lett 48:105–109. http://dx.doi.org/10.1128/AEM.71.8.4906-4909.2005.

16. Mellbye et al. 2016. Complete cluster genes in *Nitrobacter* species, p 267–293. In Ward BB, Arb D, Klotz MG (ed), Nitrification. ASM Press, Washington, DC.

17. Barrett AL, Taylor TA, Beatty JT, Greenberg EP. 2002. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. J Bacteriol 184:6515–6521. http://dx.doi.org/10.1128/JB.184.18.6515-6521.2002.

18. Rebes A, Rudert M, Bock E. 1987. Growth of *Nitrobacter* by dissipatory nitrate reduction. FEMS Microbiol Lett 48:105–109. http://dx.doi.org/10.1128/AEM.71.8.4906-4909.2005.

19. Carlson JM, Chakravarty A, DeZiel CE, Gross RH. 2007. SCOPe: a web server for practical de novo motif discovery. Nucleic Acids Res 35: W239–W246. http://dx.doi.org/10.1093/nar/gkm310.

20. Park JY, Lee YN. 1988. Solubility and decomposition kinetics of nitrates and nitrite in an aqueous solution. *J Phys Chem* 92:6294–6302. http://dx.doi.org/10.1021/j100333a025.

21. Poughon I, Dussap CG, Gros JB. 2001. Energy model and metabolic flux analysis for autotrophic nitrifiers. Biotechnol Bioeng 72:416–433.

22. Schreiber F, Wunderlin P, Udart KM, Wells GF. 2012. Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. Front Microbiol 3:372. http://dx.doi.org/10.3389/fmicb.2012.00372.

23. Perez-Garcia O, Chandran K, Villas-Boas SG, Singhal N. 2016. Assessment of nitric oxide (NO) redox reactions contribution to nitrous oxide formation during nitrification using a multispecies metabolic network model. Biotechnol Bioeng 113:1214–1236. http://dx.doi.org/10.1002/bit.25880.

24. Daims B, Bock E. 1990. Energy conservation in *Nitrobacter*. *FEMS Microbiol Lett* 66:157–162. http://dx.doi.org/10.1111/j.1574-6968.1990.tb03989.x.

25. Feltner JB, Wolter DJ, Pope CE, Groleau M, Smalley NE, Greenberg EP. 2006. Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255. *Appl Environ Microbiol* 72:1155–1164. http://dx.doi.org/10.1128/AEM.01223-13.

26. Freitag A, Rudert M, Bock E. 1987. Growth of *Nitrobacter* by dissipatory nitrate reduction. FEMS Microbiol Lett 48:105–109. http://dx.doi.org/10.1128/AEM.71.8.4906-4909.2005.

27. Welsh MA, Blackwell HE. 2016. Chemical probes of quorum sensing: from compound development to biological discovery. *FEMS Microbiol Rev* 40:774–794. http://dx.doi.org/10.1093/femsre/fuw009.

28. Kester RA, De Boer W, Laanbroek HJ. 1997. Production of NO and N2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration. Appl Environ Microbiol 63:3872–3877.

29. Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY. 2016. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarcheota. *ISME J* 10:1836–1845. http://dx.doi.org/10.1038/ismej.2016.2.

30. Beauumont HJ, Lens SI, Reijnders WN, Westerhoff HV, van Spanning RJ. 2004. Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrK, a novel nitrite-sensitive transcription repressor. *Mol Microbiol* 54:356–357. http://dx.doi.org/10.1111/j.1365-2958.2004.04248.x.

31. Beauumont HJ, Lens SI, Westerhoff HV, van Spanning RJ. 2005. Novel nirK cluster genes in *Nitrosomomas europaea* are required for NirK-dependent tolerance to nitrite. *J Bacteriol* 187:6849–6851. http://dx.doi.org/10.1128/JB.187.19.6849-6851.2005.

32. Su H, Cheng Y, Oswald R, Behrendt T, Trebs I, Meixner FX, Andreade MO, Cheng P, Zhang Y, Pöschl U. 2011. Soil nitrite as a source of...
atmospheric HONO and OH radicals. Science 333:1616–1618. http://dx.doi.org/10.1126/science.1207687.

43. Oswald R, Behrendt T, Ermel M, Wu D, Su H, Cheng Y, Breuninger C, Moravek A, Mougin E, Delon C, Loubet B, Pomerening-Rösler A, Sörgel M, Pöschl U, Hoffmann T, Andreass MO, Meixner FX, Trebs I. 2013. HONO emissions from soil bacteria as a major source of atmospheric reactive nitrogen. Science 341:1233–1235. http://dx.doi.org/10.1126/science.1242266.

44. Kwiatkowski AV, Laratta WP, Toffanin A, Shapleigh JP. 1997. Analysis of the role of the nmrR gene product in the response of Rhodobacter sphaeroides 2.4.1 to exogenous nitric oxide. J Bacteriol 179:5618–5620.

45. Bartnikas TB, Wang Y, Bobo T, Veselov A, Scholes CP, Shapleigh JP. 2002. Characterization of a member of the NnrR regulon in Rhodobacter sphaeroides 2.4.3 encoding a haem-copper protein. Microbiology 148:825–833. http://dx.doi.org/10.1099/00221287-148-3-825.

46. Vaccaro BJ, Thorgersen MP, Lancaster WA, Price MN, Wetmore KM, Poole FL, II, Deutschbauer A, Arkin AP, Adams MW. 2016. Determining roles of accessory genes in denitrification by mutant fitness analyses. Appl Environ Microbiol 82:51–61. http://dx.doi.org/10.1128/AEM.002602-15.

47. Nowka B, Daims H, Spieck E. 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. Appl Environ Microbiol 81:745–753. http://dx.doi.org/10.1128/AEM.02734-14.

48. Andrews JH, Harris RF. 1986. r- and K-selection and microbial ecology. Adv Microb Ecol 9:99–147. http://dx.doi.org/10.1007/978-1-4757-0611-6_3.

49. Zhu J, Chai Y, Zhong Z, Li S, Winans SC. 2003. Agrobacterium bioassay strain for ultrasensitive detection of N-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in Mesorhizobium huakuii. Appl Environ Microbiol 69:6949–6953. http://dx.doi.org/10.1128/AEM.69.11.6949-6953.2003.

50. Joelsson AC, Zhu J. 2006. LacZ-based detection of acyl-homoserine lactone quorum-sensing signals. Curr Protoc Microbiol 1:Unit 1C 2. http://dx.doi.org/10.1002/0470010109.mc101c02s3.

51. Hood-Nowotny R, Umana NH, Inselbacher E, Oswald-Lachouani P, Wanek W. 2010. Alternative methods for measuring inorganic, organic, and total dissolved nitrogen in soil. Soil Sci Soc Am J 74:1018–1027. http://dx.doi.org/10.2136/sssaj2009.0389.

52. Mellbye BL, Giguere A, Chaplen F, Bottomley PJ, Sayavedra-Soto LA. 2016. Steady-state growth under inorganic carbon limitation conditions increases energy consumption for maintenance and enhances nitrous oxide production in Nitrosomonas europaea. Appl Environ Microbiol 82:3310–3318. http://dx.doi.org/10.1128/AEM.00294-16.

53. Robinson MD, Smyth GK. 2008. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics 9:321–332. http://dx.doi.org/10.1093/biostatistics/kxm030.

54. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. http://dx.doi.org/10.1093/bioinformatics/btp166.

55. Erickson HE, Perakis SS. 2014. Soil fluxes of methane, nitrous oxide, and nitric oxide from aggrading forests in coastal Oregon. Soil Biol Biochem 76:268–277. http://dx.doi.org/10.1016/j.soilbio.2014.05.024.