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Acyl-Homoserine Lactone Production in Nitrifying Bacteria of the Genera *Nitrosospira*, *Nitrobacter*, and *Nitrospira* Identified via a Survey of Putative Quorum-Sensing Genes

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ABSTRACT The genomes of many bacteria that participate in nitrogen cycling through the process of nitrification contain putative genes associated with acyl-homoserine lactone (AHL) quorum sensing (QS). AHL QS or bacterial cell-cell signaling is a method of bacterial communication and gene regulation and may be involved in nitrogen oxide fluxes or other important phenotypes in nitrifying bacteria. Here, we carried out a broad survey of AHL production in nitrifying bacteria in three steps. First, we analyzed the evolutionary history of AHL synthase and AHL receptor homologs in sequenced genomes and metagenomes of nitrifying bacteria to identify AHL synthase homologs in ammonia-oxidizing bacteria (AOB) of the genus *Nitrosospira* and nitrite-oxidizing bacteria (NOB) of the genera *Nitrococcus*, *Nitrobacter*, and *Nitrospira*. Next, we screened cultures of both AOB and NOB with uncharacterized AHL synthase genes and AHL synthase-negative nitrifiers by a bioassay. Our results suggest that an AHL synthase gene is required for, but does not guarantee, cell density-dependent AHL production under the conditions tested. Finally, we utilized mass spectrometry to identify the AHLs produced by the AOB *Nitrosospira multiformis* and *Nitrosospira briensis* and the NOB *Nitrobacter vulgaris* and *Nitrospira moscoviensis* as *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-3-hydroxy-tetradecanoyl-L-homoserine lactone (3-OH-C_{14}-HSL), a monounsaturated AHL ($C_{10:1}$-HSL), and *N*-octanoyl-L-homoserine lactone (C_8-HSL), respectively. Our survey expands the list of AHL-producing nitrifiers to include a representative of *Nitrospira* lineage II and suggests that AHL production is widespread in nitrifying bacteria.

IMPORTANCE Nitrification, the aerobic oxidation of ammonia to nitrate via nitrite by nitrifying microorganisms, plays an important role in environmental nitrogen cycling from agricultural fertilization to wastewater treatment. The genomes of many nitrifying bacteria contain genes associated with bacterial cell-cell signaling or quorum sensing (QS). QS is a method of bacterial communication and gene regulation that is well studied in bacterial pathogens, but less is known about QS in environmental systems. Our previous work suggested that QS might be involved in the regulation of nitrogen oxide gas production during nitrite metabolism. This study characterized putative QS signals produced by different genera and species of nitrifiers. Our work lays the foundation for future experiments investigating communication between nitrifying bacteria, the purpose of QS in these microorganisms, and the manipulation of QS during nitrification.

KEYWORDS *Nitrobacter*, *Nitrosospira*, *Nitrospira*, acyl-homoserine lactone, ammonia oxidation, nitrification, nitrite oxidation, quorum sensing

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Nitrification, the aerobic sequential oxidation of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-), has a profound impact on environmental nitrogen (N) cycling (1). In particular, nitrification contributes to N availability for plants, N oxide gas emissions, and NO_2^- and NO_3^- leaching that leads to eutrophication (1, 2). Both the rate of nitrification and the coupling of NH_3 oxidation to NO_2^- oxidation may influence the above-mentioned processes. During nitrification, high NH_3 oxidation rates are associated with the increased production of nitric oxide (NO) and the greenhouse gas nitrous oxide (N_2O), collectively called N oxide gases (3–5). The transient accumulation of NO_2^- is also associated with N oxide emissions and potential toxic effects (6–9). Finally, the accumulation of NO_3^- often leads to leaching and eutrophication (2). A greater understanding of the nitrification process is important for efficient fertilization of agricultural crops, wastewater treatment, and control of N oxide emissions.

Diverse microorganisms participate in nitrification. NH_3 oxidation is generally carried out by NH_3-oxidizing bacteria (AOB) and NH_3-oxidizing archaea (AOA), while NO_2^--oxidizing bacteria (NOB) carry out nitrite oxidation (1, 10–13). In addition, complete ammonia oxidation to nitrate (comammox) was discovered in *Nitrospira* lineage II, a subset of the NOB (14, 15). Genome sequencing of many nitrifying bacteria revealed genes associated with bacterial cell-cell signaling or quorum sensing (QS), via *N*-acyl-homoserine lactone (AHL) chemical signals (also referred to as autoinducers) (14–21). The role of AHLs in nitrifying bacteria is largely unknown, but a quorum-quenching transcriptome-sequencing (mRNA-Seq) study on the NOB *Nitrobacter winogradskyi* suggested that AHL QS regulates N oxide fluxes during the metabolism of NO_2^- (22).

Bacterial QS is a widespread process that uses diffusible chemical signals to coordinate gene expression in response to cell density, diffusion dynamics, and spatial distribution (23–25). QS through the production of AHLs, the best-studied chemical signal or autoinducer, can control a variety of different cooperative and stress-associated phenotypes, such as exoenzyme secretion, exopolysaccharide production, biofilm formation, luminescence, conjugation, and adaptation to starvation (25–27). AHL QS generally employs a LuxI homolog autoinducer synthase and a LuxR homolog signal receptor/transcription factor (24). The continuous basal expression of the autoinducer synthase produces AHLs that initially diffuse or are transported out of the cell. When a critical concentration is reached, AHLs make their way back into the cell and are bound by the signal receptor. The receptor/transcription factor activates a variety of genes, generally including the autoinducer synthase. This process often creates a feed-forward loop that coordinates gene expression in whole populations of cells (25).

AHL production has been demonstrated for only a few nitrifying bacteria. In the AOB *Nitrosomonas europaea*, AHLs were detected in the culture supernatant, but its genome lacks any known AHL synthase or AHL receptor homologs (28, 29). The genome of the AOB *Nitrosospira multiformis* contains putative QS genes, and AHLs were previously identified by heterologous expression, but that study was unable to detect AHLs in pure cultures (17, 30). The NOB *Nb. winogradskyi* was shown to produce AHLs and express a QS-controlled phenotype in previous studies (22, 31). A subsequent study identified the same AHLs in *Nb. winogradskyi* and observed lower concentrations of AHLs during mixotrophic growth (32). In addition, a metagenomic clone of an AHL synthase potentially from the phylum *Nitrospirae*, which includes NOB and comammox bacteria, produced AHLs (33). Due to the previous discrepancies between genomic and phenotype evidence, some confusion remains about which genes are responsible for AHL production and how ubiquitous the production of AHLs is in nitrifying bacteria.

In this study, we investigated putative AHL QS genes and AHL production in diverse nitrifying bacteria through genomic analyses and screening of pure cultures. We thoroughly screened genomic databases for AHL autoinducer synthase LuxI homologs and autoinducer receptor/transcription factor LuxR homologs in nitrifying bacteria and analyzed the evolutionary history of LuxI and LuxR homologs in nitrifiers. Next, we addressed the discrepancy between genomic and phenotypic evidence by screening pure cultures of nitrifying bacteria both with and without putative QS genes. Finally, we

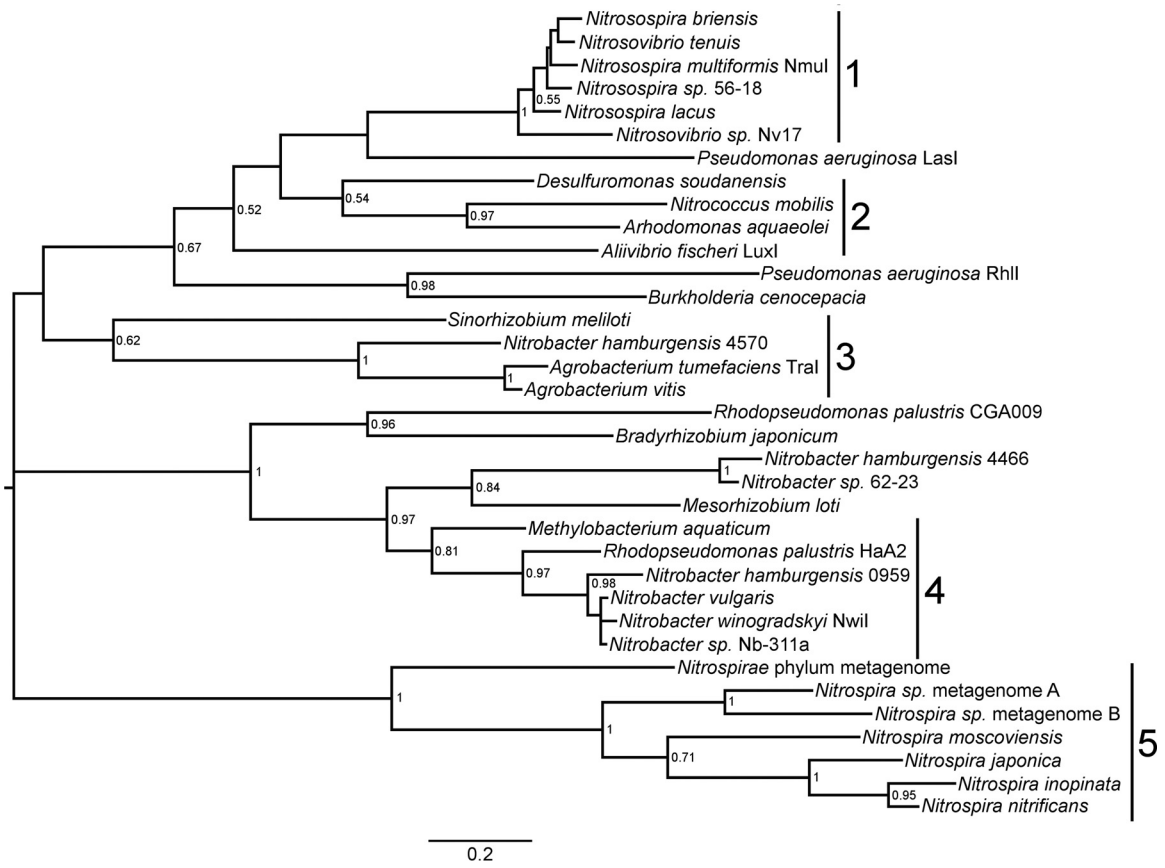

FIG 1 Phylogenetic tree of selected LuxI homologs from nitrifiers and other bacteria. The bar indicates the mean number of substitutions per site. Bootstrap values from 500 resamplings are shown only for nodes with values of 0.5 or higher. Numbered bars indicate clades 1 through 5, as described in Results. The protein name or gene number of the LuxI homolog follows the genus, species, and strain designation, if applicable.

identified AHLs produced by species of the genera *Nitrosospira*, *Nitrobacter*, and *Nitrospira* by ultraperformance liquid chromatography-mass spectrometry (UPLC-MS).

RESULTS

AHL QS genes form distinct clades in AOB, NOB, and comammox bacteria. In order to identify putative genes associated with AHL QS, *Nb. winogradskyi* LuxI/R homologs (NwiI/R) were used to perform a comprehensive BLAST search of nitrifier genomes in the KEGG and NCBI databases. This search identified both novel putative QS genes and previously characterized genes of the genera *Nitrosospira*, *Nitrococcus*, *Nitrobacter*, and *Nitrospira* (see Data Set S1 in the supplemental material). The identification of QS gene homologs in the genus *Nitrococcus* and in *Nitrospira* lineage II was particularly interesting, as AHL QS genes had not been reported previously for these genera in the literature. The amino acid sequences of the identified QS genes were aligned with MUSCLE, and MEGA was used to construct a phylogenetic tree of LuxI and LuxR homologs identified in nitrifying bacteria along with several well-known QS-proficient bacteria (Fig. 1, Fig. S1, and Data Set S1). Phylogenetic analysis of LuxI autoinducer synthase homolog sequences showed better overall bootstrap support than that for LuxR homolog sequences, so we focused our analysis on autoinducer synthase genes (Fig. 1 and Fig. S1). As previously shown for other QS-proficient bacteria, the evolutionary history of LuxI and LuxR homologs in nitrifiers loosely resembles the 16S rRNA phylogeny, suggesting an ancient origin of AHL QS genes in these bacteria (34).

The autoinducer synthase genes identified in nitrifying bacteria formed five distinct clades with important distinguishing features (Fig. 1). Clade 1 included members of the

TABLE 1 Bacterial strains and plasmids

Genus and species	Type of bacterium[a]	QS[b]	Strain designation	Reference(s)
Nitrosospira multiformis	AOB	+/+	ATCC 25196^T	17
Nitrosospira briensis	AOB	+/+	C-128	20
Nitrosomonas europaea	AOB	−/−	ATCC 19718	29
Nitrosomonas eutropha	AOB	−/−	C91	62
Nitrosococcus oceani	AOB	−/−	ATCC 19707	63
Nitrobacter hamburgensis	NOB	+/−	X14	18, 64
Nitrobacter vulgaris	NOB	+/+	AB_1	21, 65
Nitrococcus mobilis	NOB	+/−	Nb−231	48
Nitrospina gracilis	NOB	−/−	3/211	48, 66
Nitrospira defluvii	NOB	−/−	A17	40, 67
Nitrospira moscoviensis	NOB	+/+	M1	19, 41
Agrobacterium tumefaciens		−/−	KYC55(pJZ372)(pJZ384)(pJZ410)	36, 54

[a]Ammonia-oxidizing bacterium (AOB) or nitrite-oxidizing bacterium (NOB).

[b]Data indicate whether the species genome contains a putative AHL synthase (LuxI homolog) and a putative AHL receptor (LuxR homolog) and if AHLs were detected in pure cultures by a bioassay, respectively. For example, +/+ indicates that the genome contains QS genes and that AHLs were detected.

genera *Nitrosospira* and *Nitrosovibrio* and was the only clade that included AOB with putative AHL QS genes (Fig. 1). Clade 1 LuxI homologs showed on average 28 to 30% identity to *Nb. winogradskyi* NwiI. LuxI/R homologs were previously identified in *Nss. multiformis* but have not been characterized in other AOB (30). Clade 2 contained only one known nitrifying bacterium, the marine NOB *Nitrococcus mobilis* (Fig. 1). In our analysis, the *Nc. mobilis* autoinducer synthase gene grouped with autoinducer synthase genes from QS-proficient halophiles and sulfur-reducing bacteria (*Arhodomonas aquaeolei* and *Desulfuromonas soudanensis*, respectively). The *Nc. mobilis* LuxI homolog was 24% similar to NwiI and has not been characterized previously. Clade 3 contained LuxI homologs associated with the conjugation of the Ti plasmid commonly associated with *Agrobacterium tumefaciens* (35) (Fig. 1). The genome of *Nitrobacter hamburgensis* contains three plasmids possessing some conjugation genes similar to those on the Ti plasmid and one autoinducer synthase belonging to clade 3, showing 28% identity to NwiI (18). Clade 4 included all known *Nitrobacter* species, including *Nb. winogradskyi*, *Nb. hamburgensis*, and *Nb. vulgaris*, and also includes metabolically diverse members of the *Rhizobiales*, such as *Rhodopseudomonas palustris* (Fig. 1). Although the LuxI homologs from these NOB showed 86 to 100% identity to NwiI, two autoinducer synthase genes from a *Nb. hamburgensis* plasmid and a metagenome formed a small separate clade with only 39 to 44% identity. Finally, clade 5 contained LuxI homologs belonging to the phylum *Nitrospirae* (Fig. 1). Our search identified several autoinducer synthase genes belonging to *Nitrospira* lineage II NOB and the two recently identified complete-ammonia-oxidizing (comammox) bacteria (Fig. 1). These LuxI homologs showed 25 to 28% identity to NwiI, and AHL QS genes have not been reported previously for *Nitrospira*.

Production of AHLs by *Nitrosospira*, *Nitrobacter*, and *Nitrospira* is cell density dependent during batch culturing. Pure cultures of nitrifiers with sequenced genomes and uncharacterized autoinducer synthase genes were selected from each clade to screen for AHL production (Table 1). We also chose to screen the AOB *Nss. multiformis* since a previous characterization study was unable to detect AHLs from pure cultures (30). Because previous research reported AHL production by the NH_3 oxidizer *Nm. europaea*, whose genome does not contain known autoinducer synthase or receptor genes, we also evaluated several nitrifiers with sequenced genomes that do not contain LuxI/R homologs (28) (Table 1).

To initially screen nitrifying pure cultures for AHL production, we used a broad-range, ultrasensitive *A. tumefaciens* bioassay previously shown to detect a wide range of AHLs with diverse acyl chain lengths and saturation and oxidation states (36, 37). As shown in Table 1, AHLs were detected in pure cultures of the NH_3 oxidizers *Nss. multiformis* and *Nitrosospira briensis* and the NO_2^- oxidizers *Nb. vulgaris* and *Nitrospira*

moscoviensis (Fig. 2). Under the batch culture conditions tested, we were unable to detect AHLs from cultures of the NH_3 oxidizers *Nm. europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus oceani* and the NO_2^- oxidizers *Nb. hamburgensis*, *Nc. mobilis*, *Nitrospina gracilis*, and *Nitrospira defluvii* (Table 1). The results of this screen suggested that the presence of AHL synthase genes is required for the production of AHLs, yet the presence of putative AHL synthase and receptor genes does not guarantee the production of detectable AHLs under the applied conditions (Table 1). In all AHL-positive nitrifiers except *Ns. moscoviensis*, AHLs accumulated in batch cultures, and there were statistically significant increases as the optical density at 600 nm (OD_{600}) approached 0.01 (Fig. 2A to C). *Ns. moscoviensis* showed a statistically significant increase in the AHL concentration at an extremely low OD_{600} of 0.005, and AHL concentrations also increased significantly at an OD_{600} of 0.01 (Fig. 2D). Since cell density, measured as the optical density, was often below an OD_{600} of 0.01, we also measured nitrite accumulation and consumption for AOB and NOB, which corresponded to cell density during batch culturing (Fig. 2; see also Fig. S2 in the supplemental material). In addition, there was a statistically significant increase in the amount of AHLs produced per cell, based on the OD_{600}, on days 2, 4, 4, and 10 for *Nm. multiformis*, *Nm. briensis*, *Nb. vulgaris*, and *Ns. moscoviensis*, respectively (Fig. 2). A particularly interesting result was the accumulation of approximately 100-fold-higher relative concentrations of AHL in *Ns. moscoviensis* despite lower cell densities (Fig. 2D). However, all of our bioassay results were normalized to *N*-decanoyl-L-homoserine lactone (C_{10}-HSL) standards. The enhanced detection of AHL in *Ns. moscoviensis* suggested the production of a different AHL that the bioassay was extremely sensitive to, as previously reported (36).

Identification of AHLs by UPLC-MS. AHLs from AHL-positive nitrifiers were extracted from the batch culture supernatant and concentrated for identification by mass spectrometry. UPLC-MS analysis detected a different AHL from each species of nitrifying bacteria (Table 2 and Fig. 3). AHLs were identified based on the presence of a peak at *m/z* 102.055 in the extracted-ion chromatogram (XIC), which corresponds to the protonated lactone moiety. A compound with molecular ion $[M + H]^+$ of *m/z* 256.1869 corresponding to C_{10}-HSL was detected in the *Nss. multiformis* supernatant (Table 2 and Fig. 3A). The *Nss. briensis* supernatant contained a compound with $[M + H]^+$ of *m/z* 328.2456, which corresponded to *N*-3-hydroxy-tetradecanoyl-L-homoserine lactone (3-OH-C_{14}-HSL) (Table 2 and Fig. 3B). A compound with $[M + H]^+$ of *m/z* 254.1719 corresponding to the unsaturated AHL $C_{10:1}$-HSL was identified in the *Nb. vulgaris* supernatant (Table 2 and Fig. 3C). The *Ns. moscoviensis* supernatant contained a compound with $[M + H]^+$ of *m/z* 228.1589, corresponding to *N*-octanoyl-L-homoserine lactone (C_8-HSL) (Table 2 and Fig. 3D).

Further analysis of the XICs of each AHL confirmed the identity of the chemical structures. The *m/z* values listed below are rounded to the nearest whole number. The structures of C_{10}-HSL and $C_{10:1}$-HSL were compared to previously reported XICs with similar fragmentation patterns (31). The XIC for C_{10}-HSL showed characteristic $[M + H - H_2O]^+$, $[M + H - 101]^+$, and $[M + H - 101 - H_2O]^+$ peaks at *m/z* 238, 155, and 137, respectively (Fig. 3A). The XIC of $C_{10:1}$-HSL showed peaks at *m/z* 236, 153, and 135, corresponding to $[M + H - H_2O]^+$, $[M + H - 101]^+$, and $[M + H - 101 - H_2O]^+$, respectively, representing the loss of two H molecules due to the carbon-carbon double bond (C═C bond) (Fig. 3C). The position of the C═C bond in the acyl chain was determined to be between carbon 9 and carbon 10 based on the ion fragmentation model presented in a previous study (38). However, we were unable to clarify the isomeric form of the C═C bond of $C_{10:1}$-HSL. While the XIC for 3-OH-C_{14}-HSL differed from previously reported results, both XICs showed *m/z* 209, corresponding to $[M + H - 101 - H_2O]$, and different MS methods might account for other differences (Fig. 3B) (39). The XIC of the *Ns. moscoviensis* AHL C_8-HSL showed $[M + H - H_2O]^+$, $[M + H - 101]^+$, and $[M + H - 101 - H_2O]^+$ at *m/z* 210, 127, and 109, respectively, matching previously reported results (Fig. 3D) (39). In addition, a previous study showed that the *A. tumefaciens* bioassay used to quantify AHL concentrations is

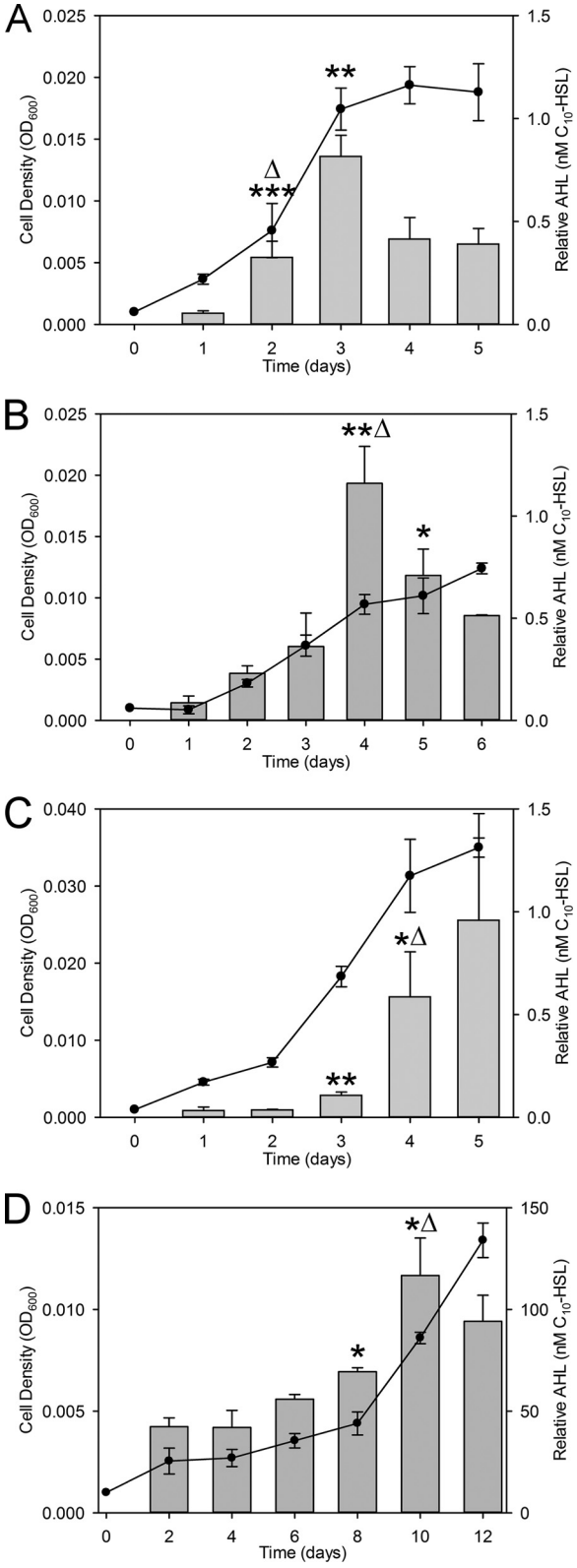

FIG 2 AHL production during growth in batch culture. AHLs were detected by a bioassay during batch culture of the NH_3 oxidizers *Nss. multiformis* (A) and *Nss. briensis* (B) and the NO_2^- oxidizers *Nb. vulgaris* (C) and *Ns. moscoviensis* (D). Circles indicate the cell density (OD_{600}) (left *y* axis), and bars represent the relative AHL concentration detected (equivalent to nanomolar concentrations of C_{10}-HSL) (right *y* axis) over time (days) (*x* axis). Relative AHL concentrations were quantified based on C_{10}-HSL standards reported previously, but actual concentrations vary depending on the AHL being measured (22, 36).

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TABLE 2 Chromatographic and mass spectrometric data for AHLs identified by UPLC-MS

Bacterial species	AHL	Chemical formula	Exact mass (Da)	r_T (min)[a]	$[M + H]^+$ (m/z)[b]	Error (ppm)
Nss. multiformis	C_{10}-HSL	$C_{14}H_{25}NO_3$	255.18344	2.35	256.1869	−3.2
Nss. briensis	3-OH-C_{14}-HSL	$C_{18}H_{32}NO_4$	327.24096	3.02	328.2456	−3.2
Nb. vulgaris	$C_{10:1}$-HSL	$C_{14}H_{23}NO_3$	253.16779	2.15	254.1719	−2.1
Ns. moscoviensis	C_8-HSL	$C_{12}H_{21}NO_3$	227.15214	2.16	228.1589	−4.8

[a]r_T, retention time.
[b]Experimental m/z values of protonated molecules.

particularly sensitive to C_8-HSL, which explains the enhanced detection of AHLs in *Ns. moscoviensis* batch cultures (36).

DISCUSSION

Overview. In this study, genomic databases were searched for AHL synthase and receptor genes to comprehensively screen for the distribution of putative AHL QS genes in nitrifying bacteria. Based on our search and subsequent phylogenetic analyses, we selected 10 nitrifying bacteria, both with and without putative AHL synthase genes, to screen for AHL production during chemolithoautotrophic growth. Our results suggested that the presence of an AHL synthase gene homolog is required for, but does not guarantee, the production of AHLs by nitrifying bacteria under the conditions tested. In particular, we were unable to detect AHLs from pure cultures of *Nm. europaea*, an AOB without an AHL synthase gene, despite previous reports of AHL production, and we were also unable to detect AHLs from pure cultures of *Nc. mobilis* and *Nb. hamburgensis*, NOB that have AHL synthase genes (18, 28). Finally, we identified AHLs produced by *Nss. multiformis*, *Nss. briensis*, *Nb. vulgaris*, and *Ns. moscoviensis* and measured cell density-dependent increases in AHL production during batch culturing. Our results suggest that these nitrifiers may have functional AHL QS systems. Our work confirmed and expanded on previous reports of AHL QS genes and phenotypes for the genera *Nitrosospira* and *Nitrobacter*, identified AHL production in the genus *Nitrospira*, and suggested that AHL QS genes may be widespread in nitrifying bacteria.

Evolution of AHL QS in nitrifying bacteria. Our analysis of the evolutionary history of putative autoinducer genes in nitrifying bacteria has expanded on previous work focused on *Nb. winogradskyi* and QS-proficient *Rhizobiales* (31). Similar to previous work, our analysis identified both a *Nitrobacter*-specific clade (clade 4) and a Ti plasmid-associated clade (clade 3) of autoinducer synthase homologs (31). By expanding our phylogenetic analysis to other genera and species of AOB and NOB, we identified several more clades specific to each group of nitrifiers and their environments (clades 1, 2, and 5). Our analysis suggested an ancient origin of QS genes in nitrifying bacteria, without recent horizontal gene transfer, since the autoinducer synthase gene phylogeny closely resembles the 16S rRNA phylogeny (34).

The discovery of putative AHL synthase and receptor genes within the genus *Nitrospira* is particularly interesting, as AHL QS genes had not been previously characterized for these NOB in the literature. Surprisingly, we found putative QS genes only within *Nitrospira* species belonging to the previously described lineage II that includes both NOB and comammox bacteria (14, 15, 40, 41). Our findings suggest that a fundamentally different selective pressure led to AHL QS genes being only either acquired or maintained in one of the six lineages of *Nitrospira*. Future experiments are needed to determine the purpose of putative AHL QS genes in *Nitrospira* and confirm if other less-studied *Nitrospira* lineages also contain putative AHL QS genes.

FIG 2 Legend (Continued)
Asterisks indicate statistically significant changes in AHL concentrations compared to previous measurements (*, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 5e{-}4$ [versus all previous measurements, as determined by a two-tailed t test]). Delta indicates a statistically significant increase in the amount of AHLs produced per OD_{600} compared to the amount measured the previous day (Δ, $P \leq 0.05$). Values are the means of data from three independent biological replicates. Error bars indicate the standard deviations of the means.

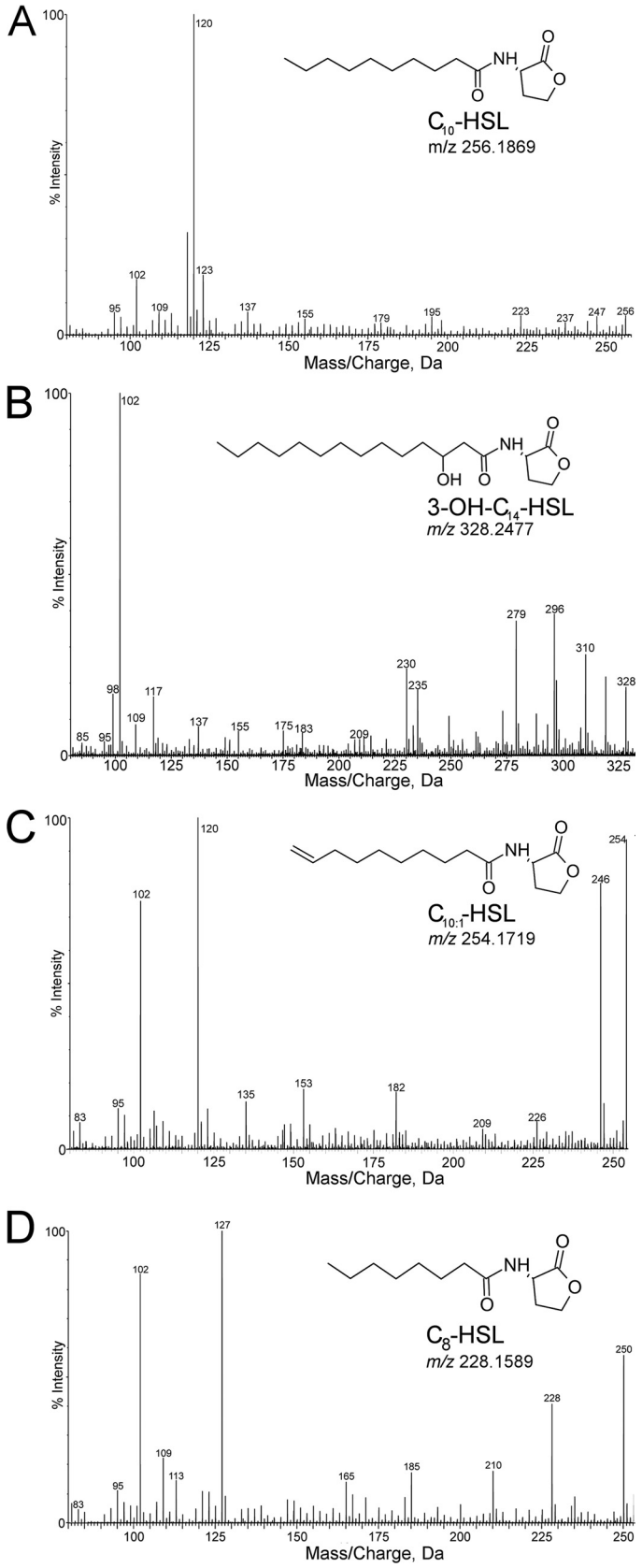

FIG 3 UPLC-MS chromatograms and structures of AHLs produced by NH_3-oxidizing and NO_2^--oxidizing nitrifying bacteria. Shown are extracted-ion chromatogram (XIC) fragmentation pattern spectra for protonated molecules ($[M + H]^+$), chemical structures, and *m/z* of C_{10}-HSL produced by the AOB *Nss*.

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Determination of whether chemical language exists during nitrification. Our survey of AHL QS in nitrifying bacteria forms a foundation for future studies on chemical communication in environmental bacteria. While many studies have focused on model heterotroph bacteria that are amenable to genetic manipulation, many important environmental bacteria, such as nitrifiers, are understudied (24). The goal of this study was to identify which nitrifying bacteria produce AHLs and which AHLs are produced. By screening nitrifiers closely related to species with previously characterized AHL QS genes (*Nitrosospira* and *Nitrobacter* species), our study identified potential themes in the chemical signaling of clades 1 and 4. *Nss. multiformis* and *Nss. briensis* produced drastically different AHLs, suggesting that although their AHL synthases show 88% identity at the amino acid level, their AHL synthases function differently, possibly due to different fatty acid pools within the cell (42). In addition, based on data from previous studies, the *Nss. multiformis* AHL synthase NmuI produced different AHLs in pure culture (C_{10}-HSL) than when the gene was expressed in *Escherichia coli* (C_{14}-HSL and 3-oxo-C_{14}-HSL) (30). On the other hand, *Nb. vulgaris* produced the same unsaturated AHL, $C_{10:1}$-HSL, as previously characterized for *Nb. winogradskyi*, and the *Nb. vulgaris* AHL synthase gene is 94% identical (31). These results suggest the likelihood that there is an amino acid identity threshold for AHL synthases to produce the same AHLs.

A coupled AHL synthase gene and an AHL receptor gene generally suggest a within-species function for QS (43). However, if different species of bacteria produce similar AHLs, we hypothesize that they could also perceive each other's AHLs when growing in close proximity. Previous work suggested that AHLs with similar tail lengths can be identified by noncognate receptor proteins (43). Of the AHL-producing nitrifiers known, AHL cross talk between the AOB *Nss. multiformis* and the NOB *Nb. winogradskyi* and *Nb. vulgaris* may be possible, as they all utilize a similar AHL with a 10-carbon acyl tail and can be found in soil environments (12, 13, 17, 31). In addition, AHL receptor homologs vary in signal specificity, so possible cross talk with a *Nitrospira* species that produces C_8-HSL, similar to *Ns. moscoviensis*, may also be possible (43).

Implications for future studies of AHL QS in environmental and engineered systems. In environmental systems such as soils, AHL QS offers an attractive model for potential chemical communication between AOB and NOB. We are particularly interested in how AOB and ammonia-oxidizing archaea remain coupled with NOB to avoid the accumulation of nitrite in the environment, especially considering the role of nitrite in the production of nitrogen oxide gases (7–9, 44). AHL QS may serve to facilitate nitrification coupling or, alternatively, may contribute or reduce nitrogen oxide gas emissions indirectly. Research has shown that nitrite accumulates transiently in soils under some circumstances, and whether or not there might be a role for AHL QS in the recovery of NO_2^- consumption is unknown (7–9). Indeed, in a previous study involving quorum-quenching mRNA-Seq techniques, the results suggested that AHL QS might play a role in nitrogen oxide emissions from NO_2^- metabolism by the NOB *Nb. winogradskyi* (22).

There is also an opportunity for future studies of the contribution of AHL QS to engineered systems such as wastewater treatment systems. Most wastewater treatment systems depend on biofilm consortiums of heterotrophs and nitrifiers to effectively treat wastewater, and QS can play an important role in biofilm development (45). In addition, previous studies suggested a role for AHL QS in mixed populations carrying out anaerobic ammonium oxidation (anammox) (46, 47). Although there has been recent interest in cycles of AHL production and destruction, via quorum quenching, in wastewater treatment systems, most of those studies focused on heterotrophs (45). Future studies that focus on the potential for AHL QS in nitrifying bacteria are needed.

FIG 3 Legend (Continued)
multiformis (A), 3-OH-C_{14}-HSL produced by the AOB *Nss. briensis* (B), $C_{10:1}$-HSL produced by the NOB *Nb. vulgaris* (C), and C_8-HSL produced by the NOB *Ns. moscoviensis* (D).

MATERIALS AND METHODS

Chemicals. *N*-Decanoyl-DL-homoserine lactone (C_{10}-HSL), analytical-grade acetonitrile, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade ethyl acetate and acetic acid were purchased from EMD Chemicals (Darmstadt, Germany) and VWR International (Radnor, PA), respectively.

Bacterial strains and growth medium. Bacterial strains used in this study are outlined in Table 1. All nitrifying bacteria were cultivated in different formulations of mineral salts medium as previously described, with minor modifications (48–53). *Nitrosospira* species were cultivated at 28°C in mineral salts medium containing 2.5 to 10 mM $(NH_4)_2SO_4$, 15 mM HEPES, 3.2 mM Na_2CO_3, 0.75 mM $MgSO_4$, 1 mM KCl, 10 μM $FeCl_3$, 16.7 μM EDTA, 0.2 mM $CaCl_2$, 100 μM KH_2PO_4, 10 μM NaH_2PO_4, 1 μM $CuSO_4$, 0.6 μM Na_2MoO_4, 1.59 μM $MnCl_2$, 0.6 μM $CoCl_2$, and 0.96 μM $ZnSO_4$. The medium pH was adjusted to 8.0 with KOH. *Nitrosococcus oceani* was cultivated at 30°C in the same mineral salts medium with the addition of 0.5 mM NaCl, 50 μM KH_2PO_4, and 5 μM NaH_2PO_4, and the pH was adjusted to 7.5. The pH of actively growing cultures was adjusted with 1 M Na_2CO_3, and 0.4 μg ml^{-1} phenol red was added to monitor the pH. *Nitrosomonas* species were cultivated at 30°C in mineral salts medium containing 2.5 to 12.5 mM $(NH_4)_2SO_4$, 4.72 mM Na_2CO_3, 0.75 mM $MgSO_4$, 1 mM KCl, 10 μM $FeCl_3$, 16.7 μM EDTA, 0.2 mM $CaCl_2$, 25 mM KH_2PO_4, 2.5 mM NaH_2PO_4, 1 μM $CuSO_4$, 0.6 μM Na_2MoO_4, 1.59 μM $MnCl_2$, 0.6 μM $CoCl_2$, and 0.96 μM $ZnSO_4$, and the pH was adjusted to 8. All NOB were cultivated at 28°C, except for *Nitrospira moscoviensis*, which was cultivated at 37°C. *Nitrobacter hamburgensis* was cultivated in mineral salts medium containing 60 mM $NaNO_2$, 3.5 mM KH_2PO_4, 0.52 mM NaH_2PO_4, 0.75 mM $MgSO_4$, 10 μM $FeCl_3$, 16.7 μM EDTA, 0.28 Na_2CO_3, 0.2 mM $CaCl_2$, 1 μM $CuSO_4$, 0.6 μM Na_2MoO_4, 1.59 μM $MnCl_2$, 0.6 μM $CoCl_2$, and 0.96 μM $ZnSO_4$, and the pH was adjusted to 8.0. *Nitrobacter vulgaris*, *Nitrospira defluvii*, and *Ns. moscoviensis* were cultivated in mineral salts medium containing 100 μM to 30 mM $NaNO_2$, 70 μM $CaCO_3$, 8.6 mM NaCl, 0.2 mM $MgSO_4$, 1.1 mM KH_2PO_4, 0.2 μM $MnSO_4$, 0.8 μM H_3BO_3, 0.15 μM $ZnSO_4$, 32 nM $(NH_4)_6Mo_7O_{24}$, 3.5 μM $FeSO_4$, and 0.1 μM $CuSO_4$, and the pH was adjusted to 7.5. *Nitrococcus mobilis* and *Nitrospina gracilis* were cultivated in undefined seawater medium consisting of 70% 0.2-μm-filtered, autoclaved seawater with the addition of 34 μM $CaCl_2$, 406 μM $MgSO_4$, 3.6 μM $FeCl_3$, 6 μM EDTA, 12.5 μM KH_2PO_4, 103 nM Na_2MoO_4, 253 nM $MnCl_2$, 2.1 nM $CoCl_2$, 87 nM $ZnSO_4$, and 24 nM $CuSO_4$. Seawater medium contained 100 to 30 mM $NaNO_2$ depending on the culture activity, and the pH was adjusted to 7.5. *Agrobacterium tumefaciens* KYC55(pJZ372)(pJZ384)(pJZ410) was cultivated, preinduced, and used for the detection of AHLs as described previously (36, 54).

Growth conditions. Batch cultures of nitrifying bacteria were inoculated to an OD_{600} of 0.001 and grown in Erlenmeyer flasks in a rotatory shaker at 100 rpm in mineral salts medium as outlined above. Experimental cultures were monitored at regular intervals to check the OD_{600}, and the growth medium was analyzed for NO_2^- concentrations by the Griess assay (55) and for the presence of AHL (bioassay).

Bioinformatic analyses. The National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to identify LuxI autoinducer synthase homologs and LuxR receptor/transcription factor homologs in nitrifiers and other bacteria (see Data Set S1 in the supplemental material) (56, 57). MUSCLE was used for multiple alignments of amino acid sequences (58, 59), and phylogenetic analyses were conducted by using MEGA7 (60). The evolutionary history of LuxI and LuxR homologs in nitrifying bacteria was inferred by using the maximum likelihood method, based on the JTT matrix model (61). Phylogenetic trees were visualized by using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

AHL bioassay. AHLs were extracted by liquid-liquid extraction from 10 ml of the culture supernatant with acidified ethyl acetate and concentrated as previously described (31). Extracts were screened for AHLs by a broad-range bioassay (36, 54) and quantified as relative nanomolar concentrations of C_{10}-HSL by comparison to known concentrations of C_{10}-HSL standards, as described previously (22).

UPLC-MS. AHL structures were identified as previously described, with modifications (31). The supernatants of replicate 1-liter batch cultures were collected by centrifugation during peak AHL accumulation and extracted with acidified ethyl acetate. Extracts were evaporated under filtered air and reconstituted in water containing 20% (vol/vol) acetonitrile and 0.1% (vol/vol) formic acid.

Samples were separated on an Acquity UPLC BEH C_{18} column (2.1 by 50 mm, with a 1.7-μm particle size; Waters, Milford, MA) using an Acquity I-class UPLC instrument (Waters, Milford, MA) at a total flow rate of 0.4 ml min^{-1}. The elution program consisted of an aqueous solution of 5% (vol/vol) acetonitrile for 0.2 min, followed by a linear increase to 90% (vol/vol) acetonitrile over 3 min and a hold of 1 min at 90% acetonitrile, which was followed by reequilibration for 1 min with 5% acetonitrile. Mass spectrometry was performed by using a Synapt G2 instrument (Waters, Milford, MA) in positive-ion ionization (ES^+) mode. The instrument was operated at a source temperature of 120°C, a desolution temperature of 550°C, and a spray voltage of 2.8 kV. MS and product ion data were acquired by MS^e continuum. MS^e function 1 (low energy) was set to a trap collision energy of 4 V and a transfer collision energy of 6 V. MS^e function 2 (high energy) was set to a ramp collision energy of 20 to 30 V, and the ramp transfer collision energy was off. Product ion spectra were searched for a protonated ($[M + H^+]$) lactone moiety ($C_4H_8NO_2$ $[M + H]^+$, *m/z* 102.055) since AHLs consistently fragment to this product ion. Data were analyzed by using MassLynx V4.1 (Waters, Milford, MA). Chemical structures were drawn with ChemSketch Freeware 2012 (ACD Labs, Ontario, Canada).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01540-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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