AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Quorum Sensing in a Methane-Oxidizing Bacterium

Aaron W. Puri,[a] Amy L. Schaefer,[b] Yanfen Fu,[a] David A. C. Beck,[a,c] E. Peter Greenberg,[b] Mary E. Lidstrom[a,b]

Departments of Chemical Engineering[a] and Microbiology,[b] University of Washington, Seattle, Washington, USA; eScience Institute, University of Washington, Seattle, Washington, USA[c]

ABSTRACT Aerobic methanotrophic bacteria use methane as their sole source of carbon and energy and serve as a major sink for the potent greenhouse gas methane in freshwater ecosystems. Dissecting the molecular details of how these organisms interact in the environment may increase our understanding of how they perform this important ecological role. Many bacterial species use quorum sensing (QS) systems to regulate gene expression in a cell density-dependent manner. We have identified a QS system in the genome of *Methylobacter tundripaludum*, a dominant methane oxidizer in methane enrichments of sediment from Lake Washington (Seattle, WA). We determined that *M. tundripaludum* produces primarily *N*-3-hydroxydecanoyl-L-homoserine lactone (3-OH-C_{10}-HSL) and that its production is governed by a positive feedback loop. We then further characterized this system by determining which genes are regulated by QS in this methane oxidizer using transcriptome sequencing (RNA-seq) and discovered that this system regulates the expression of a putative nonribosomal peptide synthetase biosynthetic gene cluster. Finally, we detected an extracellular factor that is produced by *M. tundripaludum* in a QS-dependent manner. These results identify and characterize a mode of cellular communication in an aerobic methane-oxidizing bacterium.

IMPORTANCE Aerobic methanotrophs are critical for sequestering carbon from the potent greenhouse gas methane in the environment, yet the mechanistic details of chemical interactions in methane-oxidizing bacterial communities are not well understood. Understanding these interactions is important in order to maintain, and potentially optimize, the functional potential of the bacteria that perform this vital ecosystem function. In this work, we identify a quorum sensing system in the aerobic methanotroph *Methylobacter tundripaludum* and use both chemical and genetic methods to characterize this system at the molecular level.

KEYWORDS methane, methanotroph, quorum sensing, sociomicrobiology, acyl-homoserine lactone, biosynthetic gene cluster

Received 31 October 2016 **Accepted** 15 December 2016

Accepted manuscript posted online 19 December 2016

Citation Puri AW, Schaefer AL, Fu Y, Beck DAC, Greenberg EP, Lidstrom ME. 2017. Quorum sensing in a methane-oxidizing bacterium. J Bacteriol 199:e00773-16. https://doi.org/10.1128/JB.00773-16.

Editor William W. Metcalf, University of Illinois at Urbana-Champaign

Address correspondence to Aaron W. Puri, awpuri@uw.edu.

Aerobic methane-oxidizing bacteria (methanotrophs) are an important component of the carbon cycle that serve to sequester carbon from the potent greenhouse gas methane after it is produced by anaerobic microbial ecosystems (1, 2). Methanotrophs provide a carbon and energy source for communities of organisms that cannot oxidize methane themselves, thereby serving as key supporting species in the environment (3, 4). The methanotroph *Methylobacter tundripaludum* is a member of the *Gammaproteobacteria* (type I methanotroph), and it has been repeatedly identified as a dominant member of methane enrichment and stable isotope probing experiments of sediment from Lake Washington, Seattle, WA (3, 5–7). *Methylobacter* spp. have also been isolated from other geographically distinct regions, including the arctic (8), estuaries (9), and temperate wetlands (10), highlighting the diverse environments inhabited by this genus. We, therefore, use *M. tundripaludum* as part of a model system for understanding the function of aerobic methane-oxidizing bacterial communities.

Despite the critical ecosystem function performed by aerobic methanotrophs, little is known about the molecular mechanisms that mediate interactions within methane-oxidizing bacterial communities. Understanding these interactions is important in order to maintain, and potentially optimize, the functional potential of these groups. One well-studied bacterial interaction is quorum sensing (QS), which bacteria use to regulate gene expression in a density-dependent manner using diffusible signaling molecules (for review, see references 11 and 12).

In many species of *Proteobacteria*, QS systems use acyl-homoserine lactones (AHLs) produced by members of the LuxI family of AHL synthases. These signal molecules are bound by members of the LuxR family of transcription factors, which control gene expression in an AHL concentration-dependent manner. In many cases, AHLs control the production of extracellular factors, including proteases and antibiotics, which may allow resources to be dedicated to these metabolically expensive products solely under conditions in which the bacterial species has reached a sufficient density to impact its surrounding environment (13–15).

QS is thought to enable host-associated bacterial species to differentiate between low-density/free-living and high-density/host-associated states. The ecological function of QS systems that have been identified in non-host-associated species are not as well understood. Further analysis of the genes controlled by QS in these non-host-associated bacteria may help improve our understanding of how these species interact with other cells and their environment.

In this work, we identify and characterize a QS system in the methane-oxidizing bacterium *M. tundripaludum* strain 21/22. We identify the AHL signal as well as its cognate synthase and receptor. Furthermore, we determine that a secondary metabolite cluster is activated by QS in *M. tundripaludum*, and we identify an extracellular product controlled by QS. To our knowledge, a QS system has never before been characterized in a methanotroph. This characterization of the *M. tundripaludum* QS system, therefore, extends our knowledge of this widespread form of chemical communication.

RESULTS

Identification of quorum sensing genes flanking a putative nonribosomal peptide synthetase cluster in *Methylobacter tundripaludum* 21/22. We identified QS genes in the recently sequenced genome of *Methylobacter tundripaludum* strain 21/22 (Fig. 1A) (5). This included a *luxI* family member, *mbaI* (T451DRAFT_0796), as well as a *luxR* family member, *mbaR* (T451DRAFT_0820). MbaI shares 68% amino acid sequence identity with the AHL synthase from the acidophilic bacterium *Acidithiobacillus thiooxidans*, strains of which have been reported to produce primarily *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C_8-HSL) (16).

Interestingly, in *M. tundripaludum*, the QS genes flank a predicted nonribosomal peptide synthetase (NRPS) cluster (17), which we identified using the bioinformatics tool antiSMASH (antibiotics and secondary metabolite analysis shell) (Fig. 1A) (18). antiSMASH classifies several genes in this cluster as core NRPS genes, including genes encoding an acyl carrier protein, condensation and adenylation domains, and an esterase. These core genes are flanked by efflux pumps as well as additional biosynthetic genes. This cluster as well as the QS genes are also conserved in other *M. tundripaludum* isolates, including strains 31/32 (5) and SV96 (19) (see Fig. S1 in the supplemental material).

The putative NRPS cluster has a G+C content of 41%, while *mbaI* and *mbaR* more closely match the 49% G+C content of the rest of the genome (see Fig. S2 in the supplemental material). There are also several pseudogenes upstream of the cluster, including transposase fragments and an *mbaI′* pseudogene (T451DRAFT_0802) that contains several stop codons and does not contain all of the catalytic residues required for signal synthesis (Fig. 1A) (20). The genome of the related methanotroph *Methylobacter whittenburyi* also contains a QS system with *mbaI* and *mbaR*, and the products of these genes share high sequence identity with those in *M. tundripaludum* (79% and

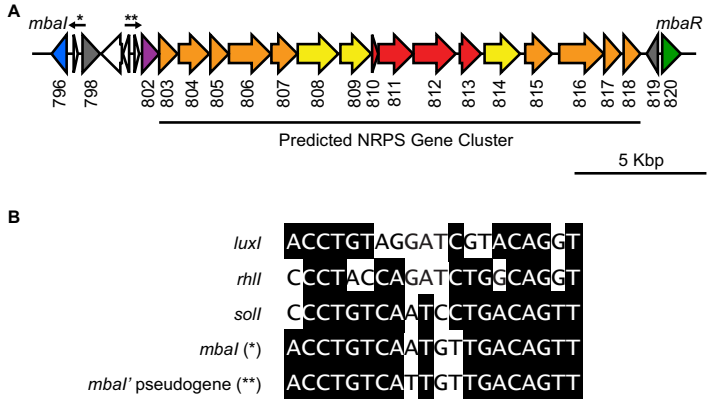

FIG 1 A quorum sensing system in *Methylobacter tundripaludum*. (A) The quorum sensing genes in *Methylobacter tundripaludum* flank a predicted nonribosomal peptide synthetase (NRPS) biosynthetic gene cluster. The arrows and asterisks identify putative MbaR-binding sites shown in Fig. 1B. The numbers below gene arrows are locus tags (T451DRAFT_0XXX) corresponding to the Joint Genome Institute Integrated Microbial Genomes (IMG) system (41). Due to space constraints, locus tags for transposase/integrase genes (white) have been omitted. Genes are colored as follows according to predicted function: *luxI* family AHL synthase gene *mbaI*, blue; *luxR* family transcription factor gene *mbaR*, green; antiSMASH-classified core NRPS genes, red (from left to right, acyl carrier protein, condensation domain, adenylation domain, and esterase genes); other biosynthetic genes, orange; efflux pump genes, yellow; transposase/integrase genes, white; *mbaI'* pseudogene, purple; and other genes, gray. (B) Comparison of putative MbaR-binding sites in front of the AHL synthase gene *mbaI* and the pseudogene *mbaI'* with known LuxR-type binding sites in the promoter sequences of *Vibrio fischeri luxI*, *Pseudomonas aeruginosa rhlI*, and *Ralstonia solanacearum solI*.

69% amino acid identity, respectively) (21) (Fig. S1). However, in *M. whittenburyi*, this locus does not contain the predicted NRPS cluster, transposase fragments, or *mbaI'* pseudogene. These observations led us to speculate that this biosynthetic gene cluster is a potential genomic island (22) that was either inserted into the genome of *M. tundripaludum* or lost in other known members of the *Methylobacter* genus.

The *Methylobacter tundripaludum* quorum sensing signal is *N*-3-hydroxydecanoyl-L-homoserine lactone. We sought to characterize the QS system in *M. tundripaludum* 21/22 by constructing a bioassay for QS signal detection. To do this, we took advantage of the fact that *luxI*-type synthase genes are often activated by QS, resulting in positive autoregulation of the system (12). We identified a candidate MbaR-binding site upstream of *mbaI* that conformed to the NNCTG-N_{10}-CAGNN pattern and dyad symmetry typically recognized by LuxR-type transcription factors (Fig. 1B) (23). We then constructed a two-plasmid reporter system in *Escherichia coli* in which *mbaR* is expressed on one plasmid under its native promoter and the *mbaI* promoter is fused to *gfp* (P_{mbaI}-*gfp*) on a separate vector.

We extracted the supernatant from a wild-type (WT) culture of *M. tundripaludum* 21/22 with ethyl acetate and added this extract to the P_{mbaI}-*gfp* reporter strain, resulting in a 4-fold increase in green fluorescent protein (GFP) fluorescence compared to a solvent control (see Fig. S3 in the supplemental material). This suggests that an AHL signal is produced by this methane-oxidizing bacterium. Furthermore, the addition of this extract to a strain containing the P_{mbaI}-*gfp* plasmid but not the *mbaR*-expressing plasmid resulted in no increase in GFP fluorescence, demonstrating that this process is MbaR dependent and that MbaR activates the expression of the synthase gene *mbaI* upon signal binding in a possible positive feedback loop (Fig. S3).

We separated the signal-containing extract by C_{18} reverse-phase high-performance liquid chromatography (HPLC) and assayed each fraction using the P_{mbaI}-*gfp* reporter strain, resulting in a single peak of activity (Fig. 2A). This activity was not present in the supernatant extract from a Δ*mbaI* mutant, confirming that MbaI is the signal synthase and suggesting that the signal is an AHL (Fig. 2A). To determine the identity of the predicted AHL, we used tandem mass spectrometry to scan for parent ions in the ethyl acetate extract while monitoring for the production of the

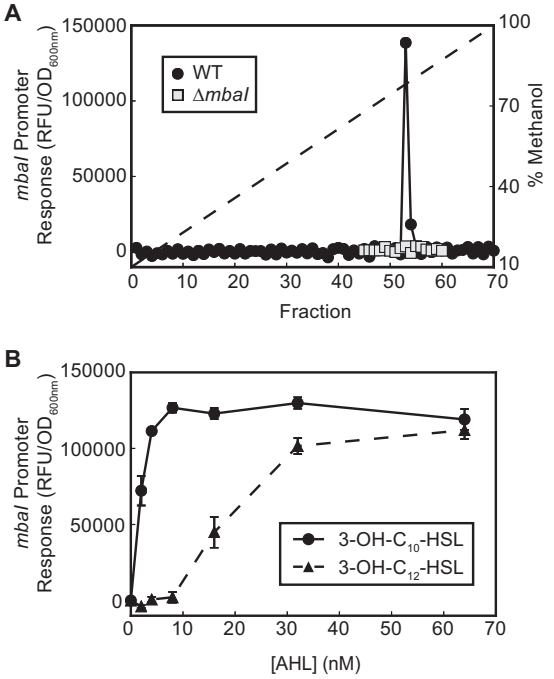

FIG 2 The *M. tundripaludum* quorum sensing system is active. (A) P_{mbaI}-*gfp* activity of HPLC-fractionated culture supernatant extracts from wild-type and Δ*mbaI M. tundripaludum* strains. The dashed line shows the methanol gradient. (B) Dose response of the P_{mbaI}-*gfp E. coli* reporter strain to 3-OH-C_{10}-HSL and 3-OH-C_{12}-HSL.

telltale lactone fragment ($[M+H]^+ = 102$). We detected two peaks that produced this fragment (Fig. 3A). The parent $[M+H]^+$ of the major peak is 272, which is consistent with the mass of *N*-3-hydroxydecanoyl-L-homoserine lactone (3-OH-C_{10}-HSL) (Fig. 3B and D). The minor peak, which is approximately an order of magnitude smaller, has a parent $[M+H]^+$ of 300, which is consistent with the mass of *N*-3-hydroxydodecanoyl-L-homoserine lactone (3-OH-C_{12}-HSL) (Fig. 3C and E). The retention times and mass spectra of these purified compounds were indistinguishable from commercial standards (data not shown).

To determine whether these AHLs have biological activity, we tested commercial standards in the P_{mbaI}-*gfp* reporter strain. MbaR is responsive to both 3-OH-C_{10}-HSL and 3-OH-C_{12}-HSL but is more sensitive to the former (half maximal activation with ~3 nM and ~25 nM signal, respectively) (Fig. 2B), which is consistent with this signal being the most abundant in the *M. tundripaludum* supernatant. Together, these results demonstrate that the QS system of the aerobic methanotroph *M. tundripaludum* 21/22 produces and responds primarily to the AHL 3-OH-C_{10}-HSL (Fig. 3D).

Kinetics and magnitude of quorum sensing signal production. In order to determine which genes are regulated by the *M. tundripaludum* QS system, we used the following approach shown to be successful in studies of other bacterial species: comparing the transcriptomes of an AHL synthase mutant grown in the presence and absence of added exogenous AHL (for example, see reference 24). To use this strategy with the *M. tundripaludum* Δ*mbaI* mutant, we determined how much 3-OH-C_{10}-HSL to add and at which time points to harvest RNA for analysis.

Because the P_{mbaI}-*gfp E. coli* reporter responds in a dose-dependent manner to the QS signal (Fig. 2B), we used it to quantify 3-OH-C_{10}-HSL levels in the supernatant of a wild-type *M. tundripaludum* culture over the course of a growth curve (Fig. 4A). We saw maximum accumulation (approximately 330 nM) of the 3-OH-C_{10}-HSL signal during stationary phase. Next, to determine the sensitivity of MbaR to 3-OH-C_{10}-HSL in its native host, we constructed an *M. tundripaludum* reporter strain wherein the signal-

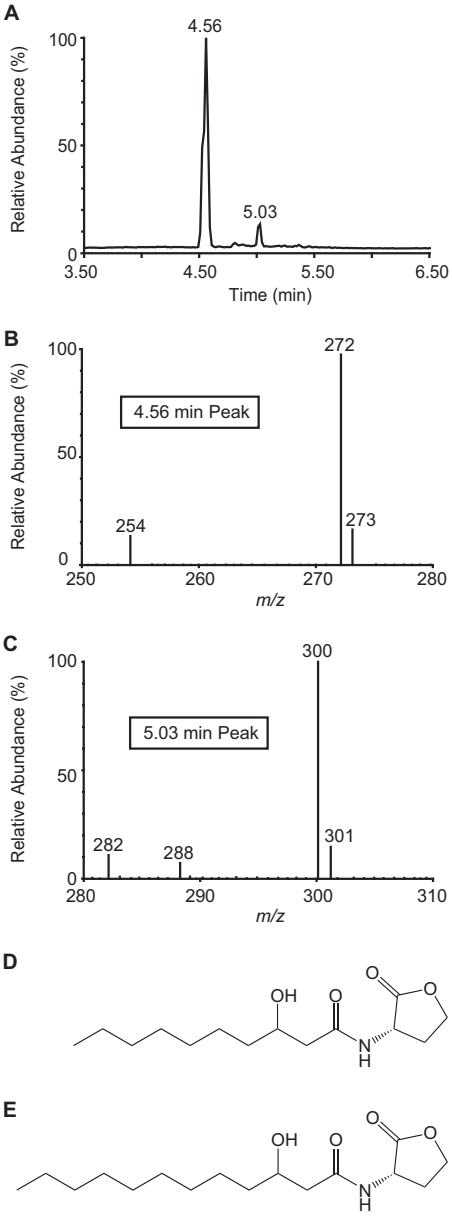

FIG 3 Tandem mass spectrometry reveals that *M. tundripaludum* produces primarily 3-OH-C_{10}-HSL. (A) Scan for parent ions of homoserine lactone fragment ($[M+H]^+ = 102$). (B, C) Parent mass spectrum for peaks at 4.56 min (B) and 5.03 min (C) in parent ion scan. (D, E) Structures of 3-OH-C_{10}-HSL (D) and 3-OH-C_{12}-HSL (E).

responsive open reading frame (ORF) *mbaI* was replaced with the catechol dioxygenase reporter gene *xylE* (25). This *mbaI*::*xylE* reporter strain was responsive to as little as 3 nM 3-OH-C_{10}-HSL (Fig. 4B).

The predicted NRPS gene cluster is regulated by quorum sensing. With these results in hand, we determined a minimal QS regulon of *M. tundripaludum* using transcriptome sequencing (RNA-seq) by analyzing differential gene expression in the Δ*mbaI* mutant in the presence or absence of 3-OH-C_{10}-HSL. We chose to add 1 μM 3-OH-C_{10}-HSL to ensure that the signal concentration would remain above the maximum concentration observed in a wild-type culture of ~300 nM throughout the course of the experiment (Fig. 4A). Based on the *mbaI*::*xylE* reporter results (Fig. 4B), we extracted RNA from late-log-phase cultures (at an optical density at 600 nm [OD_{600}] of ~0.5) when the amount of signal produced by the wild-type strain had passed the

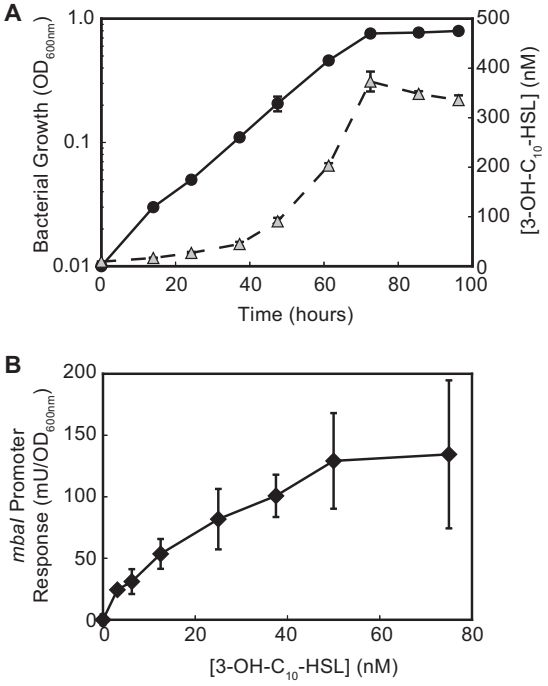

FIG 4 AHL production and detection in *M. tundripaludum*. (A) 3-OH-C_{10}-HSL accumulation (triangles) during *M. tundripaludum* growth (circles). (B) *M. tundripaludum mbaI::xylE* strain response to 3-OH-C_{10}-HSL. One unit (U) of catechol dioxygenase activity represents a change in absorbance (375 nm) per minute.

threshold required to activate the *mbaI* promoter. We also examined differential gene expression in the early stationary phase (at an OD_{600} of ~0.9), when the maximum AHL concentration occurs in a wild-type culture, to determine if additional genes are regulated at a later growth stage.

In the log-phase culture, the expression of 28 genes was ≥2.5-fold higher in the presence of the QS signal (Table 1). Many of these genes were grouped into clusters that may represent operons. The genes with the greatest increase in expression were located in the predicted NRPS gene cluster flanked by *mbaI* and *mbaR*. The expression of the first gene in the cluster, a haloacid dehalogenase (HAD) superfamily hydrolase gene (T451DRAFT_0803), was 28-fold higher in the presence of the signal. Interestingly, transcripts of the *mbaI′* pseudogene (T451DRAFT_0802) were also 4-fold higher in the presence of the signal (Table 1; see also Fig. S4 in the supplemental material). Reverse transcription-PCR confirmed that the *mbaI′* pseudogene and the hydrolase gene are transcribed together in the same operon (see Fig. S5 in the supplemental material).

We identified a potential MbaR-binding site upstream of the *mbaI′* pseudogene, centered approximately 50 bp before the transcription start site (Fig. 1B). This binding site contains only one mismatch compared to the one upstream of *mbaI* and has strong dyad symmetry, further supporting the idea that the *mbaI′* pseudogene, and consequently the predicted NRPS gene cluster, is regulated by QS. We also observed that the Δ*mbaI* mutant grows faster than the wild-type strain (9.7 ± 0.1 h versus 12.2 ± 0.5 h of generation time, respectively), suggesting that cell growth slows down after induction of these genes.

Other gene clusters with higher expression in the presence of the AHL signal included multiple efflux pump and hypothetical genes, as well as genes that encode transcriptional regulators (Table 1). However, while these gene clusters had reproducibly higher expression when signal was added, we were not able to identify putative MbaR-binding sites upstream of the approximate transcriptional start sites (see Materials and Methods for identification criteria).

TABLE 1 Genes with increased expression in *M. tundripaludum* 21/22 Δ*mbaI* in the presence of 1 μM 3-OH-C_{10}-HSL in late log phase

Gene locus (T451DRAFT_)	Product	Fold change (+AHL/−AHL)	P_{adj}[a]
0202	Hypothetical protein	10.2	2.6E−93
0203	Transcriptional regulator, TetR family	2.8	5.2E−22
0204	Hypothetical protein	3.4	4.7E−65
0796	LuxI family acyl-homoserine lactone synthase MbaI	NA[b]	NA
0802	Product of *mbaI*′ pseudogene	4.2	5.9E−31
0803	HAD superfamily subfamily IB hydrolase, TIGR01490	28.0	4.1E−239
0804	Delta-aminolevulinic acid dehydratase	18.6	2.0E−263
0805	A-factor biosynthesis repeat protein	11.8	2.8E−230
0806	2-Polyprenyl-6-methoxyphenol hydroxylase-like oxidoreductase	7.0	7.7E−71
0807	Predicted oxidoreductase, aryl-alcohol dehydrogenase-like protein	4.3	8.6E−56
0808	MFS[c] transporter, DHA2 family, multidrug resistance protein	4.3	5.9E−62
0809	Membrane fusion protein, multidrug efflux system	3.5	2.0E−59
0810	Acyl carrier protein	3.4	2.8E−27
0811	Putative polyketide synthase component	3.2	9.3E−29
0812	Long-chain acyl coenzyme A synthetase	3.2	1.2E−35
0813	Predicted hydrolase or acyltransferase of alpha/beta superfamily	2.7	1.1E−17
0814	Outer membrane protein	2.9	1.2E−44
1642	Cation/multidrug efflux pump	3.0	4.4E−34
1643	RND family efflux transporter, membrane fusion protein subunit	3.6	6.1E−40
1644	Efflux transporter, outer membrane factor lipoprotein, NodT family	3.5	4.2E−35
1645	Transcriptional regulator, TetR family	3.6	2.2E−28
1678	Hypothetical protein	2.8	5.6E−27
1875	RNA polymerase primary sigma factor	3.0	1.1E−19
1876	Membrane protease subunit HflK	2.8	6.2E−16
1972	Hypothetical protein	9.5	8.6E−128
2702	Outer membrane protein, multidrug efflux system	4.5	2.1E−112
2703	Multidrug efflux pump	6.5	6.9E−111
2704	Membrane fusion protein, multidrug efflux system	6.9	3.7E−102

[a]P_{adj}, adjusted *P* value.
[b]NA, not applicable. An accurate fold change cannot be computed for *mbaI* (T451DRAFT_0796) because these experiments were done in a Δ*mbaI* strain.
[c]MFS, major facilitator superfamily.

When maximal signal production is reached in the early stationary phase, a subset of the same genes differentially expressed during log phase still had higher expression in the presence of the signal (see Table S3 in the supplemental material). The only additional genes with higher expression when signal was added coded for two hypothetical polypeptides, both of which narrowly missed the 2.5-fold cutoff in the log-phase analysis. Neither of these genes have a predicted MbaR-binding site. Twenty-two additional genes were downregulated at least 2.5-fold as well at this later time point, including multiple gene clusters related to redox reactions (see Table S4 in the supplemental material). Together, these results demonstrate a QS regulon in the aerobic methanotroph *M. tundripaludum*.

A product with strong UV absorbance is present in *M. tundripaludum* supernatants in a quorum-sensing-dependent manner. In the process of separating the ethyl acetate extract of the *M. tundripaludum* supernatant over an HPLC gradient to identify the AHL signal (Fig. 2), we observed a product with a large absorbance maximum of 297 nm that eluted after 48 min (Fig. 5), prior to the AHL. This product was not detectable in the supernatant from the Δ*mbaI* strain but was present when the Δ*mbaI* strain was cultured with the addition of 3-OH-C_{10}-HSL, suggesting that its production is QS dependent. The collected product had no discernible growth-

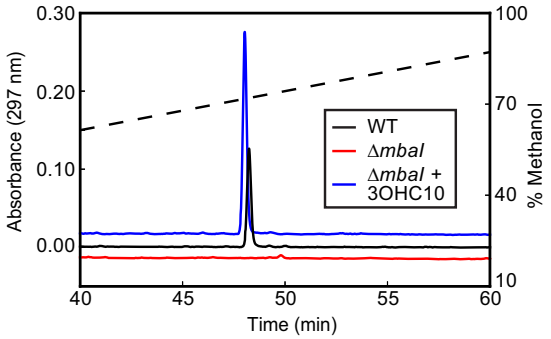

FIG 5 A product with strong UV absorbance is detected in *M. tundripaludum* culture supernatant in a quorum-sensing-dependent manner. UV absorbance (297 nm) of supernatant extract from cultures of *M. tundripaludum* wild-type (WT) and Δ*mbaI* strains and *M. tundripaludum* Δ*mbaI* with 1 μM 3-OH-C_{10}-HSL separated by HPLC. The dashed line shows the methanol gradient. The absorbance traces are vertically offset for clarity.

inhibitory activity against *E. coli* MG1655 or *Bacillus subtilis* PY79. Together, these results suggest that the QS system in *M. tundripaludum* regulates the production of an extracellular factor with strong UV absorbance.

DISCUSSION

Many species use QS systems to regulate gene expression in a cell density-dependent manner; however, this form of cellular communication has not been extensively studied in methanotrophs. Here, we describe an AHL-based QS system in *M. tundripaludum*. *M. tundripaludum* produces primarily 3-OH-C_{10}-HSL, a signal that is also produced by environmental isolates of other bacterial species, including *Burkholderia thailandensis* (14) and *Pseudomonas fluorescens* (26). This opens up the possibility of cross talk between bacterial species in methane-oxidizing communities, as both *Burkholderiales* and pseudomonads have also been identified in enrichments from Lake Washington sediment (3, 7).

We discovered that in *M. tundripaludum* QS controls primarily the expression of a predicted NRPS gene cluster flanked by *mbaI* and *mbaR* in the genome. It is notable that the *mbaI'* pseudogene immediately upstream of this biosynthetic gene cluster is also transcribed and that there is a predicted MbaR-binding site upstream of this pseudogene that is nearly identical to the one upstream of the bona fide *mbaI* gene (Fig. 1B; see also Fig. S4 in the supplemental material). It is possible that *mbaI* and its upstream region were duplicated and that this led to the control of the biosynthetic gene cluster by QS.

Although the *mbaI'* pseudogene is transcribed, any resulting gene product does not appear to have AHL synthase activity, as there is no detectable AHL activity produced by the Δ*mbaI* mutant strain (Fig. 2A). This does not rule out another possible function for the product of *mbaI'*. In *Methylobacterium extorquens* AM1, which can utilize C_1 compounds, including methanol and methylamine but not methane, the product of a truncated I gene termed *tslI* controls the expression of the AHL synthase gene *msaI* and, therefore, indirectly regulates QS in this bacterium (27).

The product of the predicted NRPS cluster and its function are unknown. We detected an ethyl acetate-extractable factor with strong UV absorbance in the *M. tundripaludum* supernatant that is produced in a QS-dependent manner. As the putative NRPS genes are upregulated in the presence of a QS signal and there are multiple genes encoding efflux pump systems in the cluster, these results are consistent with the extracellular factor being the product of the biosynthetic gene cluster. However, additional studies will be needed to confirm this link as well as to determine the structure and function of this factor.

QS systems often regulate the production of extracellular products (13), such as antibiotics (14, 15) and proteases (28). One hypothesis is that this regulation prevents

these products from being produced at low cell densities, when the fitness cost for each individual bacterium may outweigh any benefit of the small amount of produced good (12). In *M. tundripaludum*, we observed a measurable fitness defect under laboratory conditions from the expression of QS-regulated clusters, as the AHL synthase Δ*mbaI* mutant grows approximately 20% faster than the wild type. Increased growth and respiration rates have been observed in QS-null mutants in *Burkholderia* spp. that may be explained by the abrogated production of QS-controlled goods or metabolic slowing under QS (29, 30). Increased growth rates have also been observed in *Vibrio harveyi* QS-null mutants that can be partially explained by alleviation of QS-controlled bioluminescence (31). The fact that the predicted NRPS gene cluster is conserved in *M. tundripaludum* strains isolated from such disparate geographic regions as Svalbard, Norway (strain SV96) (8), and Seattle, WA (strains 21/22 and 31/32) (5), suggests that there may be an ecological role for this gene cluster at high cell densities (see Fig. S1 in the supplemental material).

Several other gene clusters are more highly expressed in the presence of the QS signal; however, we were unable to identify strong candidates for MbaR-binding sites upstream of any of these clusters. One possible explanation for this is that we do not know all of the criteria for identifying sequence motifs that are bound by MbaR. However, another possibility is that these genes are indirectly regulated by QS, such as through activity of the putative NRPS gene cluster product. There are several examples of multiple layers of gene regulation via bioactive signaling molecules in one organism, such as in *Pseudomonas* and *Burkholderia* spp., which can have multitiered AHL-mediated QS systems that also regulate the production of signaling molecules such as 4-quinolones (32). Furthermore, the genes that we identified with lower expression in the presence of the signal in stationary phase may also be the result of the indirect effects of QS.

Identification of other genes with differential expression in the presence of 3-OH-C_{10}-HSL may be due to technical aspects of the system we used to determine the *M. tundripaludum* QS regulon. For example, we cannot rule out that adding 1 μM exogenous 3-OH-C_{10}-HSL signal to *M. tundripaludum* cultures triggered the activation of the clusters of efflux pump genes, such as T451DRAFT_1642 through T451DRAFT_1644 and T451DRAFT_2702 through T451DRAFT_2704, as a response to pump excess amounts of this molecule out of the cell. These genes would need to be tested individually before further conclusions can be made.

The predicted NRPS biosynthetic gene cluster may be the only set of genes activated directly by QS in *M. tundripaludum*. There is precedence for such a restricted QS regulon. Only a small subset of genes is activated in the photosynthetic bacterium *Rhodopseudomonas palustris* (33), as well as in the plant pathogen *Pseudomonas syringae* (34). This is in stark contrast to the QS systems of *Pseudomonas aeruginosa*, which regulate hundreds of genes (35, 36). This may reflect the difference in niches that can be occupied by these bacterial species. *Pseudomonas aeruginosa* is well known to occupy a diverse array of environmental niches, while aerobic methanotrophs, such as *M. tundripaludum*, are found mainly in the relatively restricted niche of opposing counter gradients of oxygen and methane in sediment (37). As these methanotrophs are generally not thought to be host associated, the difference in regulon size may also be the result of whether or not QS is needed to differentiate between a low-density planktonic environment and a high-density host-associated environment.

If there is indeed only one set of genes directly controlled by QS in *M. tundripaludum*, it brings up the question of what QS controls in other methanotrophs, such as *M. whittenburyi*, which has a QS system highly homologous to the one found in *M. tundripaludum* but does not have the predicted NRPS genes or virtually any other synteny at this locus. It is possible that there is no general function of QS in these methanotrophs but rather that this system represents a specific trait acquired for certain environmental niches. More studies will be needed to determine the role of QS in this ecologically significant group of bacteria.

Sequence data. Normalized counts and computed ±3-OH-C_{10}-HSL pairwise fold changes for the log- and stationary-phase RNA-seq experiments are available in Tables S5 and S6, respectively, in the supplemental material.

MATERIALS AND METHODS

Plasmid construction. All plasmids were constructed using Gibson assembly (38), with the exception of the reporter vector pAWP112, which was constructed by inserting the 400 bp upstream of *mbaI* into the promoter probe plasmid pPROBE-GFP[LVA] (39) using the SacI and EcoRI restriction sites. Plasmids used in this study are listed in Table S1 in the supplemental material. Primers used in this study are listed in Table S2 in the supplemental material.

Strain growth and genetic manipulation. Strains used in this study are listed in Table S1. *E. coli* and *B. subtilis* strains were grown in Luria-Bertani (LB) medium at 37°C. *M. tundripaludum* 21/22 was cultured in an atmosphere of 25% methane in air. Plates were incubated at room temperature in sealed jars (Oxoid Limited, Hampshire, United Kingdom), while liquid cultures were grown at 18°C in 250-ml glass serum bottles (Kimble Chase, Vineland, NJ) or 18- by 150-mm tubes (Bellco Glass, Vineland, NJ) sealed with rubber stoppers and aluminum seals (Wheaton, Millville, NJ) and shaken at 200 rpm. Cultures were grown in nitrate mineral salts (NMS) medium (10) containing 0.2 g/liter $MgSO_4 \cdot 7H_2O$, 0.2 g/liter $CaCl_2 \cdot 6H_2O$, 1 g/liter KNO_3, and 30 μM LaCl as well as 1× trace elements; 500× trace elements contain 1.0 g/liter Na_2-EDTA, 2.0 g/liter $FeSO_4 \cdot 7H_2O$, 0.8 g/liter $ZnSO_4 \cdot 7H_2O$, 0.03 g/liter $MnCl_2 \cdot 4H_2O$, 0.03 g/liter H_3BO_3, 0.2 g/liter $CoCl_2 \cdot 6H_2O$, 0.6 g/liter $CuCl_2 \cdot 2H_2O$, 0.02 g/liter $NiCl_2 \cdot 6H_2O$, and 0.05 g/liter $Na_2MoO \cdot 2H_2O$. A final concentration of 5.8 mM phosphate buffer (pH 6.8) was added immediately before use.

Plasmids were introduced via electroporation (for Δ*mbaI*) as previously described (40) or via conjugation using *E. coli* donor strain S17-1 (for *mbaI*::*xylE*). For conjugation, the *M. tundripaludum* biomass was spread on an NMS plate and grown under methane. After 2 days, an equal volume of donor biomass containing the plasmid of interest was added, and the mixture was grown under methane at room temperature for an additional 2 days. For both electroporation and mating, successful integrants (single crossovers) were selected on NMS plates containing kanamycin (50 μg/ml). Subsequent sucrose counterselection was performed as previously described (25).

antiSMASH analysis. Putative biosynthetic gene clusters were identified using antiSMASH version 3.0.5 (18) with default settings using genomes downloaded from the Joint Genome Institute (JGI) Integrated Microbial Genomes (IMG) system (41).

Supernatant extraction and HPLC fractionation. Two milliliters of *M. tundripaludum* culture supernatant was routinely extracted twice with an equal volume of ethyl acetate containing 0.01% acetic acid. For HPLC fractionation, dried extract from 15 ml of stationary-phase culture supernatant was resuspended in 150 μl of 1:1 methanol-water and separated using a Nucleosil C_{18} column (5 μm; 250 mm by 4.6 mm) (Supelco, Bellefonte, PA) at 0.75 ml/min using a linear gradient of 10% to 100% methanol in water over 70 min. A 5-μl aliquot of each 0.75-ml fraction was analyzed using the P_{mbaI}-*gfp* assay described below.

AHL analysis using *E. coli* P_{mbaI}-*gfp* reporter strain. Acyl-homoserine lactone signals were detected as previously described (42). Briefly, an overnight culture of *E. coli* reporter strain containing plasmids pAWP112 and pAWP113 was subcultured to an OD of 0.1 in LB with kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml). Subsequently, 500 μl was added to 1.5-ml tubes containing dried signal, and these tubes were shaken at 37°C for 4 h. Cultures were then pelleted and resuspended in 500 μl of 50 mM Tris, pH 7.5, and 100 μl was measured for GFP fluorescence (485-nm excitation, 510-nm emission) in a 96-well plate (Nunc black optical bottom) by using a plate reader (Tecan Infinite F500). Signal *N*-3-hydroxydecanoyl-L-homoserine lactone was purchased from Cayman Chemical (Ann Arbor, MI), and *N*-3-hydroxydodecanoyl-DL-homoserine lactone was purchased from Sigma-Aldrich (St. Louis, MO).

Catechol dioxygenase activity assay. A log-phase *M. tundripaludum mbaI*::*xylE* culture was subcultured to an OD_{600} of 0.02, and 5-ml aliquots were added to tubes containing dried signal or supernatant extract. The cultures were grown to the early stationary phase (OD_{600} of ~0.9), at which point catechol dioxygenase activity was assayed in whole cells by measuring the rate of change in absorbance at 375 nm in the presence of 1 mM catechol with a plate reader (Molecular Devices SpectraMax 190) as previously described (43).

Mass spectrometry analysis of AHL signal. Extracted samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters Xevo LC-MS system, consisting of an Acquity ultraperformance liquid chromatography (UPLC) system and a Xevo triple-quadrupole mass spectrometer (Waters, Milford, MA). An HSS T3 column (1.8 μm; 2.1 mm by 100 mm; Acquity UPLC) was used for liquid chromatography. The LC gradient started with 10% methanol in water and increased to 100% methanol at 5 min, with 50% methanol at 5.1 min, 10% methanol at 5.5 min, and a hold for 1 min at a flow rate of 0.45 ml/min.

AHLs were initially identified using a parent ion scan monitoring for the lactone fragment ($[M+H]^+ = 102$). Multiple reaction monitoring (MRM) parameters for 3-OH-C_{10}-HSL as well as 3-OH-C_{12}-HSL were determined by tuning commercially available standards with the Xevo mass spectrum in positive mode. For biological samples, both MRM scan and MS scan (mass range from 250 to 350) were used.

RNA preparation, RNA-seq, and data analysis. A log-phase *M. tundripaludum* Δ*mbaI* culture was subcultured to an OD_{600} of 0.01 and added to bottles containing dried 3-OH-C_{10}-HSL (or solvent control), resulting in a final concentration of 1 μM signal. RNA was extracted at the desired time points as previously described (43). cDNA library preparation and RNA sequencing were performed by Genewiz (South Plainfield, NJ) using Illumina HiSeq 2500 1 × 50 (single-ended) reads. The raw reads from the

sequencing facility were aligned to the *M. tundripaludum* strain 21/22 genome (genome identification number 2563366535) as downloaded from JGI's IMG system on 13 January 2016 (41). Alignment was performed using BWA version 0.7.12-r1044 with the BWA-MEM algorithm and default parameters (44). The alignments were postprocessed into sorted BAM files with SAMTools version 1.2-232-g87cdc4a (45). Reads were attributed to open reading frames (ORFs) as well as the *mbaI'* pseudogene T451DRAFT_0802 using the *htseq-count* tool from the HTSeq framework version 0.6.1p1 in the intersection-nonempty mode (46). Differential abundance analysis was performed with DESeq2 1.2.10 (47, 48) using R 3.3.0.

Genes were considered to be differentially expressed if there was an average change of ≥2.5-fold in comparisons of normalized counts (those in the presence versus the absence of 3-OH-C_{10}-HSL), as well as an adjusted *P* value of less than 1E−05 (47).

MbaR-binding site identification. To identify possible MbaR-binding sites upstream of differentially expressed gene clusters, approximate transcription start sites were first determined by visualizing the aligned RNA-seq reads using Integrative Genomics Viewer (IGV) (49). A sequence was considered a putative MbaR-binding site if (i) it was centered 40 to 80 bp upstream of the approximate transcription start site, (ii) it contained less than 10 mismatches compared to the MbaR-binding site upstream of *mbaI* (ACCTGTCAATGTTGACAGTT), (iii) it matched the general NNCTG-N_{10}-CAGNN pattern with one mismatch or less, and (iv) eight or more of its base pairs had dyad symmetry (23).

Growth inhibition assay. Ten milliliters of molten 1% LB agar was inoculated with 100 μl of stationary-phase *E. coli* MG1655 or *B. subtilis* PY79, and this mixture was overlaid onto spots of *M. tundripaludum* (pregrown for 7 days on an NMS1 agar plate) or onto a new 1.5% LB agar plate. In the latter case, 5 μl of the HPLC fraction containing the UV absorbent compound (containing compound from the equivalent of 65 μl of *M. tundripaludum* stationary-phase culture supernatant) was spotted onto the solidified overlay. The plates were then incubated at 37°C overnight and examined for zones of growth inhibition.

Accession number(s). Normalized counts and computed ±3-OH-C_{10}-HSL pairwise fold changes for the log- and stationary-phase RNA-seq experiments have been submitted to the Gene Expression Omnibus (GEO) database under accession number GSE85617.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00773-16.

TEXT S1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

We thank members of the Lidstrom and Greenberg labs for helpful discussions, M. Sadilek for help with LC-MS/MS instrumentation, and F. Chu for help with RNA-seq sample submission and for providing *B. subtilis* strain PY79.

This work was facilitated through the use of advanced computational storage and networking infrastructure provided by the Hyak supercomputer system supported in part by the University of Washington eScience Institute.

REFERENCES

1. Nisbet EG, Dlugokencky EJ, Bousquet P. 2014. Atmospheric science. Methane on the rise—again. Science 343:493–495. https://doi.org/10.1126/science.1247828.
2. Singh BK, Bardgett RD, Smith P, Reay DS. 2010. Microorganisms and climate change: terrestrial feedbacks and mitigation options. Nat Rev Microbiol 8:779–790. https://doi.org/10.1038/nrmicro2439.
3. Beck DAC, Kalyuzhnaya MG, Malfatti S, Tringe SG, Glavina Del Rio T, Ivanova N, Lidstrom ME, Chistoserdova L. 2013. A metagenomic insight into freshwater methane-utilizing communities and evidence for cooperation between the *Methylococcaceae* and the *Methylophilaceae*. PeerJ 1:e23. https://doi.org/10.7717/peerj.23.
4. van der Ha D, Vanwonterghem I, Hoefman S, De Vos P, Boon N. 2013. Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. Antonie Van Leeuwenhoek 103:527–537. https://doi.org/10.1007/s10482-012-9835-7.
5. Kalyuzhnaya MG, Lamb AE, McTaggart TL, Oshkin IY, Shapiro N, Woyke T, Chistoserdova L. 2015. Draft genome sequences of gammaproteobacterial methanotrophs isolated from Lake Washington sediment. Genome Announc 3:e00103-15.
6. Kalyuzhnaya MG, Lapidus A, Ivanova N, Copeland AC, McHardy AC, Szeto E, Salamov A, Grigoriev IV, Suciu D, Levine SR, Markowitz VM, Rigoutsos I, Tringe SG, Bruce DC, Richardson PM, Lidstrom ME, Chistoserdova L. 2008. High-resolution metagenomics targets specific functional types in complex microbial communities. Nat Biotechnol 26:1029–1034. https://doi.org/10.1038/nbt.1488.
7. Oshkin IY, Beck DA, Lamb AE, Tchesnokova V, Benuska G, McTaggart TL, Kalyuzhnaya MG, Dedysh SN, Lidstrom ME, Chistoserdova L. 2015. Methane-fed microbial microcosms show differential community dynamics and pinpoint taxa involved in communal response. ISME J 9:1119–1129. https://doi.org/10.1038/ismej.2014.203.
8. Wartiainen I, Hestnes AG, McDonald IR, Svenning MM. 2006. *Methylobacter tundripaludum* sp. nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard Islands, Norway (78 degrees N). Int J Syst Evol Microbiol 56:109–113. https://doi.org/10.1099/ijs.0.63728-0.
9. Smith KS, Costello AM, Lidstrom ME. 1997. Methane and trichloroethylene oxidation by an estuarine methanotroph, *Methylobacter* sp. strain BB51. Appl Environ Microbiol 63:4617–4620.
10. Whittenbury R, Phillips KC, Wilkinson JF. 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61:205–218. https://doi.org/10.1099/00221287-61-2-205.
11. Ng W-L, Bassler BL. 2009. Bacterial quorum-sensing network architectures. Annu Rev Genet 43:197–222. https://doi.org/10.1146/annurev-genet-102108-134304.
12. Schuster M, Sexton DJ, Diggle SP, Greenberg EP. 2013. Acyl-homoserine lactone quorum sensing: from evolution to application. Annu Rev Microbiol 67:43–63. https://doi.org/10.1146/annurev-micro-092412-155635.
13. Bandara HMHN, Lam OLT, Jin LJ, Samaranayake L. 2012. Microbial chemical signaling: a current perspective. Crit Rev Microbiol 38:217–249. https://doi.org/10.3109/1040841X.2011.652065.

14. Duerkop BA, Varga J, Chandler JR, Peterson SB, Herman JP, Churchill MEA, Parsek MR, Nierman WC, Greenberg EP. 2009. Quorum-sensing control of antibiotic synthesis in *Burkholderia thailandensis*. J Bacteriol 191:3909–3918. https://doi.org/10.1128/JB.00200-09.
15. Hoover SE, Perez AJ, Tsui H-CT, Sinha D, Smiley DL, DiMarchi RD, Winkler ME, Lazazzera BA. 2015. A new quorum-sensing system (TprA/PhrA) for *Streptococcus pneumoniae* D39 that regulates a lantibiotic biosynthesis gene cluster. Mol Microbiol 97:229–243. https://doi.org/10.1111/mmi.13029.
16. Ruiz LM, Valenzuela S, Castro M, Gonzalez A, Frezza M, Soulère L, Rohwerder T, Queneau Y, Doutheau A, Sand W, Jerez CA, Guiliani N. 2008. AHL communication is a widespread phenomenon in biomining bacteria and seems to be involved in mineral-adhesion efficiency. Hydrometallurgy 94:133–137. https://doi.org/10.1016/j.hydromet.2008.05.028.
17. Miller BR, Gulick AM. 2016. Structural biology of nonribosomal peptide synthetases. Methods Mol Biol 1401:3–29. https://doi.org/10.1007/978-1-4939-3375-4_1.
18. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. https://doi.org/10.1093/nar/gkv437.
19. Svenning MM, Hestnes AG, Wartiainen I, Stein LY, Klotz MG, Kalyuzhnaya MG, Spang A, Bringel F, Vuilleumier S, Lajus A, Medigue C, Bruce DC, Cheng JF, Goodwin L, Ivanova N, Han J, Han CS, Hauser L, Held B, Land ML, Lapidus A, Lucas S, Nolan M, Pitluck S, Woyke T. 2011. Genome sequence of the Arctic methanotroph *Methylobacter tundripaludum* SV96. J Bacteriol 193:6418–6419. https://doi.org/10.1128/JB.05380-11.
20. Fuqua C, Greenberg EP. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol 3:685–695. https://doi.org/10.1038/nrm907.
21. Hamilton R, Kits KD, Ramonovskaya VA, Rozova ON, Yurimoto H, Iguchi H, Khmelenina VN, Sakai Y, Dunfield PF, Klotz MG, Knief C, Op den Camp HJ, Jetten MS, Bringel F, Vuilleumier S, Svenning MM, Shapiro N, Woyke T, Trotsenko YA, Stein LY, Kalyuzhnaya MG. 2015. Draft genomes of gammaproteobacterial methanotrophs isolated from terrestrial ecosystems. Genome Announc 3:e00515-15.
22. Ruzzini AC, Clardy J. 2016. Gene flow and molecular innovation in bacteria. Curr Biol 26:R859–R864. https://doi.org/10.1016/j.cub.2016.08.004.
23. Antunes LCM, Ferreira RBR, Lostroh CP, Greenberg EP. 2008. A mutational analysis defines *Vibrio fischeri* LuxR binding sites. J Bacteriol 190:4392–4397. https://doi.org/10.1128/JB.01443-07.
24. Majerczyk C, Brittnacher M, Jacobs M, Armour CD, Radey M, Schneider E, Phattarasokul S, Bunt R, Greenberg EP. 2014. Global analysis of the *Burkholderia thailandensis* quorum sensing-controlled regulon. J Bacteriol 196:1412–1424. https://doi.org/10.1128/JB.01405-13.
25. Puri AW, Owen S, Chu F, Chavkin T, Beck DA, Kalyuzhnaya MG, Lidstrom ME. 2015. Genetic tools for the industrially promising methanotroph *Methylomicrobium buryatense*. Appl Environ Microbiol 81:1775–1781. https://doi.org/10.1128/AEM.03795-14.
26. Cha C, Gao P, Chen YC, Shaw PD, Farrand SK. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol Plant Microbe Interact 11:1119–1129. https://doi.org/10.1094/MPMI.1998.11.11.1119.
27. Penalver CGN, Cantet F, Morin D, Haras D, Vorholt JA. 2006. A plasmid-borne truncated *luxI* homolog controls quorum-sensing systems and extracellular carbohydrate production in *Methylobacterium extorquens* AM1. J Bacteriol 188:7321–7324. https://doi.org/10.1128/JB.00649-06.
28. Pearson JP, Pesci EC, Iglewski BH. 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179:5756–5767. https://doi.org/10.1128/jb.179.18.5756-5767.1997.
29. An JH, Goo E, Kim H, Seo YS, Hwang I. 2014. Bacterial quorum sensing and metabolic slowing in a cooperative population. Proc Natl Acad Sci U S A 111:14912–14917. https://doi.org/10.1073/pnas.1412431111.
30. Chandler JR, Duerkop BA, Hinz A, West TE, Herman JP, Churchill ME, Skerrett SJ, Greenberg EP. 2009. Mutational analysis of *Burkholderia thailandensis* quorum sensing and self-aggregation. J Bacteriol 191:5901–5909. https://doi.org/10.1128/JB.00591-09.
31. Nackerdien ZE, Keynan A, Bassler BL, Lederberg J, Thaler DS. 2008. Quorum sensing influences *Vibrio harveyi* growth rates in a manner not fully accounted for by the marker effect of bioluminescence. PLoS One 3:e1671. https://doi.org/10.1371/journal.pone.0001671.
32. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. 2012. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 76:46–65. https://doi.org/10.1128/MMBR.05007-11.
33. Schaefer AL, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, Sufrin JR, Harwood CS. 2008. A new class of homoserine lactone quorum-sensing signals. Nature 454:595–599. https://doi.org/10.1038/nature07088.
34. Yu X, Lund SP, Greenwald JW, Records AH, Scott RA, Nettleton D, Lindow SE, Gross DC, Beattie GA. 2014. Transcriptional analysis of the global regulatory networks active in *Pseudomonas syringae* during leaf colonization. mBio 5:e01683-595–14.
35. Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J Bacteriol 185:2066–2079. https://doi.org/10.1128/JB.185.7.2066-2079.2003.
36. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185:2080–2095. https://doi.org/10.1128/JB.185.7.2080-2095.2003.
37. Auman AJ, Stolyar S, Costello AM, Lidstrom ME. 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl Environ Microbiol 66:5259–5266. https://doi.org/10.1128/AEM.66.12.5259-5266.2000.
38. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318.
39. Miller WG, Leveau JH, Lindow SE. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13:1243–1250. https://doi.org/10.1094/MPMI.2000.13.11.1243.
40. Yan X, Chu F, Puri AW, Fu Y, Lidstrom ME. 2016. Electroporation-based genetic manipulation in type I methanotrophs. Appl Environ Microbiol 82:2062–2069. https://doi.org/10.1128/AEM.03724-15.
41. Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides NC. 2014. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res 42:D560–D567. https://doi.org/10.1093/nar/gkt963.
42. Antunes LCM, Schaefer AL, Ferreira RBR, Qin N, Stevens AM, Ruby EG, Greenberg EP. 2007. Transcriptome analysis of the *Vibrio fischeri* LuxR-LuxI regulon. J Bacteriol 189:8387–8391. https://doi.org/10.1128/JB.00736-07.
43. Chu F, Lidstrom ME. 2016. XoxF Acts as the predominant methanol dehydrogenase in the type I methanotroph *Methylomicrobium buryatense*. J Bacteriol 198:1317–1325. https://doi.org/10.1128/JB.00959-15.
44. Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595. https://doi.org/10.1093/bioinformatics/btp698.
45. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
46. Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. https://doi.org/10.1093/bioinformatics/btu638.
47. Anders S, Huber W. 2010. Differential expression analysis for sequence count data. Genome Biol 11:R106. https://doi.org/10.1186/gb-2010-11-10-r106.
48. Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, Robinson MD. 2013. Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nat Protoc 8:1765–1786. https://doi.org/10.1038/nprot.2013.099.
49. Thorvaldsdóttir H, Robinson JT, Mesirov JP. 2013. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinformatics 14:178–192. https://doi.org/10.1093/bib/bbs017.