Published in final edited form as:

ACS Chem Biol. 2021 August 20; 16(8): 1332–1338. doi:10.1021/acscchembio.1c00329.

Methylotroph Quorum Sensing Signal Identification by Inverse Stable Isotopic Labeling

Dale A. Cummings Jr., Alice I. Snelling, Aaron W. Puri*

Department of Chemistry and the Henry Eyring Center for Cell and Genome Science, University of Utah, Salt Lake City, UT 84112

Abstract

Natural products are an essential source of bioactive compounds. Isotopic labeling is an effective way to identify natural products that incorporate a specific precursor; however, this approach is limited by the availability of isotopically-enriched precursors. We used an inverse stable isotopic labeling approach to identify natural products by growing bacteria on a ^{13}C -carbon source and then identifying ^{12}C -precursor incorporation by mass spectrometry. We applied this approach to methylotrophs, ecologically important bacteria predicted to have significant yet underexplored biosynthetic potential. We demonstrate this method identifies *N*-acyl homoserine lactone quorum sensing signals produced by diverse methylotrophs grown on three different one-carbon compounds. We then apply this approach to simultaneously detect five previously unidentified signals produced by a methylotroph, and link these compounds to their syntheses. We envision that this method can be used to identify other natural product classes synthesized by methylotrophs and other organisms that grow on relatively inexpensive ^{13}C -carbon sources.

Graphical Abstract

*Corresponding author: Aaron W. Puri, 315 S 1400 E Rm 2020, Salt Lake City, UT 84112, USA, (801) 213-1408, a.puri@utah.edu.
AUTHOR CONTRIBUTIONS

A.W.P. and D.A.C. designed the experiments. D.A.C., A.I.S., and A.W.P. performed the experiments. A.W.P. wrote the manuscript. A.W.P. and D.A.C. edited the manuscript. All authors read and approved of the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

METHODS

See Supporting Information.

Keywords

methylotroph; stable isotope; acyl-homoserine lactone; quorum sensing; natural products

Natural products are an important source of small molecule therapeutics,¹ agricultural compounds,² and other bioactive metabolites. Precursor feeding studies are commonly used to determine the biosynthetic route to a compound of interest, and can be used to identify previously undetected compounds produced by an organism.³⁻⁶ However, this approach is limited by the synthetic or commercial availability of an isotopically-enriched precursor. Alternatively, in an inverse stable isotopic labeling approach, a fully ¹³C-labeled organism is fed a ¹²C-biosynthetic precursor to identify natural products that incorporate this precursor. This incorporation can be detected by a negative shift in the mass-to-charge ratio (*m/z*) consistent with incorporation of all or part of the ¹²C-precursor. With this approach researchers can use any precursor without having to obtain a ¹³C-labeled version. Inverse stable isotopic labeling has been previously used to help determine the biosynthetic origins of the cofactor pyrroloquinoline quinone⁷ and an isocyanide-containing antibiotic.⁸ However, to our knowledge, this approach has not been used to systematically detect previously unidentified natural products.

Inverse stable isotopic labeling is particularly well-suited for bacteria that can grow on inexpensive and readily-available ¹³C-carbon sources, such as methylotrophs. Methylotrophs are bacteria that grow on reduced compounds with no carbon-carbon bonds, such as methane, methanol, and dimethyl sulfide.⁹ These organisms play important roles in biogeochemical cycling, bioremediation, and plant-microbe interactions.^{10,11} Genomic analysis also predicts that methylotrophs possess the biosynthetic potential to produce myriad natural products that have not yet been identified.¹² Identifying and characterizing these molecules can help scientists understand how these important organisms interact with each other and their environment, discover new drug leads, and help synthetic biologists optimize the desirable activities of methylotrophs using exogenous small molecules.

A benefit of working with methylotrophs is that ¹³C-labeled versions of their one-carbon growth substrates are relatively inexpensive. For this reason, a methylotroph was used to create ¹³C-labeled nucleic acids from ¹³C-methanol for structural studies of DNA by NMR.¹³ Stable isotopic labeling has also been used to identify active methylotrophs in the environment,¹⁴ and to create internal metabolomics standards.¹⁵ We sought to take advantage of this benefit to aid in the discovery of methylotroph natural products.

As a proof-of-concept, we applied this method to identify quorum sensing (QS) signals produced by methylotrophs. QS is a form of chemical communication used by bacteria to coordinate group behavior.¹⁶ In one well-characterized form of QS, Gram-negative proteobacteria use *N*-acyl-homoserine lactones (acyl-HSLs) produced by LuxI-family synthases. Acyl-HSL signals vary in their acyl chain, but all possess a common homoserine lactone derived from methionine via *S*-adenosyl-*L*-methionine (Figure 1A).¹⁷ These signals are detected by LuxR-family receptors, which are also transcription factors that regulate gene expression upon signal binding. Methylotrophs often possess QS systems, and several strains have three or more annotated *luxI*-family synthase genes in their genome.^{18,19} The

majority of these genes are predicted to encode CoA-utilizing enzymes known to produce noncanonical signals.²⁰ However, relatively few methylotroph acyl-HSL signals have been characterized, and many cannot be predicted based on the amino acid sequences of their synthases.¹²

Acyl-HSL QS signals are often detected using reporter assays with an engineered strain where the LuxR-family receptor activates the expression of a reporter gene upon signal binding.²¹ However, signal detection is dependent on the specificity of the receptor used, which can lead to false negatives. Reporter assays are often paired with thin layer chromatography to correlate detected signals with the retention factors of known standards, which can be inaccurate. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) can also be used to identify acyl-HSLs based on known fragmentation patterns,²²⁻²⁴ however the fragmentation patterns may be unpredictable for some acyl-HSLs. For example, the aryl-HSL *p*-coumaroyl-HSL does not produce the characteristic *m/z* 102 HSL fragment.²⁵

Detection of methionine incorporation into the common HSL portion of acyl-HSLs is a more generalizable method that has been successfully used to detect QS signals.^{6,25} ¹⁴C-labeled methionine can be used in feeding studies to indirectly identify signals by comparing radioactivity retention via HPLC to the retention times (RTs) of known acyl-HSL standards. We sought to build off of this approach by using an inverse stable isotopic labeling method with ¹²C-methionine to identify methylotroph QS signals (Figure 1B). We show this method works with three ¹³C-labeled one-carbon sources (methane, methanol, and methylamine) in two diverse methylotrophs. We then apply the method to simultaneously identify five QS signals produced by *Methylobacterium rhodinum* DSM2163²⁶ (referred to as DSM2163), including three which could not be predicted based on sequence homology to characterized QS systems. Finally, we link these signals to the synthase genes responsible for their production.

Inverse stable isotopic labeling identifies previously characterized acyl-HSL signals.

To verify the utility of the inverse stable isotopic labeling approach for identifying natural products, we began by applying it to a methylotroph that produces acyl-HSL signals that have been previously characterized. The methanol-oxidizing *Methylobacterium extorquens* PA1 produces a single LuxI-family signal synthase, MlaI, with 100% amino acid sequence identity to the characterized version found in *Methylobacterium extorquens* AM1.^{18,27} Vorholt and coworkers previously demonstrated that MlaI produces two QS signals with unsaturated acyl chains: *N*-(2-*trans*-7-*cis*-tetradecenoyl)-*L*-homoserine lactone (referred to as 2*E*,7*Z*-C_{14:2}-HSL) and *N*-(7-*cis*-tetradecenoyl)-*L*-homoserine lactone (7*Z*-C_{14:1}-HSL) (Figure 2).²⁷

In order to streamline the inverse stable isotopic labeling procedure for future screening of diverse bacteria with potentially different growth rates, we chose to add the ¹²C-methionine precursor at the beginning of growth. We grew the *M. extorquens* PA1 *cel* strain CM2730 (PA1)²⁸ with ¹³C-methanol and different concentrations of ¹²C-methionine. We extracted the supernatant with acidified ethyl acetate, analyzed the extract by LC-MS, and detected

features using the program MZmine2.²⁹ We identified two features with m/z values of 322 and 324, which correspond to protonated versions of $2E,7Z\text{-}C_{14:2}\text{-HSL}$ and $7Z\text{-}C_{14:1}\text{-HSL}$, respectively, that have incorporated the four carbons of ^{12}C -methionine but are otherwise fully ^{13}C -labeled (Figure S1 and Table 1). When we cultured PA1 with ^{12}C -methanol as a control, we identified features with the same RTs but with m/z values of 308 and 310, which correspond to the protonated ^{12}C -versions of $2E,7Z\text{-}C_{14:2}\text{-HSL}$ and $7Z\text{-}C_{14:1}\text{-HSL}$, respectively. Together, these results show that the inverse stable isotopic labeling approach can be used to correctly identify acyl-HSLs.

The methionine titration indicated that the optimal concentration of the ^{12}C -methionine precursor was 0.5 mM when added at the beginning of growth (Figure S1). Higher-than-necessary label concentrations may result in slowed growth or nonspecific incorporation of ^{12}C -precursor carbons due to toxicity or methionine metabolism, respectively. We did not observe any fully ^{13}C -labeled acyl-HSLs using our workflow upon addition of ^{12}C -methionine at any tested concentration. This indicates that for acyl-HSL natural products it is necessary to include a separate ^{13}C -carbon source condition with no precursor as a benchmark to identify ^{12}C -enriched features in the ^{13}C -carbon source + ^{12}C -methionine condition.

In addition to detecting fully ^{13}C -labeled acyl-HSLs in the PA1 culture grown on ^{13}C -methanol without addition of the ^{12}C -precursor, we also detected several features with the same RT but stepwise decreases of one m/z unit. We also identified the same spectral pattern in the ^{13}C -methanol + ^{12}C -methionine condition, but decreased by four m/z units, corresponding to ^{12}C -methionine incorporation (Figure S2). Many methylotrophs in the alphaproteobacteria class, including *M. extorquens*, assimilate reduced one-carbon compounds such as methanol using the serine cycle.⁹ In the serine cycle, half of the carbon input is derived from carbon dioxide, which we did not label in our system and could therefore result in incomplete ^{13}C -labeling of metabolites of interest. The fact that we do observe fully ^{13}C -labeled acyl-HSLs is likely because ^{13}C -carbon dioxide is rapidly produced in the culture via central metabolism. When we grew PA1 on the carbon source ^{13}C -methylamine, we were one carbon short of complete ^{13}C -labeling in one independent replicate (Tables 1 and S1). However, this did not affect our ability to identify a decrease of four m/z units corresponding with ^{12}C -methionine incorporation, because the extent of acyl-HSL ^{13}C -labeling was consistent between the ^{13}C -methylamine and ^{13}C -methylamine + ^{12}C -methionine conditions.

We wrote a Python script to identify pairs of features with matching RTs and a decrease of four m/z units in the ^{13}C -carbon source + ^{12}C -methionine condition compared to the ^{13}C -carbon source condition. For the RTs corresponding to $2E,7Z\text{-}C_{14:2}\text{-HSL}$ and $7Z\text{-}C_{14:1}\text{-HSL}$, our script identifies several pairs of features with the desired m/z difference due to the aforementioned incomplete labeling (Table 1). Notably, out of all the feature pairs identified in the PA1 culture extract, the two acyl-HSL signals have the most identified pairs that correspond with a single feature at the same RT in the ^{12}C -carbon source control (Table S1). We can therefore use this knowledge to prioritize identified features that are more likely to be acyl-HSLs.

In order to determine if this method is more broadly applicable, we applied it to the methane-oxidizing bacterium *Methylobacter tundripaludum* 21/22 (21/22), which was previously shown to produce the QS signal *N*-(3-hydroxydecanoyl)-*L*-homoserine lactone (3-OH-C₁₀-HSL) (Figure 2).³⁰ When we grew 21/22 with ¹³C-methane, we identified a feature with an *m/z* value of 286, corresponding to protonated and fully ¹³C-labeled 3-OH-C₁₀-HSL. We detected a feature with the same RT and a decrease of four *m/z* units in the ¹³C-methane + ¹²C-methionine condition (Table 1), indicating ¹²C-methionine incorporation. These results show that the inverse stable isotopic labeling method can be broadly applied, as it works with the methane-oxidizing gammaproteobacterium 21/22 as well as the methanol- and methylamine-oxidizing alphaproteobacterium PA1.

Simultaneous identification of five acyl-HSL signals produced by the methylotroph DSM2163.

Next, we applied the inverse stable isotopic labeling method to identify the signals produced by the methanol-oxidizer DSM2163, which possesses three annotated LuxI-family acyl-HSL synthases. A previous study used a reporter assay to speculate which acyl-HSL signals are produced by this strain, but the exact signals were not identified.¹⁹ We identified several features with the signature decrease of four *m/z* units when DSM2163 was grown on ¹³C-methanol + ¹²C-methionine compared to ¹³C-methanol alone. Three of these features corresponded to the signals *N*-octanoyl-*L*-homoserine lactone (C₈-HSL), *N*-(3-oxooctanoyl)-*L*-homoserine lactone (3-oxo-C₈-HSL), and *N*-(3-hydroxyoctanoyl)-*L*-homoserine lactone (3-OH-C₈-HSL) (Figure 2 and Table 1), which we confirmed by high-resolution MS (Table S2, Figures S3-S5) as well as by verifying these compounds have identical LC-MS retention times (Figures S6A-C) and high-resolution MS/MS spectra (Tables S3-S5) to commercial standards.

Our method also identified features in the DSM2163 culture corresponding to the signals 2*E*,7*Z*-C_{14:2}-HSL and 7*Z*-C_{14:1}-HSL, which we confirmed by high-resolution MS (Table S2, Figures S7 and S8). These signals had identical LC-MS retention times (Figures S6D and S6E) and high-resolution MS/MS spectra (Tables S6 and S7) compared to the signals produced by PA1. This is consistent with the fact that one of the annotated DSM2163 acyl-HSL synthases shares 91% amino acid identity with MlaI from PA1. Notably, the characteristic *m/z* 102 HSL fragment was not detected in the MS/MS spectra of 2*E*,7*Z*-C_{14:2}-HSL, likely because it contains an α , β unsaturated acyl chain (Table S6), and would therefore not have been identified using LC-MS/MS methods that focused on this transition. Together, these results demonstrate that the inverse stable isotopic labeling method can successfully detect a total of five previously unidentified acyl-HSL QS signals produced by the methylotroph DSM2163.

Heterologous expression links acyl-HSL signals with their synthases.

As more bacterial genomes and metagenomes are sequenced, there is increasing demand for predicting how these organisms interact in the environment. Linking QS signal synthases with the signals they produce helps researchers make these predictions based on sequence homology. We constructed a strain for heterologous expression of *luxI*-family acyl-HSL

synthase genes to link synthases with their signal products. We created an unmarked deletion strain of PA1 called AWP227 that no longer produces acyl-HSL signals by knocking out the acyl-HSL receptor gene *mIaR* and the synthase gene *mIaI*. We then used AWP227 to express each of the three DSM2136 synthase genes separately on a plasmid to determine which signals are produced by each synthase.

When we heterologously expressed the DSM2163 synthase gene Ga0373200_3300, we observed features corresponding to production of C₈-HSL (Figure 3A) and 3-oxo-C₈-HSL (Figure 3B) that were not present in the no-plasmid AWP227 control. Heterologous expression of Ga0373200_1920 also resulted in production of C₈-HSL (Figure 3A), as well as 3-OH-C₈-HSL (Figure 3C). It is therefore unclear if one or both of these synthases are responsible for the C₈-HSL detected in the DSM2163 culture. We also confirmed that the synthase with 91% amino acid identity to MlaI (Ga0373200_956) produces the signals 2*E*,7*Z*-C_{14:2}-HSL and 7*Z*-C_{14:1}-HSL (Figures 3D and 3E). Vorholt and coworkers previously named a LuxI-family synthase that produces *N*-hexanoyl-*L*-homoserine lactone (C₆-HSL) and C₈-HSL MsaI for *Methylobacterium* (now reclassified *Methylorubrum*) short-chain acyl-HSLs.²⁷ We therefore name Ga0373200_1920 MsaI2 and Ga0373200_3300 MsaI3 based on our results, and also refer to Ga0373200_956 as MlaI.

The acyl-HSL signals produced by MsaI2 and MsaI3 could not be predicted on the basis of amino acid sequence alone. MsaI2 shares only 58% amino acid identity with RaiI from *Rhizobium etli* ISP42, which was reported to produce C₈-HSL and 3-OH-C₈-HSL.³¹ MsaI3 does not share >50% amino acid identity with any currently characterized LuxI-family synthases. Together, these annotations will help predict QS signal production from bacterial genome and metagenome sequences. Furthermore, our heterologous expression strain can be used in the future to identify QS signals produced by synthases from non-methylophilic species using the inverse stable isotopic labeling approach.

Natural products are a vetted source of bioactive compounds with medicinal and agricultural value. Isotopic labeling is often used to identify and characterize natural products, however in some cases the labeled precursors required for these studies are difficult to obtain. We used an inverse stable isotopic labeling approach to identify natural products via incorporation of unlabeled precursors, which expands precursor availability for natural product studies. We demonstrate that this approach can be performed using a unit-resolution LC-MS, making it accessible to chemists and microbiologists with diverse expertise. The inverse stable isotopic labeling method can be applied in the future to identify other classes of natural products based on a common precursor, including precursors which may not be readily available in an isotopically-labeled form. For example, this method could be used to identify natural products derived from the terpenoid or shikimate pathways.³² In general, feeding studies using isotopically-enriched precursors are extremely widespread, and the inverse stable isotopic labeling method is applicable in any situation where a traditional method can be used. However, this method is especially well-suited for organisms that grow on inexpensive ¹³C-carbon sources, including methanotrophs and autotrophs such as cyanobacteria, which are known to have significant biosynthetic potential.³³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work is dedicated to M.E. Lidstrom (University of Washington) for her 70th birthday. This work was supported by National Institutes of Health grant R00 GM118762 (to A.W.P.). We thank members of the Puri Lab for reading the manuscript and for helpful discussions. We thank Y. Fu (Facebook, Inc.) for useful discussions about methylotroph metabolism and isotopic labeling that led to this project. We thank N.C. Martinez-Gomez (U.C. Berkeley) for *M. extorquens* pAl *cel* strain CM2730, and M. Hammond (University of Utah) for generously sharing her LC-MS system and other lab equipment. We thank D. Petras (University of Tübingen) for sharing Python code and for helpful discussions about LC-MS analysis.

REFERENCES

- (1). Newman DJ, and Cragg GM (2020) Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J. Nat. Prod* 83, 770–803. [PubMed: 32162523]
- (2). Cantrell CL, Dayan FE, and Duke SO (2012) Natural Products As Sources for New Pesticides. *J. Nat. Prod* 75, 1231–1242. [PubMed: 22616957]
- (3). Gross H, Stockwell VO, Henkels MD, Nowak-Thompson B, Loper JE, and Gerwick WH (2007) The Genom isotopic Approach: A Systematic Method to Isolate Products of Orphan Biosynthetic Gene Clusters. *Chem. Biol* 14, 53–63. [PubMed: 17254952]
- (4). Kinnel RB, Esquenazi E, Leao T, Moss N, Mevers E, Pereira AR, Monroe EA, Korobeynikov A, Murray TF, ..., and Gerwick WH (2017) A Maldii isotopic Approach to Discover Natural Products: Cryptomaldamide, a Hybrid Tripeptide from the Marine Cyanobacterium *Moorea producens*. *J. Nat. Prod* 80, 1514–1521. [PubMed: 28448144]
- (5). May DS, Crnkovic CM, Kronic A, Wilson TA, Fuchs JR, and Orjala JE (2020) ¹⁵N Stable Isotope Labeling and Comparative Metabolomics Facilitates Genome Mining in Cultured Cyanobacteria. *ACS Chem. Biol* 15, 758–765. [PubMed: 32083834]
- (6). Schaefer AL, Harwood CS, and Greenberg EP (2018) “Hot Stuff”: The Many Uses of a Radiolabel Assay in Detecting Acyl-Homoserine Lactone Quorum-Sensing Signals, in *Quorum Sensing* (Leoni L and Rampioni G, Eds.), pp 35–47. Springer New York, New York, NY.
- (7). van Kleef MAG, and Duine JA (1988) *L*-tyrosine is the precursor of PQQ biosynthesis in *Hyphomicrobium* X. *FEBS Lett.* 237, 91–97. [PubMed: 2844590]
- (8). Brady SF and Clardy J (2005) Systematic Investigation of the *Escherichia coli* Metabolome for the Biosynthetic Origin of an Isocyanide Carbon Atom. *Angew. Chem. Int. Ed* 44, 7045–7048.
- (9). Chistoserdova L, and Lidstrom ME (2013) Aerobic Methylotrophic Prokaryotes, in *The Prokaryotes* (Rosenberg E, DeLong EF, Lory S, Stackebrandt E, and Thompson F, Eds.), pp 267–285. Springer Berlin Heidelberg, Berlin, Heidelberg.
- (10). Kumar M, Tomar RS, Lade H, and Paul D (2016) Methylotrophic bacteria in sustainable agriculture. *World J. Microbiol. Biotechnol* 32, 120. [PubMed: 27263015]
- (11). Strong PJ, Xie S, and Clarke WP (2015) Methane as a Resource: Can the Methanotrophs Add Value? *Environ. Sci. Technol* 49, 4001–4018. [PubMed: 25723373]
- (12). Puri AW (2019) Specialized Metabolites from Methylotrophic Proteobacteria. *Curr. Issues Mol. Biol* 211–224. [PubMed: 31166194]
- (13). Batey RT, Cloutier N, Mao H, and Williamson JR (1996) Improved Large Scale Culture of *Methylophilus methylotrophus* for ¹³C/¹⁵N Labeling and Random Fractional Deuteration of Ribonucleotides. *Nucleic Acids Res.* 24, 4836–4837. [PubMed: 8972874]
- (14). Radajewski S, Ineson P, Parekh NR, and Murrell JC (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* 403, 646–649. [PubMed: 10688198]
- (15). Yang S, Sadilek M, and Lidstrom ME (2010) Streamlined pentafluorophenylpropyl column liquid chromatography–tandem quadrupole mass spectrometry and global ¹³C-labeled internal standards improve performance for quantitative metabolomics in bacteria. *J. Chromatogr. A* 1217, 7401–7410. [PubMed: 20950815]

- (16). Papenfort K and Bassler BL (2016) Quorum sensing signal–response systems in Gram-negative bacteria. *Nat. Rev. Microbiol* 14, 576–588. [PubMed: 27510864]
- (17). Moré MI, Finger LD, Stryker JL, Fuqua C, Eberhard A, and Winans SC (1996) Enzymatic Synthesis of a Quorum-Sensing Autoinducer Through Use of Defined Substrates. *Science* 272, 1655–1658. [PubMed: 8658141]
- (18). Marx CJ, Bringel F, Chistoserdova L, Moulin L, Farhan Ul Haque M, Fleischman DE, Gruffaz C, Jourand P, Knief C, ..., and Vuilleumier S (2012) Complete genome sequences of six strains of the genus *Methylobacterium*. *J. Bacteriol* 194, 4746–4748. [PubMed: 22887658]
- (19). Poonguzhali S, Madhaiyan M, and Sa T (2007) Production of Acyl-Homoserine Lactone Quorum-Sensing Signals is Wide-Spread in Gram-Negative *Methylobacterium*. *J. Microbiol. Biotechnol* 17, 226–233. [PubMed: 18051753]
- (20). Liao L, Schaefer AL, Coutinho BG, Brown PJB, and Greenberg EP (2018) An aryl-homoserine lactone quorum-sensing signal produced by a dimorphic prosthecate bacterium. *Proc. Natl. Acad. Sci* 115, 7587–7592. [PubMed: 29967162]
- (21). Thornhill SG, and McLean RJC (2018) Use of Whole-Cell Bioassays for Screening Quorum Signaling, Quorum Interference, and Biofilm Dispersion, in *Quorum Sensing* (Leoni L and Rampioni G, Eds.), pp 3–24. Springer New York, New York, NY.
- (22). Morin D, Grasland B, Vallée-Réhel K, Dufau C, and Haras D (2003) On-line high-performance liquid chromatography–mass spectrometric detection and quantification of *N*-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. *J. Chromatogr. A* 1002, 79–92. [PubMed: 12885081]
- (23). Patel NM, Moore JD, Blackwell HE, and Amador-Noguez D (2016) Identification of Unanticipated and Novel *N*-Acyl L-Homoserine Lactones (AHLs) Using a Sensitive Non-Targeted LC-MS/MS Method. *PLoS One* 11, e0163469. [PubMed: 27706219]
- (24). Gould TA, Herman J, Krank J, Murphy RC, and Churchill MEA (2006) Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J. Bacteriol* 188, 773–783. [PubMed: 16385066]
- (25). Schaefer AL, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, ..., and Harwood CS (2008) A new class of homoserine lactone quorum-sensing signals. *Nature* 454, 595–599. [PubMed: 18563084]
- (26). Green PN, and Ardley JK (2018) Review of the genus *Methylobacterium* and closely related organisms: a proposal that some *Methylobacterium* species be reclassified into a new genus, *Methylorubrum* gen. nov. *Int. J. Syst. Evol. Microbiol* 68, 2727–2748. [PubMed: 30024371]
- (27). Nieto Penalver CG, Morin D, Cantet F, Saurel O, Milon A, and Vorholt JA (2006) *Methylobacterium extorquens* AM1 produces a novel type of acyl-homoserine lactone with a double unsaturated side chain under methylotrophic growth conditions. *FEBS Lett.* 580, 561–567. [PubMed: 16412429]
- (28). Delaney NF, Kaczmarek ME, Ward LM, Swanson PK, Lee M-C, and Marx CJ (2013) Development of an optimized medium, strain and high-throughput culturing methods for *Methylobacterium extorquens*. *PLoS One* 8, e62957. [PubMed: 23646164]
- (29). Pluskal T, Castillo S, Villar-Briones A, and Orešič M (2010) MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* 11, 395. [PubMed: 20650010]
- (30). Puri AW, Schaefer AL, Fu Y, Beck DAC, Greenberg EP, and Lidstrom ME (2017) Quorum Sensing in a Methane-Oxidizing Bacterium. *J. Bacteriol* 199, e00773–16. [PubMed: 27994019]
- (31). Pérez-Montaña F, Guasch-Vidal B, González-Barroso S, López-Baena FJ, Cubo T, Ollero FJ, Gil-Serrano AM, Rodríguez-Carvajal MÁ, Bellogín RA, and Espuny MR (2011) Nodulation-gene-inducing flavonoids increase overall production of autoinducers and expression of *N*-acyl homoserine lactone synthesis genes in rhizobia. *Res. Microbiol* 162, 715–723. [PubMed: 21600980]
- (32). Rinkel J, and Dickschat JS (2015) Recent highlights in biosynthesis research using stable isotopes. *Beilstein J. Org. Chem* 11, 2493–2508. [PubMed: 26734097]

- (33). Dittmann E, Gugger M, Sivonen K, and Fewer DP (2015) Natural Product Biosynthetic Diversity and Comparative Genomics of the Cyanobacteria. *Trends Microbiol.* 23, 642–652. [PubMed: 26433696]
- (34). Hoang TT, Sullivan SA, Cusick JK, and Schweizer HP (2002) β -Ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths. *Microbiology* 148, 3849–3856. [PubMed: 12480888]
- (35). Val DL, and Cronan JE (1998) In Vivo Evidence that *S*-Adenosylmethionine and Fatty Acid Synthesis Intermediates Are the Substrates for the LuxI Family of Autoinducer Synthases. *J. Bacteriol* 180, 2644–2651. [PubMed: 9573148]
- (36). Volpe JJ, and Vagelos PR (1976) Mechanisms and regulation of biosynthesis of saturated fatty acids. *Physiol. Rev* 56, 339–417. [PubMed: 6981]

Figure 1.

(A) Acyl-HSL biosynthesis incorporates methionine. *L*-Met: *L*-methionine.

MetK: *S*-adenosyl-*L*-methionine synthase. SAM: *S*-adenosyl-*L*-methionine. MTA: 5'-methylthioadenosine. (B) The inverse stable isotope labeling approach applied to acyl-HSL signals. Note that the acyl carbonyl carbon is not denoted as ¹²C or ¹³C as it can be derived from endogenous or exogenous sources and therefore may or may not be labeled when an organism is grown on a ¹³C-carbon source.

Figure 2.

Acyl-HSLs identified in this work. Stereochemistry was not determined and is inferred based on previous studies. All biologically produced acyl-HSLs that have been characterized have *L*-homoserine lactones. The hydroxyls in 3-OH-C₈-HSL and 3-OH-C₁₀-HSL are shown as 3*R* because their synthases are annotated as acyl carrier protein (ACP)-linked (KEGG Orthology term K13060) and these enzymes use acyl-ACPs from fatty acid biosynthesis^{34,35} where the stereoselective β-ketoacyl acyl carrier protein reductase FabG produces 3*R*-OH fatty acyl chains.³⁶

Figure 3.

Linking *M. rhodinum* DSM2163 LuxI-family acyl-HSL synthases with their products. Extracted ion chromatograms of supernatant extracts for the listed strains for the *m/z* values (A) 250.1419, corresponding to C₈-HSL, (B) 264.1212, corresponding to 3-oxo-C₈-HSL, (C) 266.1368, corresponding to 3-OH-C₈-HSL, (D) 330.2045, corresponding to 2E,7Z-C_{14:2}-HSL, and (E) 332.2202, corresponding to 7Z-C_{14:1}-HSL. All masses correspond to [M+Na]⁺. Mass tolerance < 5 ppm.

Table 1.

Identification of acyl-HSLs using the inverse stable isotopic labeling approach.

Strain	Carbon source	¹² C-carbon source		¹³ C-carbon source		¹³ C-carbon source + ¹² C-methionine		# Pairs detected	Identified Acyl-HSL
		RT (min)	m/z	RT (min)	Max paired m/z ^a	RT (min)	Max paired m/z		
<i>M. extorquens</i> PA1	Methanol	23.0 ^b	310	23.0	328	23.1	324	6	7Z-C _{14:1} -HSL
		22.7	308	22.7	326	22.8	322	5	2E,7Z-C _{14:2} -HSL
	Methylamine	23.1	310	23.1	327	23.2	323	5	7Z-C _{14:1} -HSL
		22.8	308	22.8	325	22.9	321	5	2E,7Z-C _{14:2} -HSL
<i>M. tundripaludum</i> 21/22	Methane	15.6	272	15.6	286	15.6	282	1	3-OH-C ₁₀ -HSL
<i>M. rhodinum</i> DSM2163	Methanol	22.9	308	22.9	326	22.8	322	5	2E,7Z-C _{14:2} -HSL
		23.2	310	23.2	328	23.1	324	5	7Z-C _{14:1} -HSL
		12.0	244	12.0	256	12.0	252	4	3-OH-C ₈ -HSL
		15.6	228	15.5	240	15.5	236	3	C ₈ -HSL
		12.7	242	12.7	254	12.7	250	1	3-oxo-C ₈ -HSL

^aMax paired *m/z* refers to the maximum feature *m/z* observed in that condition where the desired *m/z* difference of four units was observed compared to the other ¹³C-condition.

^bRT differences between PA1 and DSM2163 for 2E,7Z-C_{14:2}-HSL and 7Z-C_{14:1}-HSL were resolved when smaller injection volumes were used after initial screening (see Figure S6).