Microbial cysteine degradation is a source of hydrogen sulfide in oxic freshwater lakes

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Abstract

The sulfur-containing amino acid cysteine is abundant in the environment including in freshwater lakes. Biological degradation of cysteine can result in hydrogen sulfide (H_2S), a toxic and ecologically relevant compound that is a central player in biogeochemical cycling in aquatic environments, including freshwater lakes. Here, we investigated the ecological significance of cysteine in oxic freshwater lake environments, using model systems of isolated cultures, controlled growth experiments, and multi-omics. We screened bacterial isolates enriched from natural lake water for their ability to produce H_2S when provided cysteine. In total, we identified 29 isolates that produced H_2S and belonged to the phylum proteobacteria Bacteroidetes, Proteobacteria and Actinobacteria. To understand the genomic and genetic basis for cysteine degradation and H_2S production, we further characterized 3 freshwater isolates using whole-genome sequencing, and quantitatively tracked cysteine and H_2S levels over their growth ranges: *Stenotrophomonas maltophila*, *Stenotrophomonas bentonitica* (Gammaproteobacteria) and *Chryseobacterium piscium* (Bacteroidetes). We observed a decrease in cysteine and increase in H_2S, and identified genes involved in cysteine degradation in all 3 genomes. Finally, to assess the presence of these organisms and genes in the environment, we surveyed a five-year time series of metagenomic data from the same isolation source at Lake Mendota and identified their presence throughout the time series. Overall, our study shows that sulfur-containing amino acids can drive microbial H_2S production in oxic environments. Future considerations of sulfur cycling and biogeochemistry in oxic environments should account for H_2S production from degradation of organosulfur compounds.

Importance

Hydrogen sulfide (H_2S), a naturally occurring gas with biological origins, can be toxic to living organisms. In aquatic environments, H_2S production typically originates from anoxic (lacking oxygen) environments such as sediments, or the bottom layers of lakes. However, the degradation of sulfur-containing amino acids such as cysteine, which all cells and life forms rely on, can be a source of ammonia and H_2S in the environment. Unlike other approaches for the biological production of H_2S such as dissimilatory sulfate reduction, cysteine degradation can

occur in the presence of oxygen. Yet, little is known about how cysteine degradation influences sulfur availability and cycling in freshwater lakes. In our study, we found that multiple bacteria originating from a freshwater lake can not only produce H_2S, but do so in oxic conditions. Overall, our study highlights the ecological importance of oxic H_2S production in natural ecosystems, and necessitates a change in our outlook of sulfur biogeochemistry.

Introduction

In most natural environments, the production of hydrogen sulfide gas (H_2S) is usually attributed to defined groups of bacteria and archaea (1, 2) , and occurs primarily in anoxic environments. During the process of dissimilatory sulfate reduction, sulfate acts as a terminal electron acceptor, and is converted to hydrogen sulfide. However, other pathways for H_2S production exist, namely assimilatory sulfate production, in which H_2S contributes to cell growth and increased biomass, and the conversion of sulfur-containing amino acids such as cysteine which can lead to production of pyruvate, ammonia, and H_2S (3). It is believed that assimilatory sulfate reduction contributes to growth but does not release H_2S from the cell, while dissimilatory sulfate reduction and cysteine degradation can contribute to growth and release ecologically relevant nitrogen and sulfur compounds into the ecosystem.

The sulfur cycle is composed of several assimilatory and dissimilatory pathways, which interact in complex ways through biotic and abiotic factors. Sulfur cycling in freshwater ecosystems can have significant ecological significance, especially in places where strong redox gradients exist (4). For example, in high arctic lakes, sulfur-compounds are suggested to serve as biogeochemical hubs (5). Cysteine, a sulfur containing amino acid, has been proposed to be an overlooked carbon source (6) and is an overlooked source of sulfur. Additionally, seston (moving water that contains both living and nonliving organisms) contains organosulfur containing lipids which settle into the sediments, and contributes to the sulfur pool in lakes such as Lake Superior (7).

In seasonally stratified lakes consisting of two layers of oxygenated warm water (epilimnion) floating atop colder anoxic waters (hypolimnion), H_2S is often abundant in the hypolimnion (8, 9), due to the presence of anoxic sediment and an anoxic water column. However, an overlooked player in the pool of available H_2S is the utilization of organosulfur compounds such as cysteine by microbes. Cysteine is required for the production of proteins and is important for protein structure. It is one of the two amino acids (methionine being the other) that contains a sulfur group; however, the sulfhydryl group on cysteine is more reactive and can lead to the formation of H_2S. Like all amino acids, cysteine also contains an amine group that will form ammonia once the molecule is degraded. As such, the degradation of cysteine by microbes leads to the production of H_2S. H_2S is ecologically relevant because it can be toxic to plants and animals. During periods of anoxia, H_2S can accumulate to levels beyond the threshold for living organisms, and can cause massive fish kills (10). Unlike other sources of H_2S, cysteine degradation could occur under oxic conditions, thereby expanding the environmental scope of this sulfur pool. In addition, cysteine has been found to be able to be degraded under oxic conditions in the laboratory, but little information exists on the natural prevalence of this process in lakes. We expect that H_2S production in oxic environments (during the mixed water column periods of the year, and throughout the stratified period in the mixed epilimnion) could result from cysteine utilization by microbes.

In this study, we investigated the prevalence of organosulfur degradation in aquatic ecosystems, using both laboratory and genomic evidence, to inform our understanding of oxic sulfur cycling in freshwater lakes (Figure 1). First, we grew bacterial isolates enriched from Lake Mendota's oxic epilimnion to quantify H_2S and ammonia production, which informs the potential for organosulfur degradation in an oxygenated aquatic environment. We found 18 isolates producing H_2S under oxygenic conditions. We selected three H_2S-producing isolates for detailed characterization using full-genome sequencing and chemical analyses to track cysteine and H_2S production over their growth: *Stenotrophomonas maltophila*, *Stenotrophomonas S. bentonitica* (Gammaproteobacteria) and *Chryseobacterium piscium* (Bacteroidetes). In all three isolates, cysteine decreased when H_2S increased over their exponential growth curve under oxic conditions. Finally, we contextualized our laboratory results using a time-series of metagenomic data from the same isolation source (Lake Mendota, WI), in order to inform the temporal

112 importance of organosulfur degradation. We found that genes for cysteine utilization were
113 present and abundant throughout the time-series suggesting that the ability to degrade cysteine is
114 widely distributed in Lake Mendota.
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116 Results

117 Isolates capable of H_2S production in oxic conditions

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119 To answer the question of whether bacteria could produce H_2S in the presence of oxygen, we
120 grew pure culture isolates originally isolated from the water column of temperate eutrophic Lake
121 Mendota. We grew the isolates under control and treatment conditions (addition of cysteine), and
122 tracked H_2S production after 24 hours **(Figure 2, Table S1)**. Using qualitative H_2S
123 measurements, we found that 18 isolates produced H_2S when grown with cysteine. We
124 performed 16S rRNA sequencing on the 29 isolates that produced H_2S. Isolates that produced
125 both H_2S and ammonia were identified as *Stenotrophomonas rhizophila*, *Stenotrophomona*
126 *maltophilia* (Betaproteobacteria), *Citrobacteria gillenii* (Gammaproteobacteria) and
127 *Chryseobacterium sp.* (Bacteroidetes), whereas those producing H_2S but not ammonia were
128 identified as *Pseudomonas arsenicoxydans, Pseudomonas mandelii, Pseudomonas migulae,*
129 *Pseudomonas thivervalensis,* and *Microbacterium flavescens*.
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131 Detailed microbiological, chemical and genomic characterization in selected isolates

132

133 Next, we selected 3 isolates (#43, #13 and #66) representing distinct species that produced H_2S
134 for further characterization. These detailed characterizations include OD_{600}-based growth rates,
135 and paired quantitative measurements of cysteine and H_2S. The addition of cysteine resulted in
136 concommital production of H_2S over time (**Figure 3A, Table S2**). All organisms used L-cysteine
137 preferably to D-cysteine (**Figure 3B, Table S3**).
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Next, we also performed full-genome sequencing using combined short-read and long-read sequencing on these 3 isolates (**Table 1**). We performed full-genome sequencing because functional information such as gene content cannot be predicted reliably from 16S rRNA sequencing of the isolates alone. The full genome of Isolate 43 was assembled into a single circular genome, with estimated completeness of 100%, and taxonomically assigned to *Stenotrophomonas maltophila*. Unlike the 16S rRNA sequence which assigned it to *S. rhizophila*, the full-genome was actually closer to *S. maltophila*. The full genome of Isolate 13, could be assembled into 2 long contigs, but also with completeness of 100% and taxonomically assigned to *S. bentonitica*. The *Chryseobacter* genome was assembled in one circular genome, and 100% complete, and assigned to *Chryseobacterium piscium*. Overall, the 16S rRNA amplicon sequencing performed prior agreed with full-genome sequencing assignment in some cases, and in others, the whole-genome sequencing assignment allowed finer taxonomic resolution (such as in the case of Isolate 13), and overall provides more information about the genetic content of the genomes. The genomic content was then used to inform how or why H_2S might be produced in oxic environments, as shown in the laboratory experiment.

Using genome-level gene annotations of the 3 isolates, we identified the presence of genes involved in cysteine utilization. We identified genes involved in the degradation of cysteine to ammonia, pyruvate and H_2S: metC, malY, tnaA, cysM, cysK (these 5 start from L-cysteine), and dcyD (which uses D-cysteine as a starting substrate) (**Table S4**). However, we note that these genes may have other enzymatic activities, such as cysteine biosynthesis instead of degradation (**Table S5**). (**Figure 4**)

Leveraging the full-genomic content of the 3 isolates (**Table S6**), we proposed a cellular map of these isolates (**Figure 5A**). All three isolates had genes related to central carbon metabolism: including the TCA cycle, glycolysis, gluconeogenesis, the pentose phosphate pathway and the glyoxylate cycle. They could both generate fatty acids using fatty acid biosynthesis, and utilize fatty acids through the beta-oxidation pathway. As expected, they had genes for the cysteine metabolism, including cysteine biosynthesis pathways from homocysteine and serine, as well as other amino acid utilization, such as methionine degradation pathway.

In spite of these similarities, the three isolates also have distinguishing characteristics amongst them (**Table S6, Figure 5B**). For example, while all have genes for sulfur oxidation (sulfur dioxygenase), genes for thiosulfate oxidation were present in the two *Stenotrophomonas* isolates but not *Chryseobacterium*. Isolate #66 (*Chryseobacter piscium*) contained a urease suggesting the use of organic nitrogen in the form of urea but this was absent in the two *Stenotrophomonas* isolates.

Finally, many more genes for sugar utilization were identified in the two *Stenotrophomonas* isolates in the comparison to *Chryseobacterium*.

Presence of cysteine-degrading organisms and genes in a five-year metagenomic environmental time-series

To put these laboratory results and lab-grown organisms into a natural environment context, we leveraged previously published genomic time-series of Lake Mendota spanning 2008-2012 in the oxygenated epilimnion to search for the presence of cysteine degradation genes in metagenomic data.

First, we searched the time-series to see if organisms in our study were also present in the time series. To do this, we linked the 16S rRNA sequences of the isolated organisms to the assembled metagenomes from the time series. We found that while the 16S rRNA sequences were also present in the time series (**Table S7, S8, S9**), and broadly distributed over time, these scaffolds were not part of binned genomes. Therefore, little information about these isolates would be gathered from metagenomic data only. As such, the full-genome sequencing we performed was particularly helpful in understanding the full genomic structure of the H_2S-producing organisms.

Second, we searched for the 6 genes associated with cystine degradation and production of H_2S (**Table S5**) (in binned and unbinned scaffolds). In total, we searched over 22 million amino acid sequences and identified 1882 hits to 5 genes, since no dcyD homologs (involved in the D-cysteine to pyruvate, ammonia and hydrogen sulfide degradation pathway) were found (**Figure 6, Table S10**). cysK and malY were the genes with most corresponding matches at any time

point, followed by metC and cysM. Only 2 scaffolds contained tnaA. Overall, after correcting for the genome size, there was no visible temporal trend of the genes, although genes were found throughout the 5-year time series.

Among these cysteine-degrading gene sequences, several were identified in binned MAGs (**Figure 6B, Table S11**), therefore taxonomy could be assigned to them. Overall, 139 genes were distributed in genomes of Actinobacteria, Bacteroidetes, Chloroflexota, Cyanobacteria, Planctomycetes, Proteobacteria, and Verrucomicrobia, which are common freshwater bacteria, and broadly distributed in freshwater lakes. tnaA was only present in Bacteroidetes, but other genes were more broadly distributed.

Discussion

Different types of H_2S production: the fate of cysteine and the origin of the H_2S

The H_2S producing isolates identified by us fell into two groups, ammonia-producing and ammonia-consuming, when grown in media with cysteine. We hypothesize that those that produced ammonia and H_2S in the presence of cysteine, but not under "controlled" conditions were those that were potentially contributing to the H_2S pool. However, it is possible that the isolates that consume ammonia also conduct the cysteine degradation pathway, but do not excrete ammonia from the cell. Instead, since ammonia is an important biological compound, it could be used by the organism instead of being released into the media and measured.

We do not believe that this occurs with H_2S, and that most, if not all, of the isolates that produce H_2S in the presence of oxygen will excrete it from the cell. H_2S is toxic to organisms that undergo aerobic cellular respiration (11). There is evidence that some bacteria use H_2S as a protective compound against antibiotics, but it also creates a large amount of stress on the cell making it useful only in extreme situations (12). While it is possible that organisms may use H_2S

internally as a source of sulfur, we did not identify any sulfide quinone oxidoreductases, flavocytochrome c dehydrogenases, or other genes for the oxidation or transformation of H_2S. All 3 isolates were obligate aerobes based on laboratory assays.

Of the 20 amino acids, little is known about the cysteine in the environment. One of the difficulties in studying the fate of cysteine in oxic environmentsis that it can be oxidized into cystine (13), which *E.coli* has been shown to uptake (14). In a study of *E.coli* K-12 that lacked a cysteine transporter, cysteine was found to be able to enter the cell through transporters dedicated to other amino acids that worked best when no amino acids alternatives other than cysteine were present in the medium (15). It is likely that the majority of H_2S produced by the cell in the three isolates originates from cysteine given the demonstrated reduction in cystine concentrations when the isolates are grown with added cysteine (**Figure 3**), coupled with the release of H_2S and production of ammonia.

Genomic structure of the H_2S-producing isolates

Overall, the three isolates selected for whole genome sequencing revealed genes for cysteine degradation into H_2S. Based on laboratory studies, they were able to produce H_2S in the presence of oxygen. All genomes were obligate aerobes, which brings interesting questions about the life history of these organisms.

Little information is known about the ecology of *Stenotrophomonas maltophilia*, *Stenotrophomonas Bentonitica*, and *Chryseobacterium piscium* in the natural environment. *S. bentonitica* was originally characterized in bentonite formations, and was predicted to have high tolerance to heavy metals (16), and has been observed in arctic seawater (17). *C. piscium* was also isolated from a fish in the arctic ocean (18), but its ecological significance in the oceans remains unknown. This previously described *C. piscium* strain LMG 23089 was not reported to produce H_2S yet our genetic analyses suggest that it has the enzymatic machinery to degrade cysteine.

One possible explanation for this discrepancy is that LMG 23089 was previously grown on SIM medium to test H_2S, which is lower resolution than the modern H_2S probes which measure µM concentrations, and because SIM medium uses thiosulfate as a sulfur source. As a side test on isolate #66, H_2S was not produced when thiosulfate was provided, but H_2S was produced when cysteine was provided.

One particular finding of this study was that none of the 6 genes searched for cysteine degradation into H_2S and ammonia was common to *all* three isolates, despite all three isolates showing the same cysteine-decrease, ammonia-increase and H_2S-increase over time. This could be explained by alternative, perhaps less straightforward pathways for H_2S production. One pathway is led by a gene named cystathionine gamma-lyase ("CTH" or "CSE"). In some bacteria and mammals, this enzyme is involved in H_2S production (19). A HMM search for this enzyme showed that it was present in Isolate #13, 43 and #66. While it was not initially included in the initial methods and study, this could hint to another commonality among oxic H_2S producing organisms.

Challenges associated with measuring oxic H_2S production from organosulfur in the environment

Extrapolating these laboratory results to widespread distribution of organosulfur degradation in the natural environment necessitates several steps, namely because of the major gaps that exist concerning the sulfur cycle in freshwater lakes, and because bridging the gap between cultivation-based, omics-based (20) , and field-based experiments is needed. Foremost, the identity, distribution, and availability of organosulfur compounds broadly across lakes globally is currently mostly unknown. Cysteine is notoriously difficult to measure, and many previous studies characterizing the amino acid composition of the water column only measure the sulfur-containing organosulfur compound taurine (21, 22).

Organic sulfur in the form of cysteine is an important organosulfur amino acid, and is important in protein folding and function (23). As such, there is a difference in the fates of cysteine when it exists bound in cell walls, versus when cysteine is free in the water column and available for

degradation by bacteria. While cysteine has been shown to contribute to the carbon pool and carbon flow in lakes (6), more quantitative field measurements are necessary to support whether cysteine also serves as a sulfur pool. Yet, other forms of organosulfur have important significance in aquatic environments. In marine environments for example DMSP (dimethylsulfoxonium propionate) is a critical component of the marine organosulfur cycle (24).

Additionally, current differences between computational gene homology searches versus *in vivo* enzymatic functions are challenging to assess for the genes responsible for the degradation of cysteine into pyruvate, ammonia and H_2S. One reason is that the enzymatic activity of the gene has mostly been described in model organisms such as *E. coli*, and it has been shown that gene activity can be induced by genetic factors, or environmental factors such as metals (25). At least 6 genes have been proposed to have this enzymatic activity, yet, each gene may serve different functions *in situ*, and it is difficult to assert directionality of enzymatic function based on metagenomic or genomic analyses only. To this end, the isolated bacterial strains from this study, which are non-model organisms, and originate from the natural freshwater lake environment, may be used for further detailed biochemical, physiological, and microbiological studies. Further characterization of these bacterial isolates using gene-knockoutsand gene expression studies may inform the functional activity of these genes in nature.

Implications of oxic H_2S production by microbes in freshwaters

This study demonstrates that the production of H_2S by microbes in lake ecosystems occurs in the presence of oxygen, using evidence from pure culture bacterial isolates, and screening of long-term time series of metagenomic lake data. By combining lake-to-laboratory experiments, we show that multiple bacterial strains spanning Gammaproteobacteria, Betaproteobacteria, Actinobacteria and Bacteroidetes are all able to produce H_2S under oxic conditions, and at temperatures that would be ecologically relevant for surface lake water during the summer. Surface water temperatures in Lake Mendota can reach up to 27°C, and the top few meters of water surface are saturated in oxygen. Long-term ecological data shows such trends. Worldwide, maximum lake surface temperature can range between 23 to 31°C (26) (**Table S12**).

Unlike dissimilatory sulfate reduction, cysteine utilization by bacteria to generate ammonia, pyruvate and H_2S, is not dependent on sulfate as an initial reaction substrate. Increased sulfate concentrations are shown to lead to higher sulfide reduction rates in shallow eutrophic freshwater, the sulfur originating from algal decay for example (27). While Lake Mendota is a low-iron and high sulfate lake (28), not all lakes have elevated sulfate levels, and therefore, H_2S production might previously not have been thought of as relevant to study. However, sulfur-containing amino-acids can have many origins. In lakes, concentrations of amino acids (free and combined) often reflect the input and outputs of the lake (29, 30). For example, amino acids (although cysteine was not measured) contribute a detectable amount to the nitrogen cycle, and are facilitated by bacterial activity (30).

There are several implications for aerobic H_2S production in oxygenated aquatic ecosystems. In the carbon cycle, once aerobic methanotrophs were identified as early as 1900's, further aerobic methanotrophs from various phyla of bacteria were found to be ubiquitous in aquatic ecosystems, and have had implications for the cycling of methane, a potent greenhouse gas that is 25X more potent than CO_2. Likewise, the discovery of aerobic bacterial methanogenesis (31), contributes to resolving the "methane paradox", in which methane was often measured in oxygenic surface waters, yet the molecular paradigm explaining it previously was strictly known to occur in anoxic environments. In these two cases, the observation of the processes (methanogenesis and methane consumption) that were typically thought to occur in anoxic environments only, but in oxic environments now, are crucial for the understanding of the carbon (biogeochemical cycle). In a similar vein, much remains to be discovered about the sulfur cycle. In lakes, H_2S has typically been associated with anoxic environments, and from processes that are oxygen-sensitive such as dissimilatory sulfate reduction. Here, we show hydrogen-sulfide production to not only occur in oxic conditions but also by several guilds of bacteria, and in detectable quantities.

Freshwater lakes which are dimictic can become stratified in temperature and oxygen during the summer, and oxygen concentrations vary throughout the year. In the fall and spring, oxygen is abundant, and cysteine degradation into H_2S could be a relevant process for the sulfur pool, and

the fluxes of H_2S to the atmosphere could be significant since wind is prevalent. Under ice during the winter, where oxygen is plentiful, H_2S could be produced but could be consumed or oxidized, but gases would be trapped under ice. During summer, the anoxic hypolimnion and sediments are known sources of H_2S due to dissimilatory sulfate reduction, but density gradients would prevent H_2S from reaching the atmosphere. However, during the summer, the oxygenated mixed epilimnion could be sources of H_2S through organosulfur degradation. If we consider the importance of oxic hydrogen sulfide production, which could occur year-round, the pool of H_2S and the scope of sulfur transformations may be greater than anticipated, if we focus solely on the anoxic hypolimnion (**Figure 7**). Future work aiming to understand the broader distribution of sulfur-containing amino acids and other organosulfur compounds in freshwaters, their fates and transformations, as well as their contribution to H_2S production, will inform global sulfur biogeochemical cycling.

Methods

Enrichment cultures of isolates from a temperate freshwater lake

Lake Mendota (43°06′24″ N 89°25′29″ W) is a temperate eutrophic lake in South-Central Wisconsin, in Madison, WI, USA. Lake Mendota is part of the Long-Term Ecological Research Network North Temperate Lakes (NTL-LTER, https://lter.limnology.wisc.edu/about/lakes). Lake Mendota encounters annual stratification and annual seasonal anoxia in the hypolimnion. Lake water was collected on September 14, 2018 from an integrated water sample (0m to 12m) from Lake Mendota at the Deep Hole location (43°05'54", 89°24'28"), where the maximum depth is 23.5m. The lake water was collected in pre-acid washed 2L sampling bottles using a flexible PVC tubing, and brought back on shore within hours for immediate processing. The water samples were collected during stratification and the oxygenated epilimnion. Serial dilution was performed and grown on PCB (plate count media broth) agar media, at room temperature (~21^{o}C), in the lab. The PCB media was made of: 1L water, 5g/L of yeast extract, 10g/L of

tryptone and 2g/L of dextrose/D-glucose. If grown on solid media, 10g of agar per 1L media was added. Enrichment resulted in about 60 isolates.

Screening for cysteine degradation into H_2S and ammonia

Isolates were able to grow on PCB and R2A media. R2A media is a culture medium for bacteria that typically grow in water. It is less "nutrient rich" than PCB media, and therefore slightly closer to natural lake water. For the screening of the isolates for H_2S production, we grew them on R2A media. Each isolate had two treatments: grown in R2A media without cysteine for the control, and grown in R2A media with amended cysteine as the treatment.

R2A media consisted of (1L of water): 0.5g of casein, 0.5g of dextrose, 0.5g of starch, 0.5g of yeast extract, 0.3g of K_2HPO_4, 0.3g of sodium pyruvate, 0.25g of peptone, 0.25g of beef extract, 0.024g of $MgSO_4$, and autoclave. To make the same media for plates, we added 15g of agar before autoclaving. For controls, isolates were grown in the media without cysteine amendments. For "treatments", 2mM cysteine was added.

To assess the amount of cysteine degradation into H_2S and ammonia, we screened each 60 isolates on whether they produce H_2S and/or ammonia. To test H_2S production, we grew the strains individually in liquid media, and used lead acetate test strips (Fisher Scientific, USA) to qualitatively assess H_2S production. A darkening of the strip shows that H_2S was produced. To test ammonia concentrations after 24 hours, we measured samples at time zero, and 24 hours using Ammonia Salicylate Reagent Powder Pillows and Ammonia Cyanurate Reagent Powder Pillows (Hatch Reagents) and used spectrophotometry at the 655nm wavelength.

Identification of H_2S producing bacteria using 16S rRNA sequencing

Colony PCR and DNA extractions were conducted using the EtNa Crude DNA Extraction and ExoSAP-ITTM PCR Product Cleanup protocols on the isolates that tested positive for producing H_2S (10). Full length 16S rRNA products were generated for sequencing using universal 16S rRNA primers (27f, 1492r). DNA concentration yields were measured using the qBit dsDNA HS

assay kit (QuBit). DNA was sequenced at the University of Wisconsin Madison Biotechnology Center (Madison, WI, USA) The program 4Peaks (32) was used to clean the base pairs by quality-checking followed by homology search using BLASTn against the NCBI Genbank database (accessed December 2019) (33) to identify the sequences.

Detailed characterization of 3 hydrogen-sulfide producing isolates

We selected 3 isolates that could aerobically produce H_2S for further detailed characterization. We selected these isolates because some of the 18 isolates had identical 16S rRNA sequences, therefore we chose isolates that had distinct 16S rRNA sequences for full-genome sequencing. Additionally, using 16S rRNA sequencing of the isolate, one only was assigned to *Stenotrophomonas sp.*, and we believed that full-genome sequencing would enable us to get a higher taxonomic confirmation and more complete information.

We performed DNA extraction using the PowerSoil Powerlyzer kit (Qiagen) without protocol modifications, and sent the genomes for full genome sequencing at Microbial Genome Sequencing Center (MIGS) (Pittsburg, PA) for combined short read illumina and long read nanopore sequencing. The data was processed by MIGS to assemble the short-reads (Illumina Next Seq 2000) and long-reads (Oxford Nanopore Technologies) into full-genomes. Quality control and adapter trimming was performed with bcl2fastq (Illumina) and porechop (https://github.com/rrwick/Porechop) for Illumina and ONT sequencing respectively. Hybrid assembly with Illumina and ONT reads was performed with Unicycler (34)**.** Genome annotation of the 3 isolates was done with Prokka v.1.14.5(35), using the --rfam setting.

Genome completeness and contamination were estimated using CheckM v.1.1.3 (36) *lineage_wf*. Taxonomic classification was conducted using GTDB-tk v.0.3.2 (37) with the database release r95. The full-genome taxonomic classification agreed with the prior 16S rRNA sequencing results, but we were further able to identify Isolate 43 as *Stenotrophomonas bentonitica*. We ran METABOLIC-G v.4.0 (38) to identify genes associated with cysteine degradation and other metabolic pathways.

Growth measurements of the three isolates were measured using OD600 with a spectrophotometer, with measurements every 1 hour. The isolates were grown in R2A broth media, shaken in an incubator at 27°C. Aliquots were collected over the growth range for cysteine measurements. A H_2S microsensor (Unisense) was used to measure H_2S over time.

Methods to measure Cysteine

Cysteine concentrations were measured as cystine, as described in (39) (https://osf.io/9k8a6/). One of the reasons for measuring cystine instead of cysteine is that in oxic environments, cysteine is oxidized rapidly into cystine (13, 14). Additionally, unless LC-MS is used, cysteine can be difficult to measure directly. Samples were diluted 5:4:1 Sample:DI H_2O:DMSO and left at room temperature for at least 24 hours. Chromatographic analysis was performed on an Agilent 1260 Infinity IIwith a Agilent Zorbax Eclipse Plus C18 RRHT 4.6x50mm, 1.8µm, with Guard column. Column temperature was maintained at 40°C using an Agilent 1260 TCC (G1316A).

Gradient elution was performed using Mobile Phase A (MPA) consisting of 10mM Na_2HPO_4, 10mM Sodium Tetraborate Decahydrate, in DI H_2O, adjusted to pH 8.2 with HCl, filtered to 0.45µm. Mobile Phase B (MPB) consisted of 45:45:10 Acetonitrile:Methanol:DI H_2O. Gradient used for elution was as follows: 0 minutes 98% MPA, 2% MPB; 0.2 minutes 98% MPA, 2% MPB; 6.67 minutes 46% MPA, 54% MPB; 6.77 minutes 0% MPA, 100% MPB; 7.3 minutes 0% MPA, 100% MPB; 7.4 minutes 98% MPA, 2% MPB; 8 minutes 98% MPA, 2% MPB. Flow rate was 2.0mL/min. Pump used was a Agilent Infinity Series G1311B Quat Pump. Pre-column derivatization was performed using an Agilent 1260 ALS (G1329B) with an injector program. Detection was performed using an Agilent 1260 Infinity II MWD (G7165A) at 338nm with 10nm bandwidth. Reference was 390nm with 20nm bandwidth.
Recovery was tested during method development. Recoveries of Cystine ranged from 87.2-101.5%, with an average of 92.1%.

Methods to measure H_2S using a microsensor

Aliquots of at least 1mL were taken from cultures at desired times after inoculation. H_2S concentrations were measured by suspending the H_2S probe (Unisense) in the aliquot and leaving it in place until measurement stabilized. Measurements were manually edited to exclude data gathered while the probe was stabilizing in the sample.

Generation of Metagenome-assembled genomes

Sequencing of the Lake Mendota time series for 2008-2012 was previously done at the Joint Genome Institute (40), containing 97 times points (and therefore 97 metagenomic datasets). Metagenomics data was processed in-house. In summary, each metagenome was quality filtered using fastp (41), and individually assembled using metaSPAdes. Each metagenome was mapped to each individual assembly using BBMap v38.07 (42) version with 95% sequence identity cutoff. Differential coverage mapping to all samples was used to bin contigs into metagenome-assembled genomes (MAGs) using Metabat2 v.2.12.1 (43). Bins were quality assessed with CheckM v.1.1.2 (36), dereplicated with dRep v.2.4.2 (44), and classified with GTDB-tk v.0.3.2 (45). This resulted in a total of 116 MAGs from Lake Mendota (**Table S13**) (https://osf.io/qkt9m/).

Searching for the presence of the three cultured isolates presence in the Lake Mendota time-series

To determine whether the isolates that were cultured in the laboratory were present in the natural environment, we performed a homology search using Blastn v.2.6.0+ (33) of the 16S rRNA, using an e-value threshold of 1e-6. 16S rRNA sequences were queried against a custom database of the scaffolds in the assembly (-db), with a further search against the binned scaffolds in the 116 MAGs (-db). The comprehensive database containing all scaffolds of the assembly comprised 16,599,321 scaffolds, whereas the final set of MAGs contained 28,395 scaffolds.

Searching for cysteine genes and isolates presence in time-series

Genes for cysteine degradation were identified using HMMsearch v3.1b2 (46) . HMMs were downloaded from KoFam (47), accessed May 2020). The KO numbers for the six cysteine degradation genes are: metC (K01760), cysK (K01738), cysM (K12339), malY (K14155), tnaA (K01667), and dcyD (K05396) (**Table S4**). Our HMM files are available in **Supplementary File 1** but are the same published by KoFAM, with the modification of manual addition of the TC threshold. HMM-based homology searches were conducted on the 97 Lake Mendota metagenomes assemblies as described above.

Data availability

The 16S rRNA sequences for the 29 H_2S producing isolates, the full genome sequences (nucleotides and amino acids) for isolates #13, #43 and #66 are available on OSF: https://osf.io/g25eq/ during the peer-review process. The sequences will be deposited in NCBI prior to publication.

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Contributions

P.Q.T, S.C.B., J.C.H, K.K and K.A contributed to study design and conceptualization. P.Q.T, S.C.B, J.C.H and K.K conducted experiments on the isolates. J.C.H performed the chemical analyses of the isolates. P.Q.T, S.C.B and J.C.H conducted genomic analyses. P.Q.T, S.C.B and E.A.M conducted metagenomic analyses. P.Q.T, S.C.B and J.C.H analyzed the data and generated figures. P.Q.T, S.C.B, J.C.H and K.A drafted and edited the manuscript. All authors provided feedback and suggestions.

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Table S10. Cysteine hits the Assembly.

Table S11. Cysteine hits to MAGS

Table S12. MAG characteristics binned from LMTS.

Table S13. Global minimum and maximum lake surface temperatures (ref. Sharma et al., 2015)

List of Supplementary Files

File S1. HMM profiles.zip

Tables

Table 1. Genome characteristics of 3 selected isolates that produce H2S in the presence of oxygen and cysteine

Name	Genome Size (bp)	Completeness[1]	GC content	Taxonomy[2]	NCBI Project ID	Number of contigs
Isolate13-LM-B-02-08	4,188,104	100%	66.8%	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;**s__Stenotrophomonas maltophilia_O**	TBD	1
Isolate43-LM-B-01-03	4,325,715	100%	66.5%	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;**s__Stenotrophomonas bentonitica**	TBD	2
LM_BA_5.2	1,375,102	100%	33.7%	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Weeksellaceae;g__Chryseobacterium;**s__Chryseobacterium piscium**	TBD	7

[1] Using CheckM (See Methods) [2] Using GTDB-tk (See Methods)

Figures

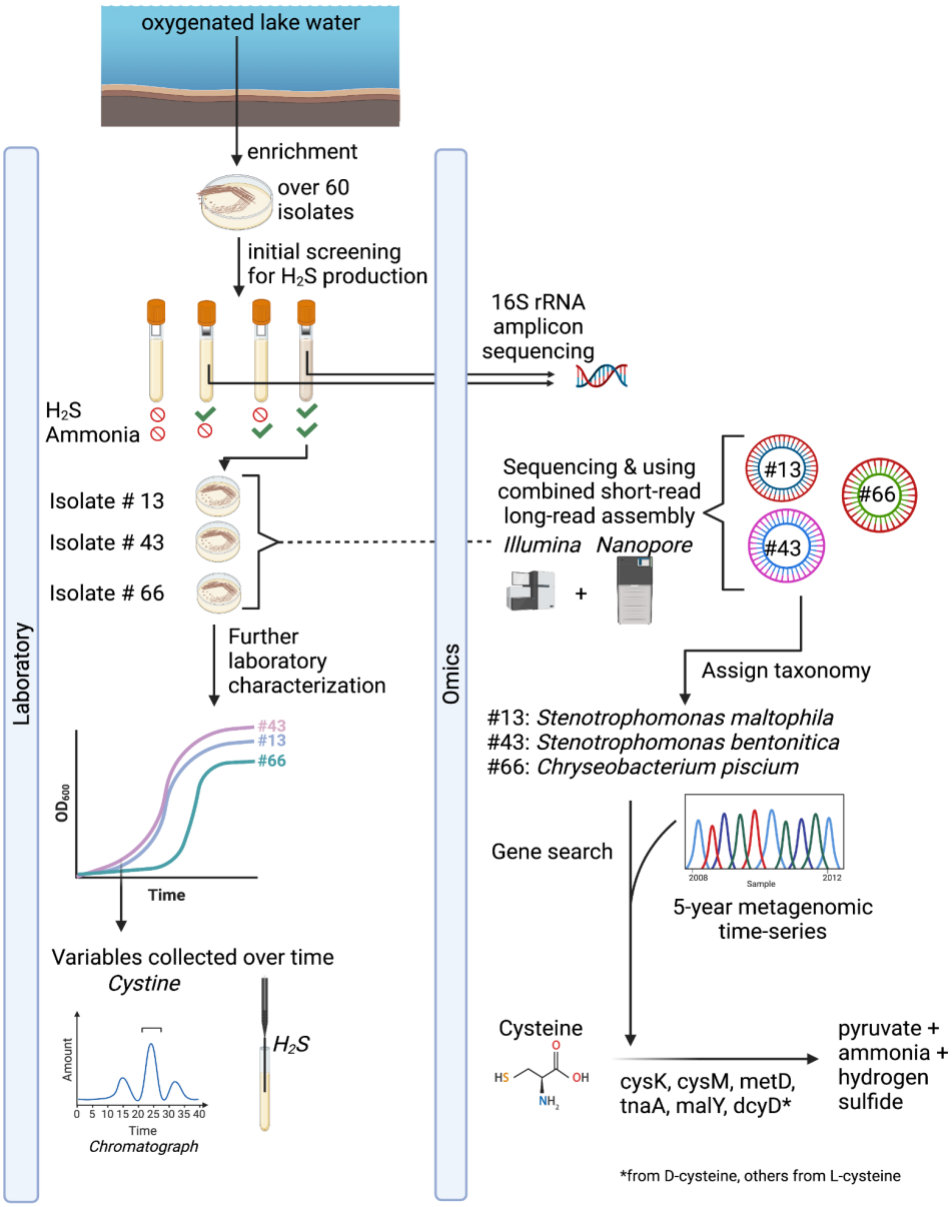


Figure 1. Methods overview of our study. Isolates were enriched from oxygenated lake water. Isolates were screened for H_2S and ammonia production, and those that produced H_2S were selected for 16S rRNA sequencing. Then based on the results, three isolates were selected for whole-genome sequencing using a combination of short and long-reads. Genome characterization of functional potential and taxonomic classification was conducted on the isolates. Genes involved in cysteine degradation were searched in the isolates, and a five-year metagenomic time series of Lake Mendota (2008-2012).

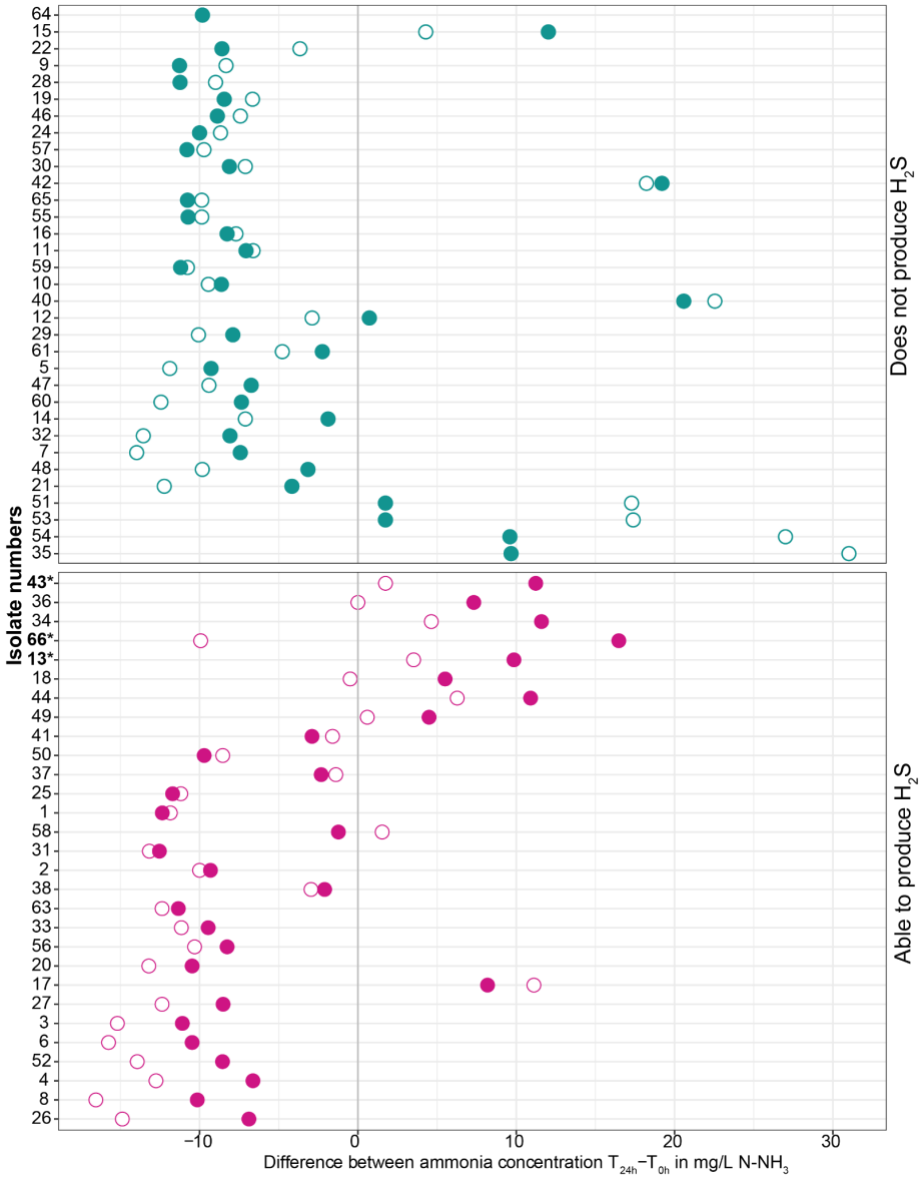
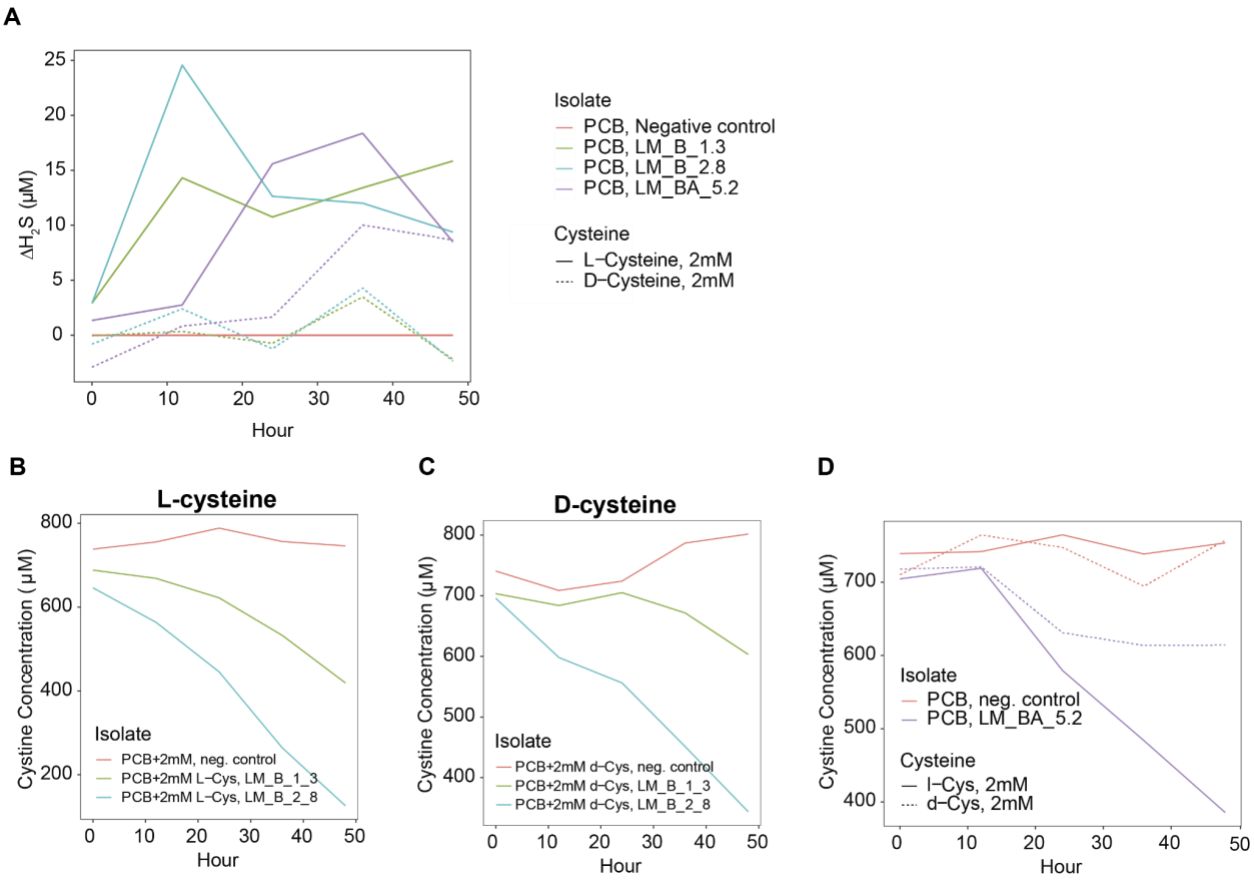


Figure 2. Qualitative production of hydrogen sulfide among microbial isolates enriched from a freshwater lake water column. Filled circles are isolates when grown with cysteine, and open circles are isolates when grown without cysteine. The vertical line represents values corrected for the control (natural ammonia production/consumption in the negative control). All points to the right of the vertical lines indicate a production of ammonia, and all points to the left of the vertical lines refer to those that consumed ammonia after 24 hours. The isolates #43, #13 and #66 were selected for further analysis.



733

734 **Figure 3. Further characterization of the three isolates and demonstration of cysteine**
735 **degradation and H_2S production.** A. Higher amounts of H_2S was produced by the three isolates
736 over 50 hours compared to negative controls. B, C, D. Identification of different forms of
737 cysteine that can be degraded. L-cysteine decreased in all isolates compared to the control (B,
738 D). D-cysteine also decreased over time in all except the negative control, however, the net
739 amount decreased was less compared to L-cysteine. Cysteine concentrations were measured as
740 Cystine as described in the methods. Because Isolate #13 and #43 were assessed in another
741 experiment than #66, but using the exact same instruments and methods, plots are separated by
742 HPLC runs. Due to large sample volume it was not possible to test all isolates and conditions in
743 one HPLC run.

744

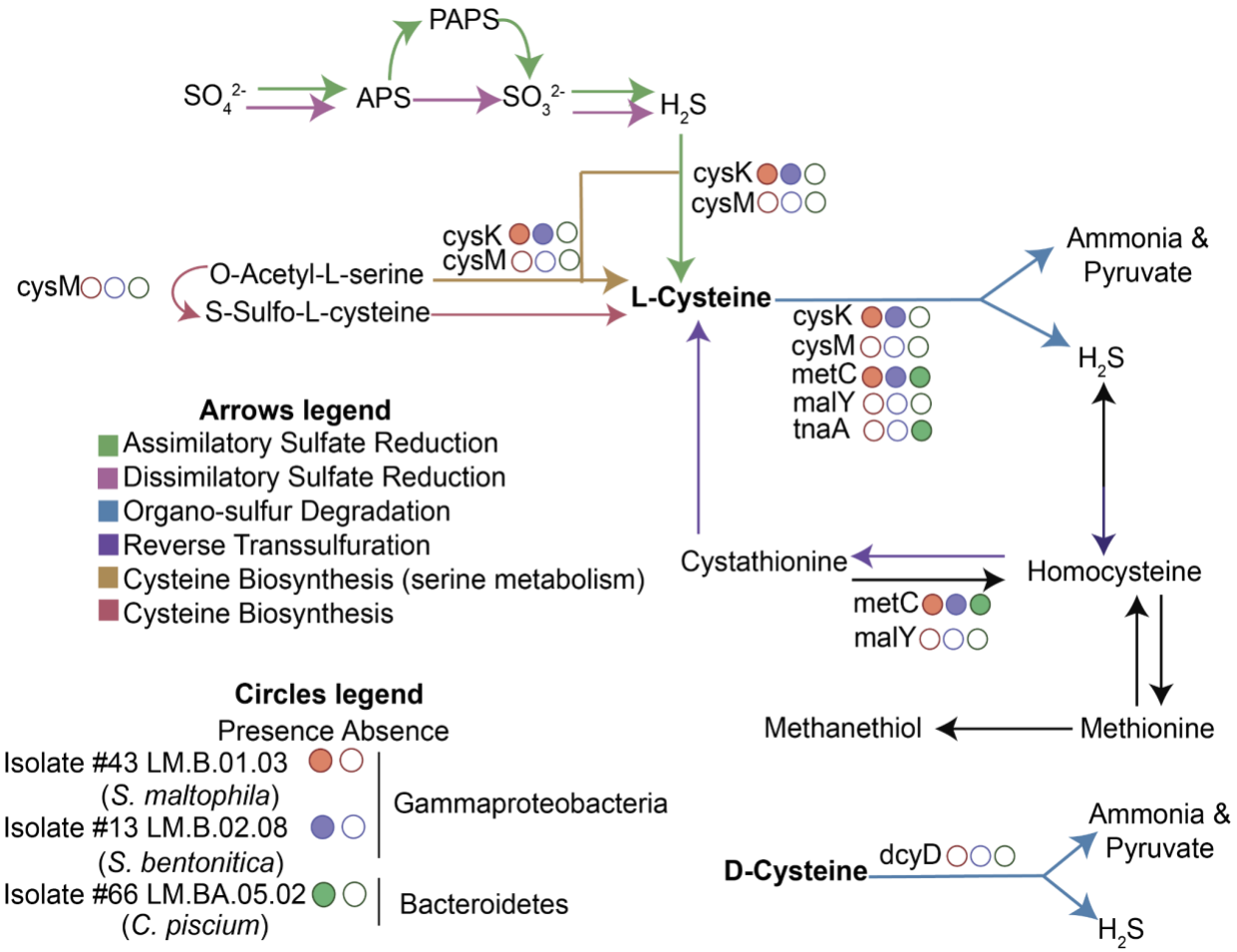
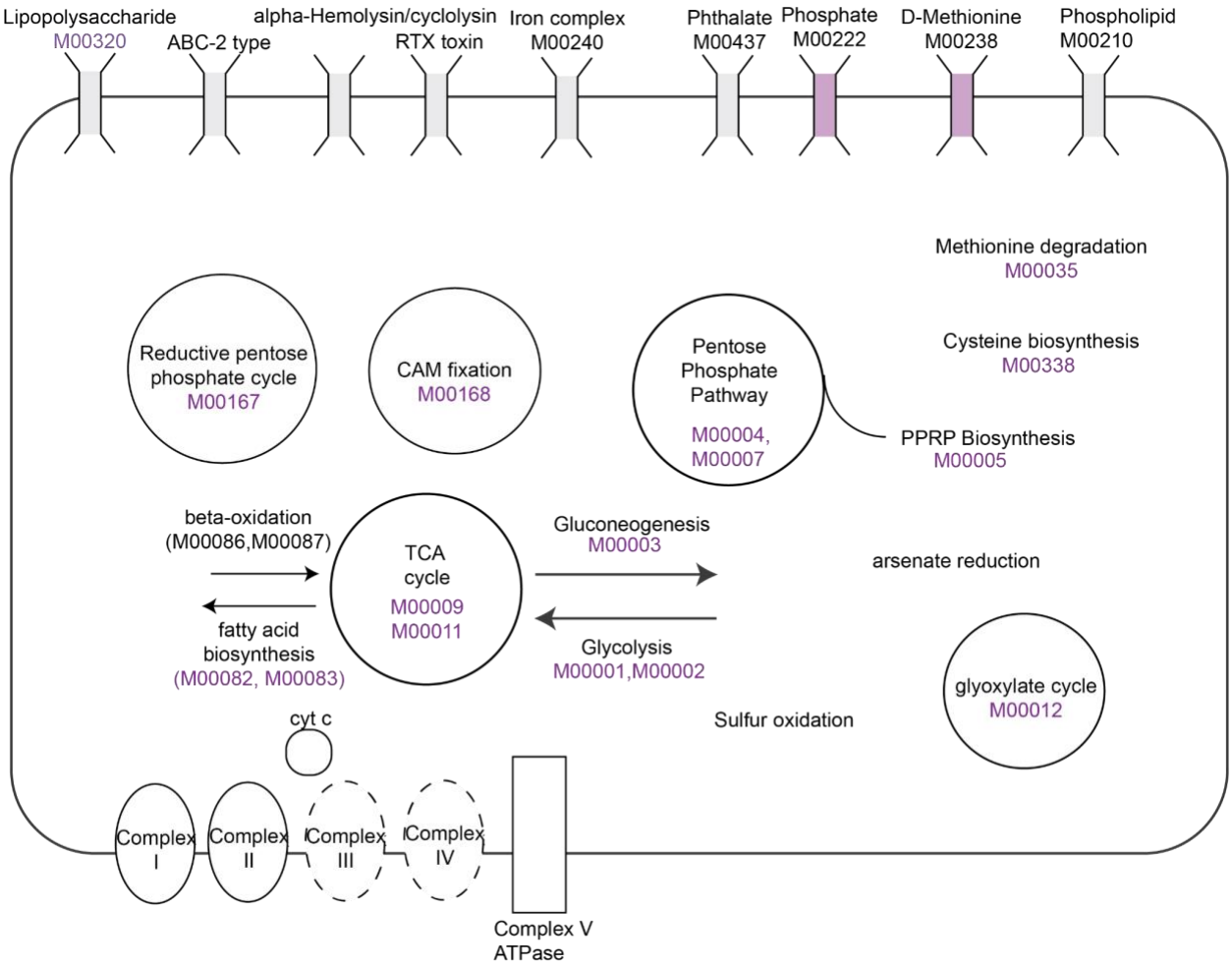


Figure 4. Diagram showing pathways for sulfur and organosulfur metabolism. Several pathways for hydrogen sulfide and cysteine production exist in microorganisms. The presence/absence of key genes (cysK, cysM, malY, cysM, metC, tnaA, sseA, aspB, and dcyD along the blue arrow) in the three isolate's genomes are shown by filled (present) circles, and clear (not present) circles.



754

755 **Figure 5A. Cellular map showing the pathways, genes, cycles and transporters which were**
756 **common to all three isolates.** A complete list is found in **Table S6**. The KEGG module
757 identifiers are listed in purple whenever relevant.

758

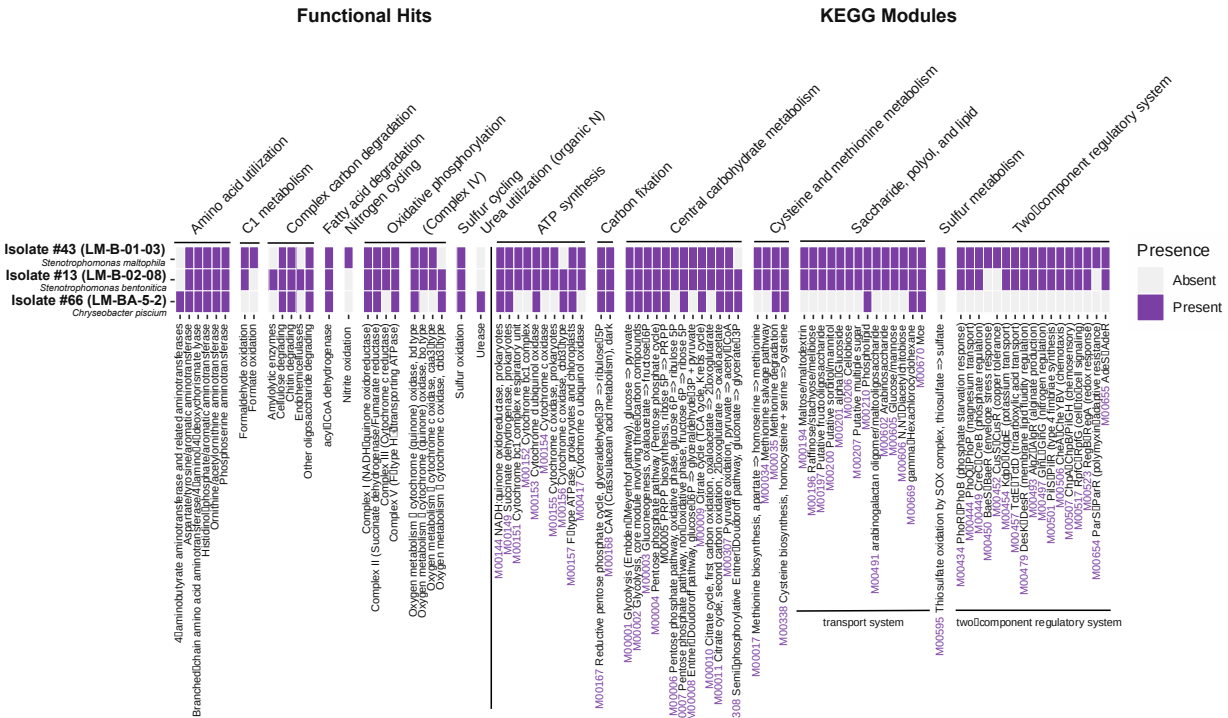
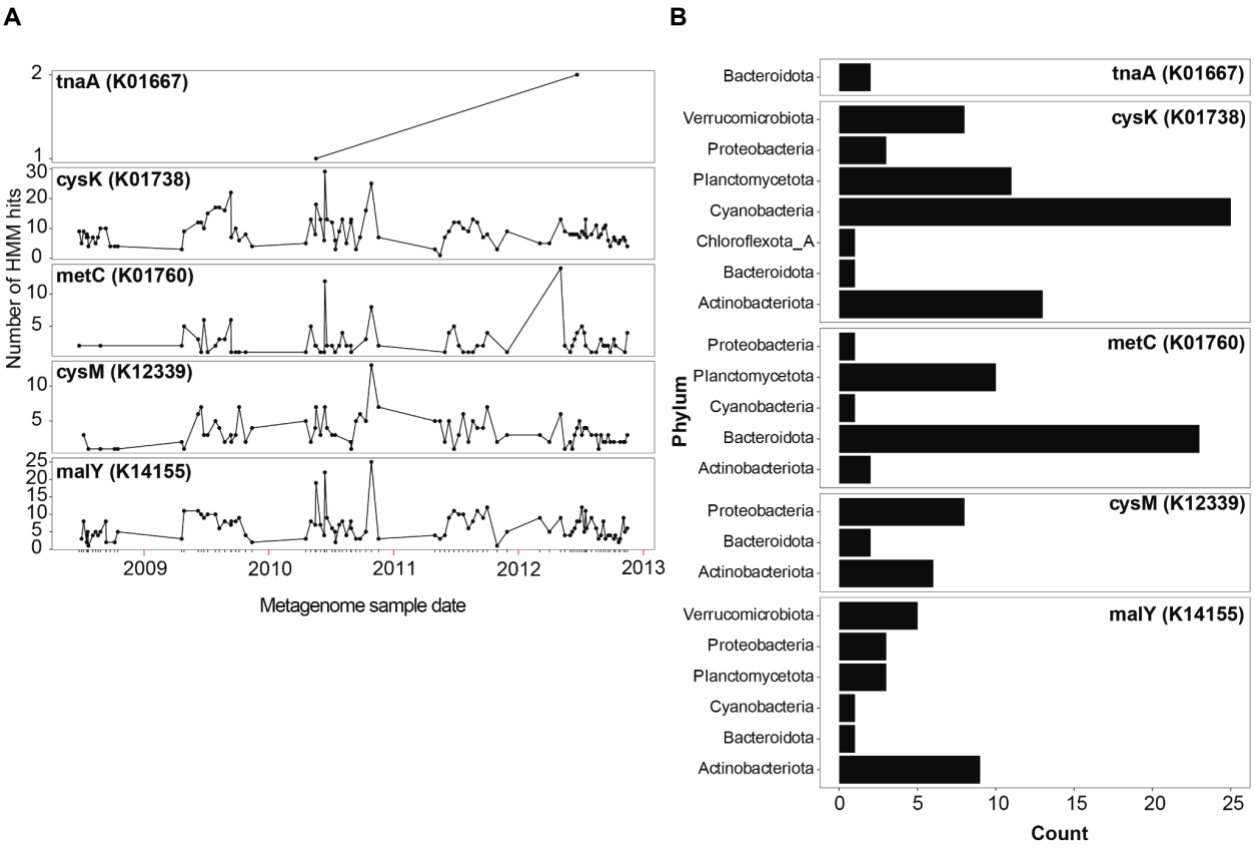


Figure 5B. Heat map showing selected metabolic functions and pathways in the three isolates. A complete list is found in **Table S6**.



764

765 **Figure 6.** A. **Number of amino acid coding sequences matching one of the cysteine genes**
766 **searched (5 genes) which may have the enzymatic activity of degrading cysteine into**
767 **ammonia, pyruvate and hydrogen sulfide**. We also searched for dcyD (6th gene) but did not
768 identify it in any sample. B. **Presence of these 5 genes in the binned metagenome-assembled**
769 **metagenomes of the same time series**.

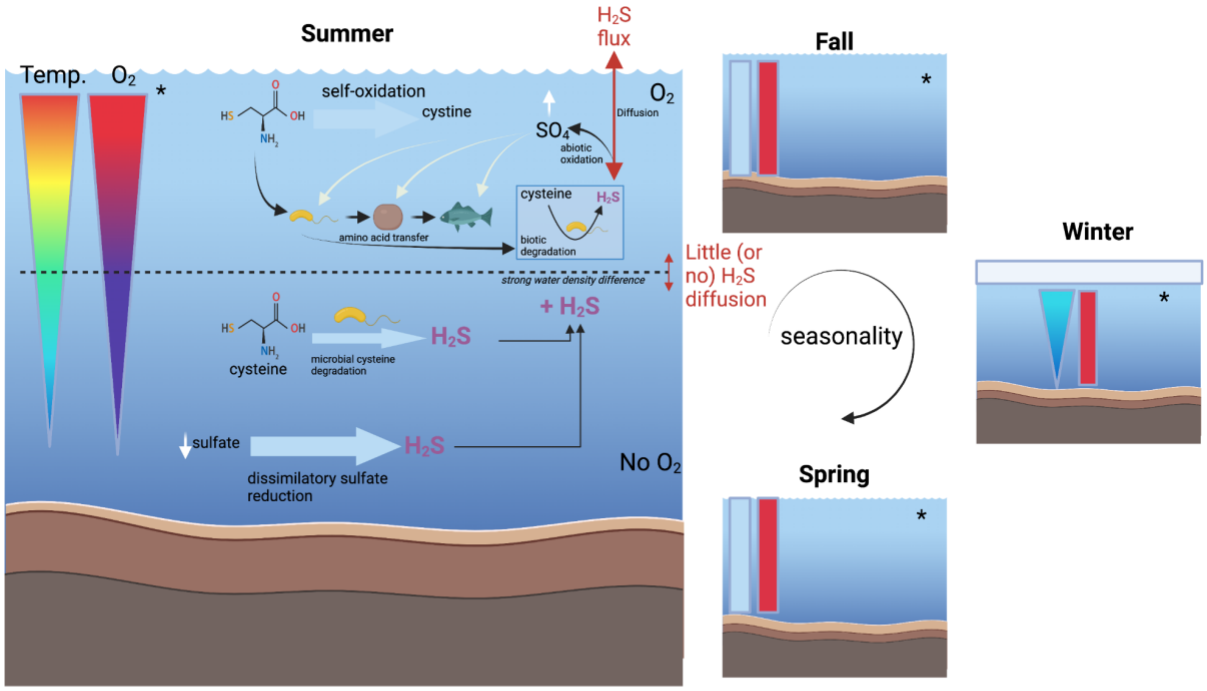


Figure 7. Conceptual figure showing the potential relevance of cysteine degradation in a freshwater lake environment, with respect to oxygen availability and seasonality. Oxygenated seasons and part of the lake water columns are shown with an asterix. Significant research gaps include cysteine concentrations in the natural environment over time, H_2S fluxes across different layers in the lake water column, and contribution of different H_2S sources in the hypolimnion.