Wetland sediments host diverse microbial taxa capable of cycling alcohols

Running title: alcohol cycling in wetland sediments

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Abstract

Alcohols are commonly derived from the degradation of organic matter, and yet are rarely measured in environmental samples. Wetlands in the Prairie Pothole Region (PPR) support extremely high methane emissions and the highest sulfate reduction rates reported to date, likely contributing to a significant proportion of organic matter mineralization in this system. While ethanol and isopropanol concentrations up to 4-5 mM in PPR wetland pore fluids have been implicated in sustaining these high rates of microbial activity, the mechanisms that support alcohol cycling in this ecosystem are poorly understood. Here we leveraged metagenomic and transcriptomic tools to identify genes, pathways and microorganisms potentially accounting for alcohol cycling in PPR wetlands. Phylogenetic analyses revealed diverse alcohol dehydrogenases and putative substrates. Alcohol dehydrogenase and aldehyde dehydrogenase genes were encoded...
in 62 metagenome-assembled genomes (MAGs) affiliated to 16 phyla. The most frequently encoded pathway (in 30 MAGs) potentially accounting for alcohol production was a *Pyrococcus furiosus*-like fermentation which can involve pyruvate:ferredoxin oxidoreductase (PFOR). Transcripts for 93 out of 137 PFOR genes encoded in these MAGs were detected, as well as for 158 out of 243 alcohol dehydrogenase genes retrieved from these same MAGs. Mixed acid fermentation and heterofermentative lactate fermentation were also frequently encoded. Finally, we identified 19 novel putative isopropanol dehydrogenases in 15 MAGs affiliated to *Proteobacteria, Acidobacteria, Chloroflexi, Planctomycetes, Ignavibacteria, Thaumarchaeota,* and the candidate divisions KSB1 and Rokubacteria. We conclude that diverse microorganisms may use uncommon and potentially novel pathways to produce ethanol and isopropanol in PPR wetland sediments.

**Importance**

Understanding patterns of organic matter degradation in wetlands is essential for identifying the substrates and mechanisms supporting greenhouse gas production and emissions from wetlands, the main natural source of methane to the atmosphere. Alcohols are common fermentation products but are poorly studied as key intermediates in organic matter degradation in wetlands. By investigating genes, pathways and microorganisms potentially accounting for the high concentrations of ethanol and isopropanol measured in Prairie Pothole wetland sediments, this work advanced our understanding of alcohol fermentations in wetlands linked to extremely high greenhouse gas emissions. Moreover, the novel alcohol dehydrogenases and microbial taxa potentially involved in alcohol metabolism may serve biotechnological efforts in bioengineering
commercially valuable alcohol production, and in the discovery of novel isopropanol producers or isopropanol fermentation pathways.

Introduction

Fermentation is a primary mode of organic matter degradation. Fermentative pathways can both result in carbon mineralization and generate substrates that fuel anaerobic respiration, contributing to methane (CH₄) and carbon dioxide (CO₂) emissions. The relevance of these interconnected processes in soils and sediments has been previously reported across a range of ecosystems. For example, Wrighton and collaborators (1) suggested that fermentation was a major route of carbon turnover in a shallow alluvial aquifer, resulting in the generation of a range of labile substrates including hydrogen, ethanol, formate, acetate, lactate and butyrate. In this system these intermediates were inferred to support nitrate, sulfate, and iron reduction. In boreal fens, the fermenter community differed between high CH₄- and high CO₂-producing peat slurry incubations, indicating that varying fermentative pathways may impact the CO₂/CH₄ ratio in greenhouse gas emissions (2). In marine sediments, temperature perturbation experiments revealed a close coupling between fermentation and sulfate reduction (3), while iron reduction and methanogenesis were inferred to be supported by fermentation products in Arctic tundra soils (4).

Although alcohols – particularly ethanol, but also 1-propanol, isopropanol (2-propanol), and butanol – are common fermentation products, their role in stimulating sedimentary carbon cycling has received little attention. Indeed, knowledge gaps associated with the magnitude and fate of ethanol produced in wetlands represent a fundamental constraint in estimating global carbon
budgets (5). Technical issues have frequently precluded the measurement of alcohols in complex environmental samples. For instance, pore water samples for proton-Nuclear Magnetic Resonance (1H-NMR) are often concentrated using alcohols, which prohibits the measurement of natural alcohol abundances in the sample (6). However, there are some instances of alcohols being measured in sedimentary samples; in 2005, Metje & Frenzel reported ethanol concentrations up to 10.5 mM in methanogenic peat soil incubations (7), while Zhuang and collaborators developed pre-treatment techniques to measure ethanol and methanol with gas chromatography in marine sediment pore waters, reporting ethanol concentrations that ranged from 3 to 62 µM (8). The same technique was used to measure concentrations of ethanol ranging from 11 to 2,535 nM in freshwater sediments (9).

The Prairie Pothole Region (PPR) of North America is the tenth largest wetland ecosystem in the world (10). Using non-concentrated sediment pore waters, ethanol and isopropanol concentrations up to 4.5 mM have been measured with 1H-NMR in PPR wetland sediments (11), suggesting that fermentation may play a key role in organic matter degradation into these alcohols. This ecosystem is carbon rich, with pore fluid dissolved organic carbon concentrations reaching ~180 mg/L (12). In addition, extremely high methane fluxes (~160 mg CH4/m2/h) and the highest sulfate reduction rates ever reported to date (~22 µmol/cm3/day) have been measured in PPR wetlands (11). The depletion of alcohols during a period of high sulfate reduction, and the identification of candidate sulfate-reducing bacteria genomes encoding alcohol dehydrogenases, suggested a possible role for these substrates in driving sulfate reduction in this system. Moreover, the detection of F420-dependent alcohol dehydrogenases and mcrA genes affiliated with alcohol-utilizing Methanofollis species indicated that methanogenesis may also be directly supported by these fermentation
Despite the potential importance of alcohols in supporting biogeochemical activity in PPR sediments, the microbial members and the pathways responsible for alcohol fermentation in this system remain unknown.

Here, we used metagenomics and metatranscriptomics to investigate putative alcohol-cycling microorganisms in PPR wetland sediments using sequencing data obtained for samples previously analyzed (11, 13). We have examined genome-encoded alcohol dehydrogenases and pathways that could result in ethanol and isopropanol production in Prairie Pothole wetlands. Our results indicate that known and novel pathways for alcohol cycling are active across phylogenetically diverse microbial groups in this ecosystem, and that a variety of novel alcohol dehydrogenases have yet to be characterized. These results have both environmental relevance - in the context of carbon cycling and greenhouse gas emissions - and industrial importance, given the decades of efforts in engineering microorganisms for the production of these alcohols (14, 15).

**Results**

**Putative alcohol-cycling microorganisms are phylogenetically diverse and encode a variety of alcohol dehydrogenases**

Depth-resolved metagenomic datasets were obtained from sediments in two characteristic wetlands near Jamestown, North Dakota, USA, and processed as previously described (13). These wetlands are rich in dissolved organic carbon (12) and sulfur compounds (16, 17) due to the local hydrological regime and the underlying pyrite-rich glacial till (18). Dynamic shifts in redox
conditions occur in these wetlands due to annual and seasonal rainfall and temperature changes, storm events that transport agricultural runoff into the wetlands, and prairie winds, which can mix and oxygenate the shallow (1-3 m) water column (19). Samples analyzed in this study had been previously characterized for pore water concentrations of methane, sulfate, sulfide, ethanol, methanol, isopropanol, and acetate, as well as for sulfate reduction rates, dissolved organic carbon compounds, and 16S rRNA gene-based microbial communities (11). Remarkably, extremely high ethanol and isopropanol concentrations (up to 4-5 mM), methane concentrations (up to 6 mM) and sulfate reduction rates (up to 22 µmol/cm³/day) were measured in these sediments, with substrates depleted from spring to summer when methane emissions and sulfate reduction rates were highest (11).

Metagenome-assembled genomes (MAGs) were screened for the presence of both an aldehyde dehydrogenase and an alcohol dehydrogenase (ADH) with potential for primary or secondary alcohol fermentation or oxidation, which excluded short chain, aryl, and polyvinyl ADHs, as well as choline, sugar-alcohol and phosphonate catabolism-related ADHs. Out of 449 MAGs recovered from our metagenomic datasets, 62 had at least one gene encoding each enzyme and estimated contamination levels of less than 13%, and thus were selected for further analyses. Known pathways for alcohol fermentation (Figure 1) were investigated in order to determine the potential for ethanol and isopropanol production.

An overview of the MAGs selected for this study is provided in Figure 2. Detailed MAG information on genome and investigated genes is provided in Supplemental Table 1. The selected MAGs had abundances (Supplemental Table 1) varying between 0.12 and 0.62 total coverage.
normalized per Gbp of metagenome (average 0.34 and median 0.31), which is well within general trends observed for the entire 449 MAG dataset (minimum coverage of 0.004 and maximum of 2.5, with average 0.26 and median 0.25).

The selected MAGs representing candidate alcohol-cycling microorganisms spanned 16 phylum-level taxonomic groups (Figure 3): Proteobacteria (20 MAGs), Chloroflexi (8), Acidobacteria (6), Ignavibacteriae (5), Actinobacteria (4), Planctomycetes (4), Bacteroidetes (2), Thaumarchaeota (2), Cyanobacteria (1), Nitrospirae (1), Spirochaetes (1), Verrucomicrobia (1), Calditrichaeota (1), the candidate phyla KSB1 (1), Rokubacteria (1), and Latescibacteria (1). Three bacterial MAGs were unclassified.

A wide variety of alcohol dehydrogenases with potential for alcohol fermentation or oxidation were recovered from PPR draft genomes, varying in number between 1 and 30 per MAG. Overall, 366 ADHs were identified, with 334 being at least 100-amino acids long. Phylogenetic analyses of these sequences (Figure 4) indicated that ADHs formed clusters primarily based on cofactor, with a major segregation between iron-, zinc-, and pyrroloquinoline quinone (PQQ)-type ADHs, and secondarily based on putative substrate preference. A monophyletic cluster of 91 poorly annotated ADHs was determined to contain NADH:quinone oxidoreductases and uncharacterized medium chain dehydrogenase superfamily members (collapsed branch in Figure 4), which excluded the corresponding genes from further analyses. From the remaining 243 ADH genes, 158 were inferred to be expressed within the microbial community via metatranscriptomic data (Supplemental Table 2).
Reference isopropanol dehydrogenases included in the phylogenetic analysis formed a monophyletic group with 36 sequences in total, allowing the identification of 19 binned ADHs as putative isopropanol dehydrogenases. These sequences belonged to 15 MAGs affiliated to the betaproteobacterial order Burkholderiales, the alphaproteobacterial family Hyphomicrobiaceae, the phyla Acidobacteria, Ignavibacteriae, Chloroflexi, Plancomycetes, and Thaumarchaeota, candidate divisions KSB1 and Rokubacteria, and one unclassified bacterial genome. Out of these 19 putative isopropanol dehydrogenase genes, 14 were detected in metatranscriptomic data (Supplemental Table 2).

Another monophyletic group contained mostly propanol-preferring ADHs (71 sequences) plus only 4 ethanol-preferring reference ADHs (both fermentative and oxidative ADHs from \textit{Saccharomyces cerevisiae}, ADH-I from \textit{Streptococcus pneumoniae}, and the fermentative ADH-I from \textit{Zymomonas mobilis}). Similarly, PQQ-type methanol/ethanol dehydrogenases (34 sequences) also formed a monophyletic group that also contained 2 zinc-type and 2 iron-type ADHs. Interestingly, 11 lanthanide-dependent methanol dehydrogenases (including two unbinned sequences from our metagenomes annotated as XoxF) formed a monophyletic group within the PQQ-type ADH branch (Figure 4).

Finally, 99 sequences formed a branch of mostly inferred ethanol dehydrogenases. Bifunctional acetaldehyde/ethanol dehydrogenases formed two monophyletic groups; a sub-branch of sequences almost exclusively identified in this work contained only two reference sequences (the propanediol utilization propanol dehydrogenase PduQ from \textit{Enterococcus faecalis} and the oxidative ADH-II from \textit{Zymomonas mobilis}), while a second sub-branch contained only 3
sequences from this study, and 16 reference sequences (9 butanol dehydrogenases, 4 ethanol dehydrogenases, 2 propanediol utilization propanol dehydrogenases, and 1 methanol dehydrogenase), with 4 of these reference sequences obtained from *Clostridium* species.

**Known, unusual and novel pathways may be used for ethanol and isopropanol fermentation in Prairie Pothole Wetland sediments**

In this study we additionally analyzed central carbon metabolism pathways in the context of alcohol fermentations. Respiratory processes were also examined in order to determine if MAGs could represent facultative or obligate fermenters (Supplemental Table 1). Investigated fermentation pathways are represented in Figure 1 and MAG genomic potential is summarized by taxa in Figure 5 (with a detailed gene presence/absence report provided in Supplemental Table 1). Detailed descriptions of inferred metabolisms in each MAG are presented as Supplemental Text.

The majority of the MAGs (59 of 62) encoded sugar utilization systems, the machinery for Embden–Meyerhof–Parnas (EMP) glycolysis (58 of 62), and the pentose phosphate pathway (52 of 62). Moreover, 50 of the MAGs encoded a pyruvate dehydrogenase complex, and 49 contained tricarboxylic acid (TCA) cycle genes (2 *Thaumarchaeota* MAGs contained an incomplete TCA cycle). Only one MAG encoded the Entner–Doudoroff pathway.

The potential for alcohol fermentation was encoded across a range of pathways. Overall, 42 out of 62 MAGs had potential for at least one of the fermentative pathways investigated, although all encoded at least one acetaldehyde dehydrogenase and one alcohol dehydrogenase, suggesting that novel pathways may exist in these microorganisms. *P. furiosus*-like fermentation was the most
frequent fermentation pathway present in our MAGs (30 out of 62), with 68% of PFOR genes in these MAGs identified in metatranscriptomic data (Supplemental Table 3). In total, 48 MAGs encoded pyruvate:ferredoxin oxidoreductase (PFOR); 46, 2-oxoglutarate:ferredoxin oxidoreductase (OGFOR); 29, indolepyruvate:ferredoxin oxidoreductase (IFOR); 3, 2-oxoisovalerate:ferredoxin oxidoreductase (OIFOR); 49, acetyl-CoA synthetase (ACS), 22, aldehyde:ferredoxin oxidoreductase (AFOR); and 30, ferredoxin:NADP oxidoreductase. Additionally, 18 MAGs contained the functional potential for mixed acid fermentation, and 11 MAGs for heterofermentative lactate fermentation. Despite previous detection of acetone and isopropanol in PPR wetland pore fluids (11), none of the MAGs reconstructed here contained the minimal set of genes (see Materials and Methods) to determine potential for acetone/isopropanol-butanol-ethanol fermentation.

Only one Chloroflexi MAG (metabat2.725) encoded a glyceraldehyde-3-phosphate:ferredoxin oxidoreductase; therefore, we infer that the conversion of glucose to pyruvate does not proceed as in *P. furiosus* (20), but as in the conventional EMP glycolysis in the majority of the MAGs in this study. From pyruvate (or indolepyruvate, 2-oxoglutarate, and 2-oxoisovalerate), the pathway may involve the reactions described by Ma and collaborators (21) as displayed in Figure 1. The PFOR reaction would generate acetyl-CoA and acetaldehyde. Acetyl-CoA would be converted into acetate by ACS, while acetaldehyde would be converted into acetate by AFOR, or into ethanol by an alcohol dehydrogenase.

Of the 14 MAGs encoding putative isopropanol dehydrogenases, only 3 also encoded other genes involved in isopropanol-butanol-ethanol fermentation. The KSB1-affiliated MAG (metabat2.380;
~95% complete) encoded a putative isopropanol dehydrogenase, phosphotransbutyrylase, and butyrate kinase and represents the most likely microorganism involved in isopropanol-butanol-ethanol fermentation in this study. Despite this, no transcripts for its putative isopropanol dehydrogenases were detected. Similarly, the Acidobacteria MAG maxbin2.0013 (~61% complete) encoded a putative isopropanol dehydrogenase and a butyraldehyde dehydrogenase, while the Alphaproteobacteria MAG metabat2.370 (~30% complete) encoded both acetoacetate decarboxylase and a putative isopropanol dehydrogenase. However, in contrast to the KSB1 MAG, transcripts matching putative isopropanol dehydrogenases were detected in both of these MAGs. Although we acknowledge that genome incompleteness may play a role in the detection of truncated pathways for isopropanol-butanol-ethanol fermentation, we note that even relatively complete genomes (~95%) such as the Burkholderiales MAG metabat2.802 and the Ignavibacteriae MAG metabat2.334 lacked other genes in this fermentation pathway aside from a putative isopropanol dehydrogenase, suggesting that these microorganisms may use novel pathways for isopropanol production or consumption. Additionally, 5 of the 14 putative isopropanol dehydrogenases that were inferred to be active (via mRNA transcripts) were encoded in Acidobacteria MAGs and 2 in Planctomycetes MAGs – taxa that are not currently known to play a role in isopropanol cycling. Together, these data indicate that novel isopropanol-producing pathways may await discovery, and that phyla that were previously unrecognized in playing a role in isopropanol metabolism may be important in this process in Prairie Pothole wetlands.

Organisms with the ability to cycle alcohols were implicated in reducing a range of oxidized substrates. Seven of the MAGs contained marker genes for sulfate reduction, while 31 were potentially able to catalyze dissimilatory nitrate reduction to ammonium (DNRA) (Figure 5).
Although none of the MAGs contained genes encoding the complete denitrification pathway, 38 contained a truncated pathway. Reflecting the metabolic versatility of these microorganisms, the majority (54 of 62) of MAGs were also predicated to perform oxygen reduction. Only 2 MAGs did not have potential for any of these respiratory processes. *Thaumarchaeota* maxbin2.0428 had no NADH dehydrogenase or cytochrome c reductase, and lacked a complete TCA cycle. Both *Thaumarchaeota* MAGs encoded amoBC, but not amoA. *Actinobacteria* maxbin2.0055 contained an NADH dehydrogenase, but lacked a TCA cycle or cytochrome c reductase; however, given that the genome was only ~50% complete, additional undetected respiratory terminal reductases may be affiliated with this microorganism (Supplemental Table 1).

**Discussion**

This study aimed to investigate potential microbial genes, taxa and pathways implicated in the production of ethanol and isopropanol in Prairie Pothole wetlands, as these alcohols have been previously measured in millimolar concentrations in sediment pore waters (11). Metagenome-assembled genomes (MAGs) were obtained from sediment samples in which these alcohols were measured and screened for alcohol metabolism genes, resulting in the selection of 62 MAGs for further analyses in this study.

Out of 16 phylum-level groups, the potential for ethanol production was detected in 13, while candidate isopropanol dehydrogenases were identified in 8 (Supplementary Text). This potential for isopropanol metabolism should be considered as putative, given that enzymatic studies are needed to confirm substrate specificity of these alcohol dehydrogenases. Most of the MAGs
missing the minimal criteria for a fermentative pathway still encoded genes commonly involved
in fermentation (e.g., lactate dehydrogenase, phosphotransacetylase, acetate kinase, formate
dehydrogenase). The reconstruction of MAGs from complex sedimentary matrices is an ongoing
computational challenge, with the result that only 28 of the genomes analyzed here were more than
70% complete. Genome incompleteness may therefore explain the absence of a complete pathway
for acetone/isopropanol-butanol-ethanol fermentation in these genomes, despite the detection of
genes (or subunits) including acetoacetyl-CoA:acetate/butyrate CoA transferase, acetoacetate
decarboxylase, putative isopropanol dehydrogenase, butyraldehyde dehydrogenase, butanol
dehydrogenase, phosphotransbutyrylase, and butyrate kinase in 30 MAGs, with some genomes
encoding up to 3 of these genes (Supplemental Table 1). While we acknowledge the potential
underestimation of microbial groups implicated in alcohol cycling in Prairie Pothole wetlands, we
argue that the MAGs selected for this study had metabolic potential, abundances, and transcript
activity that support their role in alcohol cycling in this ecosystem.

The clustering of 167 MAG-encoded alcohol dehydrogenase (ADH) sequences from this study
with reference sequences allowed the inference of ADH substrates. However, a greater challenge
was determining the potential for alcohol production versus alcohol consumption based on
genomic data alone. While some alcohol dehydrogenases run preferentially in the oxidative or
fermentative direction, many are reversible and utilize a broad range of substrates (22–24).
Therefore, we cannot rule out that many of these microorganisms may utilize ethanol and/or
isopropanol as electron donors. While ethanol oxidation enters central carbon metabolism via
acetyl-CoA, isopropanol oxidation is less understood and hypothesized to follow the order
isopropanol → acetone → acetol → methylglyoxal → pyruvate in unclassified bacteria isolated from environmental samples (25).

Since the first report on isopropanol dehydrogenase activity in photosynthetic *Rhodopseudomonas* species in 1940 (26) and the confirmation that the enzyme generated acetone by direct dehydrogenation of isopropanol (27), a variety of studies have demonstrated the microbial ability to oxidize isopropanol. Hoshino reported this metabolism in *Lactobacillus brevis*, which expressed an enzyme running preferentially in the oxidation direction (28). Interestingly, methylotrophic *Bacillus* strains have been shown to oxidize not only methanol, but also ethanol, isopropanol, n-propanol, isobutanol, n-butanol, and a variety of methylated amines, sugars and organic acids (29).

Moreover, it is known that methanogens can utilize isopropanol as a hydrogen donor (30), generating acetone via F420-dependent alcohol dehydrogenase, and that acetone can be fermented to acetate by sulfate-reducing bacteria (31) or degraded by nitrate (32) and sulfate reducers (33) via different pathways (acetone decarboxylase in the first and 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase in the latter). Further research is required to elucidate the complex metabolic networks in which the putative isopropanol dehydrogenases identified in this study participate, as well as whether they act as isopropanol dehydrogenases.

Interestingly, efforts in bioengineering *P. furiosus* for industrial scale ethanol production have focused on the insertion of a heterologous acetaldehyde-utilizing alcohol dehydrogenase (AdhA) and a carbon monoxide dehydrogenase (CODH) (34), and also in deleting the aldehyde:ferredoxin oxidoreductase while expressing a heterologous bifunctional acetaldehyde/alcohol dehydrogenase (35). The first approach resulted in an ethanol yield of 20 mM. In Prairie Pothole wetland...
sediments, natural ethanol concentrations reach 4 mM, and MAGs with potential for *P. furiosus*-like fermentation encoded the genomic variations targeted in bioengineering. Moreover, the RNF complex was present in some of these MAGs and could be utilized to oxidize ferredoxin and reduce NAD\(^+\) while pumping sodium ions, subsequently utilized for ATP synthesis. Concomitant NADH formation by this RNF complex could be coupled to alcohol production by NAD-dependent ADHs. While laboratory isolation and biochemical studies are required to test these hypotheses, preliminary genomic data suggests that Prairie Pothole wetlands could be attractive sources of novel microorganisms for the industrial production of alcohols.

Given the diversity of energetically favorable respiratory metabolisms encoded within these genomes, would fermentative processes be expected in PPR sediments? We hypothesize that the heterogeneous sediment matrix allows the formation of anoxic or hypoxic pockets where electron acceptors are temporarily depleted, creating the conditions required for fermentation to occur (36). As geochemical conditions dynamically shift (e.g., sulfate upward influx from pyrite oxidation as oxygenated groundwater flows through the bedrock, nitrogen inputs from agricultural runoff during storm events, or temporary oxygenation of sediments at the water-sediment interface from wind-driven water column mixing) microorganisms may return to respiratory processes or enter into a fermentative mode. Dalcin Martins and collaborators investigated genomes of candidate sulfate-reducing bacteria in PPR wetland sediments, and similarly observed the potential for other respiratory processes – DNRA, denitrification, and oxygen reduction (37). Despite this metabolic flexibility, the extremely high measured SRRs indicate that sulfate reduction was still a critical process contributing to carbon mineralization in these sediments. We suggest that the putative fermenters identified here show similar metabolic flexibility to the candidate SRB in PPR.
sediments. Indeed, ~65% of binned ADHs were transcribed, indicating the selected MAGs represent microorganisms active in alcohol metabolism. Via this metabolic flexibility, redox chemistry is able to deviate from expected reaction order, as has been observed in other studies (1, 11, 38–40). For example, sulfate reducers may preferentially oxidize ethanol over acetate due to higher thermodynamic yields (41). Thus, the incomplete oxidation of ethanol to acetate may provide additional substrate for acetoclastic methanogens, leading to cooperation instead of competition. Moreover, high substrate concentrations may alleviate thermodynamic inhibition and allow sulfate reducers and methanogens to co-exist. High organic carbon loads have been previously hypothesized to allow for the co-occurrence of acetoclastic methanogens and sulfate reducers in coastal marine sediments (42). Alternatively, co-culture experiments indicated that H2 leaking from acetoclastic methanogens could support sulfate reducers (43), while methanogens may induce sulfate reducers to enter a fermentative mode (44). Here, 7 MAGs presented potential for sulfate reduction and also fermentation, indicating that the latter is also a potential explanation – amongst many – for the co-occurring high methane emissions and sulfate reduction rates measured in Prairie Pothole wetlands.

We conclude that PPR wetland sediments harbor a vast diversity of candidate alcohol-cycling microorganisms encoding a variety of alcohol dehydrogenases with potential for unusual, classical, and novel fermentation pathways. We have been able to assign putative substrates to alcohol dehydrogenases and better understand alcohol production in this ecosystem, which is predicted to directly support the highest sulfate reduction rates ever reported, and indirectly, via fermentation – or directly, via F420-dependent alcohol dehydrogenases – support methanogenesis that results in extremely high methane emissions (11, 45, 46). Alcohols are likely key intermediates in
sediment carbon cycling, and CO₂ and CH₄ generation, highlighting the need for systematic measurements in sediment pore waters, as well as isolation and biochemical investigations of key microorganisms implicated in alcohol cycling. Particularly, isopropanol metabolism in natural environments requires more attention, given both the industrial importance of this alcohol and the potential role as an intermediate in carbon cycling in sedimentary systems. The roles of the putative isopropanol dehydrogenases identified in this study remain to be elucidated.

**Materials and Methods**

*Sample collection, DNA extraction and sequencing, metagenome assembly and binning*

Sediment core samples were collected from two adjacent wetlands, P7 and P8, at the United States Geological Survey-managed Cottonwood Lake Study Area near Jamestown, North Dakota, USA (11). Accordingly, samples spanned wetland type (P7 and P8), season (winter, spring, summer), and depth (1-3, 10-12, and 19-21 cm). The 18 sediment samples chosen for metagenomic sequencing are the same samples previously analyzed (Additional file 1 of reference (13)), and MAGs selected for this study belong to this same MAG dataset (13). As such, 5 previously published MAGs have been reanalyzed and included in this study: maxbin2.0908, maxbin2.1011, maxbin2.0177, metabat2.783, and metabat2.793. All other 57 MAGs are analyzed in this study.

DNA was extracted using the MoBio PowerLyzer Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and quantified using a Qubit® Fluorometer (Invitrogen, Carlsbad, CA, USA). Metagenomic sequencing was performed at the DOE Joint Genome Institute. Briefly, libraries were constructed with Illumina Regular Fragment (300 bp) in tubes. For this, 100
ng of DNA was sheared to 300 bp using the Covaris LE220 and size selected using SPRI beads (Beckman Coulter, Brea, California, USA). The fragments were treated with end-repair, A-tailing, and ligation of Illumina compatible adapters (Integrated Device Technology, San Jose, California, USA) using the KAPA-Illumina library creation kit (KAPA Biosystems, Wilmington, Massachusetts, USA). The prepared libraries were quantified using KAPA Biosystem’s next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified library was then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4, and Illumina’s cBot instrument to generate a clustered flow cell for sequencing. Sequencing of the flow cell was performed on the Illumina HiSeq2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2x150 indexed run recipe.

Following read trimming and quality control with BBduk and BBMap (47) as previously described (13), metagenome assembly was performed using MEGAHIT v1.0.3 (48) using a range of kmers at default settings. The 18 assemblies were merged using the first part of the MeGAMerge pipeline (49) with default parameters. Only contigs larger than 1500 base pairs were retained. Reads were mapped back to the final contig set using Bowtie2 (50). The generated sequence mapping files were handled and converted as needed using SAMtools 1.6 (51). Metagenome binning into draft genomes was performed employing three different binning algorithms with default parameters: CONCOCT 0.4.1 (52), MaxBin2 v. 2.2.3 (53), and MetaBAT2 v. 2.10.2 (54). The three resulting bin sets were supplied to DAS Tool 1.0 (55) for consensus binning and dereplication, generating an optimized set of metagenome-assembled genomes (MAGs). A single-copy marker gene analysis was performed using CheckM 1.0.7 (56) to assess MAG quality.
Annotation and gene analyses

Contigs (contiguous DNA sequences) were gene-called and annotated using an in-house annotation pipeline as previously described (57, 58). Briefly, genes were called with Prodigal (59) and annotated based on forward and reverse blast hits (minimum 300 bit score threshold for reciprocal matches and 60 for one-way matches) to amino acid sequences in the databases UniRef90 and KEGG, while motifs were analyzed using InterProScan.

MAGs were selected for in-depth gene analyses based on the potential for alcohol cycling. At least 110 genes were searched for in each draft genome, totaling ~8,000 genes (Supplemental Table 1) involved in a variety of pathways. The minimal criteria to determine if a MAG had the potential for a pathway are presented in Table 1. The abundance of MAGs was inferred from total normalized coverage (60), calculated as the total base pairs of mapped reads (summed across all 18 metagenomes) multiplied by 1 giga base pairs divided by genome length and metagenome base pairs (summed across all 18 metagenomes).

The taxonomical classification of these selected MAGs was determined based on lineage-specific phylogenetic markers from CheckM (56). To resolve instances in which CheckM could not classify a MAG beyond domain level or to confirm taxonomy, binned rpsC sequences (encoding the ribosomal protein S3) were used to place MAGs in a phylogenetic tree containing reference sequences from the 2016 update of the tree of life (61). MAG-encoded genes annotated as alcohol dehydrogenases were used to build a phylogenetic tree containing also reference sequences retrieved from NCBI (minimum sequence length of 100 amino acids for any sequences in this
tree). For these phylogenetic trees, entire amino acid sequences were aligned with MUSCLE v 3.8.31 (62), and columns with at least 95% gaps were removed with Geneious® 9.0.5 (63). Trees were built using FastTree v. 2.1.10, which infers approximately-maximum-likelihood phylogenetic trees (64), using default parameters under the Jones-Taylor-Thorton model of amino acid evolution, and visualized with iTOL (65). Figures were edited in Adobe Illustrator version 16.0.0 (Adobe Systems Inc., San Jose, USA).

Metatranscriptomic analyses

In total, 6 sediment samples for metatranscriptomics were sent to the Environmental Molecular Sciences Laboratory (EMSL) in Richland, WA, USA: MayP7_core1_1-3cm, MayP8_core1_1-3cm, SepP7_core6_1-3cm, SepP8_core1_1-3cm, SepP7_core5_10-12, and SepP8_core2_10-12cm. These samples correspond to our previously published data (11, 13). RNA was extracted from sediments using the RNeasy Powersoil Total RNA Kit® (Qiagen, Hilden, Germany), followed by genomic DNA removal and cleaning using the Qiagen’s RNase-Free DNase Set Kit® and RNeasy Mini Kit®. Integrity of the RNA samples was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Barbara, CA, USA). RNA samples having RNA Integrity Number between 9 and 10 were used in this work. Ribo-Zero rRNA Removal Kit Plant® (Illumina, Sand Diego, CA, USA) was used for enrichment of transcripts. The SOLiD® Total RNA-Seq Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to construct template cDNA for RNA-Seq following the manufacturer’s instructions. Briefly, mRNA was fragmented using chemical hydrolysis followed by ligation with strand specific adapters and reverse transcript to generate cDNA. The cDNA fragments, 150 to 250 bp in size, were isolated and amplified through 15 amplification cycles to produce the required number of templates for the SOLiD™ EZ Bead™
system, which was used to generate the template bead library for ligation base sequencing by
5500xl SOLiD™ instrument (Thermo Fisher Scientific). The 50-base short read sequences
produced by the SOLiD sequencer were mapped in color space using the Whole Transcriptome
analysis pipeline in Life Technologies LifeScope software version 2.5 against the metagenome.
Adapter and quality trimming and quality control were performed using Lifescope’s default
settings. Because the LifeScope software does not accommodate the large number of genes present
in the metagenome, the 7471083 gene sequences were collapsed into 95 artificial chromosomes.
A companion gtf file was also created with gene locations adjusted accordingly. Since the number
of annotated genes that LifeScope is designed to handle is also limited, a placeholder gtf was
provided for the Lifescope pipeline. Output bam files were then provided as input for htsq-count
(66) for mapping of aligned reads to genes, with the ‘nonunique’ argument set to ‘all’. Lifescope
selects locations based on the best read-matching score, and randomly chooses locations when
multiple loci receive the same score. The ‘--nonunique all’ setting of htsq-count allows these
reads to be included. Reads Per Kilobase per Million mapped reads (RPKM) values for each gene
were calculated as the number of mapped reads times $10^9$ divided by the total number of reads in
that sample times the gene length in base pairs using R software (67).

Data availability and accession numbers
Raw reads, trimmed reads, individual assemblies, and quality control reports are available at the
JGI genome portal under project name “Seasonal Sulfur Cycling as a Control on Methane Flux in
Carbon-Rich Prairie Pothole Sediment Ecosystems” and JGI proposal ID 2025
(https://genome.jgi.doe.gov/portal/Seasulecosystems/Seasulecosystems.info.html). All MAGs
used in this study and metatranscriptomic data were deposited on NCBI under BioProject number
PRJNA330672. Additional files are publicly available at CyVerse (https://de.cyverse.org/de/): amino acid fasta files, annotation files, ADH sequences, and trees from this study. To access these files, users must create an account, log in, and enter the folder pathway “/iplant/home/pdalcin/PPR_alcohol_files”. The merged contig set from Dalcin Martins and collaborators (13) is available at “/iplant/home/pdalcin/microbiome_files”.

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1. Wrighton KC, Castelle CJ, Wilkins MJ, Hug LA, Sharon I, Thomas BC, Handley KM, Mullin SW, Nicora CD, Singh A, Lipton MS, Long PE, Williams KH, Banfield JF. 2014. Metabolic interdependencies between phylogenetically novel fermenters and respiratory organisms in an unconfined aquifer. ISME J 8:1452–1463.

2. Juottonen H, Eiler A, Biasi C, Tuittila ES, Yrjälä K, Fritze H. 2017. Distinct anaerobic bacterial consumers of cellulbiose-derived carbon in boreal fens with different CO2/CH4 production ratios. Appl Environ Microbiol 83.

3. Finke N, Jørgensen BB. 2008. Response of fermentation and sulfate reduction to experimental temperature changes in temperate and Arctic marine sediments. ISME J 2:815–829.

4. Herndon EM, Mann BF, Roy Chowdhury T, Yang Z, Wullsleger SD, Graham D, Liang L, Gu B. 2015. Pathways of anaerobic organic matter decomposition in tundra soils from Barrow, Alaska. J Geophys Res G Biogeosciences 120:2345–2359.

5. Kirstine W V., Galbally IE. 2012. Ethanol in the Environment: A Critical Review of Its Roles as a Natural Product, a Biofuel, and a Potential Environmental Pollutant. Crit Rev Environ Sci Technol 42:1735–1779.

6. Hornak JP. The basics of NMR.

7. Metje M, Frenzel P. 2005. Effect of temperature on anaerobic ethanol oxidation and methanogenesis in acidic peat from a Northern Wetland. Appl Environ Microbiol 71:8191–8200.
24

8. Zhuang GC, Lin YS, Elvert M, Heuer VB, Hinrichs KU. 2014. Gas chromatographic analysis of methanol and ethanol in marine sediment pore waters: Validation and implementation of three pretreatment techniques. Mar Chem 160:82–90.

9. Roebuck JA, Avery GB, Felix JD, Kieber RJ, Mead RN, Skrabal SA. 2016. Biogeochemistry of Ethanol and Acetaldehyde in Freshwater Sediments. Aquat Geochemistry 22:177–195.

10. Keddy PA, Fraser LH, Solomeshch AI, Junk WJ, Campbell DR, Arroyo MTK, Alho CJR. 2009. Wet and Wonderful: The World’s Largest Wetlands Are Conservation Priorities. Bioscience 59:39–51.

11. Dalcin Martins P, Hoyt DW, Bansal S, Mills CT, Tfaily M, Tangen BA, Finocchiaro RG, Johnston MD, McAdams BC, Solensky MJ, Smith GJ, Chin YP, Wilkins MJ. 2017. Abundant carbon substrates drive extremely high sulfate reduction rates and methane fluxes in Prairie Pothole Wetlands. Glob Chang Biol 23:3107–3120.

12. Ziegelgruber KL, Zeng T, Arnold WA, Chin Y-P. 2013. Sources and composition of sediment pore-water dissolved organic matter in prairie pothole lakes. Limnol Oceanogr 58:1136–1146.

13. Dalcin Martins P, Danczak RE, Roux S, Frank J, Borton MA, Wolfe RA, Burris MN, Wilkins MJ. 2018. Viral and metabolic controls on high rates of microbial sulfur and carbon cycling in wetland ecosystems. Microbiome 6:138.

14. Lamsen EN, Atsumi S. 2012. Recent progress in synthetic biology for microbial production of C3-C10 alcohols. Front Microbiol 3.

15. Xin F, Chen T, Jiang Y, Dong W, Zhang W, Zhang M, Wu H, Ma J, Jiang M. 2017. Strategies for improved isopropanol-butanol production by a Clostridium strain from...
glucose and hemicellulose through consolidated bioprocessing. Biotechnol Biofuels 10.

16. McAdams BC, Adams RM, Arnold WA, Chin YP. 2016. Novel Insights into the Distribution of Reduced Sulfur Species in Prairie Pothole Wetland Pore Waters Provided by Bismuth Film Electrodes. Environ Sci Technol Lett 3:104–109.

17. Zeng T, Arnold WA, Toner BM. 2013. Microscale characterization of sulfur speciation in lake sediments. Environ Sci Technol 47:1287–1296.

18. Goldhaber MB, Mills CT, Morrison JM, Stricker CA, Mushet DM, LaBaugh JW. 2014. Hydrogeochemistry of prairie pothole region wetlands: Role of long-term critical zone processes. Chem Geol 387:170–183.

19. Winter TC, Rosenberry DO. 1998. Hydrology of prairie pothole wetlands during drought and deluge: A 17-year study of the Cottonwood Lake wetland complex in North Dakota in the perspective of longer term measured and proxy hydrological records. Clim Change 40:189–209.

20. Mukund S, Adams MWW. 1995. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon Pyrococcus furiosus. J Biol Chem 270:8389–8392.

21. Ma K, Hutchins A, Sung S-JS, Adams MWW. 1997. Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, Pyrococcus furiosus, functions as a CoA-dependent pyruvate decarboxylase. Proc Natl Acad Sci 94:9608–9613.

22. Reid MF, Fewson CA. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit Rev Microbiol 20:13–56.

23. Chen J-S. 1995. Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol Rev 17:263–273.
24. Radianingtyas H, Wright PC. 2003. Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria. FEMS Microbiol Rev.

25. Taylor DG, Trudgill PW, Cripps RE, Harris PR. 1980. The Microbial Metabolism of Acetone. Microbiology 118:159–170.

26. Foster JW. 1940. The Role of Organic Substrates in Photosynthesis of Purple Bacteria. J Gen Physiol 24:123–134.

27. SIEGEL JM, KAMEN MD. 1950. Studies on the metabolism of photosynthetic bacteria. VI. Metabolism of isopropanol by a new strain of Rhodopseudomonas gelatinosa. J Bacteriol 59:693–697.

28. HOSHINO K. 1960. STUDIES ON THE MICROORGANISM PRODUCING ISOPROPANOL FROM ACETONE. J Gen Appl Microbiol 6:141–150.

29. Al-Awadhi N, Egli T, Hamer G, Wehrli E. 1989. Thermotolerant and Thermophilic Solvent-Utilizing Methylotrophic, Aerobic Bacteria. Syst Appl Microbiol 11:207–216.

30. Zellner G, Boone DR, Keswani J, Whitman WB, Woese CR, Hagelstein A, Tindall BJ, Stackebrandt E. 1999. Reclassification of Methanogenium tationis and Methanogenium liminatans as Methanofollis tationis gen. nov., comb. nov. and Methanofollis liminatans comb. nov. and description of a new strain of Methanofollis liminatans. Int J Syst Bacteriol 49:247–255.

31. Dullius CH. 2011. Physiology and biochemistry of the anaerobic biodegradation of isopropanol and acetone. University of Konstanz.

32. Dullius CH, Chen CY, Schink B. 2011. Nitrate-dependent degradation of acetone by Alicycliphilus and Paracoccus strains and comparison of acetone carboxylase enzymes. Appl Environ Microbiol 77:6821–6825.
33. Frey J, Schneider F, Huhn T, Spiteller D, Schink B, Schleheck D. 2018. Two enzymes of the acetone degradation pathway of Desulfococcus biacutus: coenzyme B12-dependent 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase. Environ Microbiol Rep.

34. Basen M, Schut GJ, Nguyen DM, Lipscomb GL, Benn RA, Prybol CJ, Vaccaro BJ, Poole FL, Kelly RM, Adams MWW. 2014. Single gene insertion drives bioalcohol production by a thermophilic archaeon. Proc Natl Acad Sci 111:17618–17623.

35. Keller MW, Lipscomb GL, Nguyen DM, Crowley AT, Schut GJ, Scott I, Kelly RM, Adams MWW. 2017. Ethanol production by the hyperthermophilic archaeon Pyrococcus furiosus by expression of bacterial bifunctional alcohol dehydrogenases. Microb Biotechnol 10:1535–1545.

36. Pedersen LL, Smets BF, Dechesne A. 2015. Measuring biogeochemical heterogeneity at the micro scale in soils and sediments. Soil Biol Biochem.

37. Dalcin Martins P, Danczak RE, Roux S, Frank J, Borton MA, Wolfe RA, Burris MN, Wilkins MJ. 2018. Viral and metabolic controls on high rates of microbial sulfur and carbon cycling in wetland ecosystems. Microbiome 6:138.

38. Maltby J, Steinle L, Löscher CR, Bange HW, Fischer MA, Schmidt M, Treude T. 2017. Microbial methanogenesis in the sulfate-reducing zone in sediments from Eckernförde Bay, SW Baltic Sea. Biogeosciences Discuss 1–45.

39. Laufer K, Nordhoff M, Roy H, Schmidt C, Behrens S, Jørgensen BB, Kappler A. 2016. Coexistence of microaerophilic, nitrate-reducing, and phototrophic Fe(II) oxidizers and Fe(III) reducers in coastal marine sediment. Appl Environ Microbiol 82:1433–1447.

40. Sela-Adler M, Ronen Z, Herut B, Antler G, Vigderovich H, Eckert W, Sivan O. 2017. Co-
existence of Methanogenesis and Sulfate Reduction with Common Substrates in Sulfate-Rich Estuarine Sediments. Front Microbiol 8:766.

41. Elferink, Luppens, Marcelis, Stams. 1998. Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge. Appl Environ Microbiol 64:2301–3.

42. Egger M, Lenstra W, Jong D, Meysman FJR, Sapart CJ, Van Der Veen C, Röckmann T, Gonzalez S, Slomp CP. 2016. Rapid sediment accumulation results in high methane effluxes from coastal sediments. PLoS One 11.

43. Ozuolmez D, Na H, Lever MA, Kjeldsen KU, Jørgensen BB, Plugge CM. 2015. Methanogenic archaea and sulfate reducing bacteria co-cultured on acetate: teamwork or coexistence? Front Microbiol 6:492.

44. Plugge CM, Zhang W, Scholten JCM, Stams AJM. 2011. Metabolic Flexibility of Sulfate-Reducing Bacteria. Front Microbiol 2.

45. Bansal S, Tangen B, Finocchiaro R. 2016. Temperature and Hydrology Affect Methane Emissions from Prairie Pothole Wetlands. Wetlands 36:371–381.

46. Tangen BA, Finocchiaro RG, Gleason RA. 2015. Effects of land use on greenhouse gas fluxes and soil properties of wetland catchments in the Prairie Pothole Region of North America. Sci Total Environ 533:391–409.

47. Bushnell B. 2016. BBMap short read aligner.

48. Li D, Liu CM, Luo R, Sadakane K, Lam TW. 2015. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.

49. Scholz M, Lo C-C, Chain PSG. 2015. Improved Assemblies Using a Source-Agnostic Pipeline for MetaGenomic Assembly by Merging (MeGAMerge) of Contigs. Sci Rep
Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–9.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.

Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, Loman NJ, Andersson AF, Quince C. 2013. CONCOCT: Clustering cONTigs on COverage and ComposiTion. Arxiv Prepr arXiv13124038v1 28.

Wu YW, Simmons BA, Singer SW. 2015. MaxBin 2.0: An automated binning algorithm to recover genomes from multiple metagenomic datasets. Bioinformatics 32:605–607.

Kang DD, Froula J, Egan R, Wang Z. 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ 3:e1165.

Sieber CMK, Probst AJ, Sharrar A, Thomas BC, Hess M, Tringe SG, Banfield JF. 2017. Recovery of genomes from metagenomes via a dereplication, aggregation, and scoring strategy. bioRxiv 107789.

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. 2015. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25:1043–1055.

Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, VerBerkmoes NC, Wilkins MJ, Hettich RL, Lipton MS, Williams KH, Long PE, Banfield JF. 2012. Fermentation, Hydrogen, and Sulfur Metabolism in Multiple Uncultivated Bacterial Phyla. Science (80-) 337:1661–1665.
58. Daly RA, Borton MA, Wilkins MJ, Hoyt DW, Kountz DJ, Wolfe RA, Welch SA, Marcus DN, Trexler R V., MacRae JD, Krzycki JA, Cole DR, Mouser PJ, Wrighton KC. 2016. Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales. Nat Microbiol 1:16146.

59. Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119.

60. Roux S, Brum JR, Dutilh BE, Sunagawa S, Duhaime MB, Loy A, Poulos BT, Solonenko N, Lara E, Poulain J, Pesant S, Kandels-Lewis S, Dimier C, Picheral M, Searson S, Cruaud C, Alberti A, Duarte CM, Gasol JM, Vaqué D, Bork P, Acinas SG, Wincker P, Sullivan MB. 2016. Ecogenomics and potential biogeochemical impacts of globally abundant ocean viruses. Nature 537:689–693.

61. Hug LA, Baker BJ, Anantharaman K, Brown CT, Probst AJ, Castelle CJ, Butterfield CN, Hernsdorf AW, Amano Y, Ise K, Suzuki Y, Dudek N, Relman DA, Finstad KM, Amundson R, Thomas BC, Banfield JF. 2016. A new view of the tree of life. Nat Microbiol 1:16048.

62. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

63. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

64. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 - Approximately maximum-likelihood
trees for large alignments. PLoS One 5.

Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 44:W242–W245.

Anders S, Pyl PT, Huber W. 2015. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169.

R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

**Figure legends**

Figure 1. Simplified summary of investigated pathways for ethanol and isopropanol production. ATP-generating reactions are indicated by closed circles; ATP-consuming, by open circles; NAD(P)H-generating reactions are indicated by closed squares; NAD(P)H-consuming, by open squares; reduced ferredoxin (Fd (red))-generating reactions are indicated by closed stars; oxidized ferredoxin (Fd (ox))-generating, by open stars. Solid arrows indicated the written reaction, while dashed arrows indicate a series of reactions not shown. In acetone/isopropanol-butanol-ethanol fermentation, red arrows indicate solventogenic phase reactions, and the green arrow and substrate indicate an additional reaction in the isopropanol-producing variation. In *Pyrococcus furiosus*-like fermentation, “R-” indicates the radical in the molecule. Enzymes are numbered as follows: 1, hexokinase; 2, glucose-6-P dehydrogenase; 3, 6-P-gluconate dehydrogenase; 4, ribulose-5-P epimerase; 5, phosphoketolase; 6, glyceraldehyde-3-P dehydrogenase; 7, 3-P-glycerate kinase; 8, 3-P-glycerate mutase; 9, enolase; 10, pyruvate kinase; 11, lactate dehydrogenase; 12, phosphotransacetylase; 13, aldehyde dehydrogenase; 14, alcohol dehydrogenase; 15,
phosphoenolpyruvate (PEP) carboxylase; 16, malate dehydrogenase; 17, fumarase; 18, succinate dehydrogenase; 19, pyruvate-formate lyase; 20, formate-hydrogen lyase; 21, acetate kinase; 22, alpha-acetolactate synthase; 23, alpha-acetolactate decarboxylase; 24, 2,3-butanediol dehydrogenase; 25, PFOR, IFOR, OGFOR or OIFOR; 26, ferredoxin hydrogenase; 27, acetyl-CoA acetyltransferase; 28, hydroxybutyryl-CoA dehydrogenase; 29, crotonase; 30, butyryl-CoA dehydrogenase; 31, phosphotransbutyrylase; 32, butyrate kinase; 33, butyraldehyde dehydrogenase; 34, butanol dehydrogenase; 35, acetoacetyl-CoA: acetate/butyrate: CoA transferase; 36, acetoacetate decarboxylase; 37, isopropanol dehydrogenase; 38, hydrogen dehydrogenase; 39, aldehyde:ferredoxin oxidoreductase; 40, acetyl-CoA synthetase.

Fermentations are not balanced, and reversible reactions are not indicated.

Figure 2. Overview of metagenome-assembled genomes selected for this study. MAG name, taxonomy, completeness, contamination and abundance are provided. Taxonomy was inferred with CheckM and phylogenetic analyses of RpsC sequences. Abundance is expressed as total normalized coverage (across all metagenomes) per Gbp of metagenome (see Materials and Methods for details).

Figure 3. Phylogeny of alcohol-cycling microorganisms spanning the archaeal and bacterial tree of life based on reference and MAG-retrieved RpsC sequences. Binned sequences were present in the color-coded clades. Only taxonomic groups containing binned RpsC sequences are labeled; however, some MAGs were lacking the rpsC gene (27/62). In these instances, taxonomy was inferred solely with CheckM (such MAGs are absent from this tree). Taxonomic groups are labeled by the branch. Abbreviations: CPR, candidate phyla radiation (collapsed clade).
Figure 4. Alcohol dehydrogenase phylogenetic tree. Zinc-type and iron-type ADHs are indicated by the outside semi-circles in red and blue, respectively. From 415 sequences in total, 76 did not cluster with any reference sequences (black color within zinc-type zone), 36 were classified as isopropanol dehydrogenases (orange), 75 as propanol/ethanol dehydrogenases (pink), 91 were NADH:quinone oxidoreductases and other medium chain reductase family members (collapsed branch), 38 formed a cluster of mostly PQQ-type ADHs (green shades), and 99 formed a cluster of mostly ethanol dehydrogenases (blue shades). Abbreviations are as follows: ADH, alcohol dehydrogenase; MDR, medium chain reductases; PQQ, pyrroloquinoline quinone; pdu, propanediol utilization alcohol dehydrogenase genes. An interactive online version of this tree (ADHs_in_62_MAGs_and.refs.tree) is available at https://itol.embl.de/shared/pdalcin.

Figure 5. A summary of metabolic potential in MAGs by taxa. The shades of blue indicate the percent of MAGs that encoded potential for each pathway. The number of MAGs in each taxa is indicated under “# MAGs”. Abbreviations are as follows: EMP, Embden–Meyerhof–Parnas glycolysis; PPP, pentose phosphate pathway; PDH, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; PFLP, P. furiosus-like fermentation; HFLF, heterofermentative lactate fermentation; MAF, mixed acid fermentation; SR, sulfate reduction; DNRA, dissimilatory nitrate reduction to ammonium; DEN, partial denitrification; OR, oxygen reduction. The asterisk (*) indicates an incomplete TCA cycle.

Tables
Table 1. Minimal criteria to determine the potential for a pathway, process, or enzyme.

| Metabolic trait                          | Criteria to determine metabolic potential                                                                 |
|-----------------------------------------|------------------------------------------------------------------------------------------------------------|
| Sugar utilization                       | At least one sugar-specific phosphotransferase component II enzyme or sugar kinase                        |
| Embden–Meyerhof–Parnas (EMP) glycolysis | 6 out of 10 genes (or 5, with one being a phosphofructokinase)                                           |
| Pentose Phosphate pathway               | 4 out of 7 genes                                                                                           |
| Entner–Doudoroff pathway                | Both 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphate-gluconate aldolase                     |
| Pyruvate or 2-oxoglutarate dehydrogenase complex | At least component E1 or component E2                                                              |
| Tricarboxylic Acid (TCA) cycle          | 5 out of 9 genes. If 2-oxoglutarate dehydrogenase was missing but 2-oxoglutarate ferredoxin oxidoreductase was present, it counted as an alternative. If both were missing, the TCA cycle was considered incomplete. For succinate dehydrogenase/fumarate reductase, at least 2 out 4 subunits needed to be present. |

*Pyrococcus furiosus*-like fermentation | pyruvate, indolepyruvate, 2-oxoisovalerate, or 2-oxoglutarate ferredoxin oxidoreductase, and 2 out of other 3 components: (i) acetyl-CoA synthetase; (ii)
| Process                                      | Potential Reactions                                                                 |
|----------------------------------------------|-------------------------------------------------------------------------------------|
| Heterofermentative lactate fermentation      | Potential for EMP, phosphoketolase, lactate dehydrogenase, aldehyde dehydrogenase, and ADH |
| Mixed acid fermentation                      | Pyruvate-formate lyase (PFL) or PFL-activating enzyme (AE), formate dehydrogenase, acetate kinase, potential for TCA or succinate dehydrogenase or lactate dehydrogenase, aldehyde dehydrogenase, and ADH |
| Butanediol fermentation                      | Aldehyde dehydrogenase, ADH, and 2,3-butanediol dehydrogenase                       |
| Acetone-butanol-ethanol (ABE) fermentation    | Acetoacetyl-CoA: acetate/butyrate CoA transferase, acetoacetate decarboxylase, phosphotransbutyrylase, butyrate kinase, aldehyde dehydrogenase and ADH |
| Isopropanol-butanol-ethanol (IBE) fermentation | Acetoacetyl-CoA: acetate/butyrate CoA transferase, acetoacetate decarboxylase, phosphotransbutyrylase, butyrate kinase, aldehyde dehydrogenase, ADH, and isopropanol dehydrogenase |
| Sulfate reduction                            | At least one subunit of the dissimilatory sulfide reductase (dsrABD) and no sox pathway |
| Dissimilatory nitrate reduction to ammonia (DNRA)| At least one ammonia-forming nitrite reductase (nirBD or nrfAH) |
Denitrification (partial)  At least one of the following: nitrate reductase (narGHI or napAB), nitrite reductase (nirKS), nitric oxide reductase (norBC), or nitrous oxide reductase (nosZ)

Oxygen respiration  At least one of the following oxygen reductases: aa3-type cytochrome c oxidase (coxABCD), cbb3-type cytochrome c oxidase (ccoNOPQ), cytochrome ba3 heme quinol oxidase, cytochrome bo3 ubiquinol oxidoreductase (cyoABCDE), cytochrome aa3-600 menaquinol oxidase (qoxABCD), cytochrome bd1 ubiquinol oxidoreductase/cyanide-insensitive oxidase (cydABX)
| Domain                  | # MAGs |
|------------------------|--------|
| Thaumarchaeota         | 2      |
| Calditrichaeota        | 1      |
| KSB1                   | 1      |
| Rokubacteria           | 1      |
| Latescibacteria        | 1      |
| Acidobacteria          | 5      |
| Actinobacteria         | 5      |
| Bacteroidetes          | 2      |
| Chloroflexi            | 8      |
| Cyanobacteria          | 1      |
| Ignavibacteriae        | 5      |
| Nitrospirae            | 1      |
| Planctomycetes         | 4      |
| Alphaproteobacteria    | 3      |
| Betaproteobacteria     | 4      |
| Deltaproteobacteria    | 7      |
| Gammaproteobacteria    | 5      |
| Zetaproteobacteria     | 1      |
| Spirochaetes           | 1      |
| Verrucomicrobia        | 1      |
| unclassified bacteria  | 3      |