

ORIGINAL ARTICLE

Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community

Mallory Embree¹, Harish Nagarajan¹, Narjes Movahedi², Hamidreza Chitsaz² and Karsten Zengler¹

¹Department of Bioengineering, University of California, San Diego, CA, USA and ²Department of Computer Science, Wayne State University, Detroit, MI, USA

Microbial interactions have a key role in global geochemical cycles. Although we possess significant knowledge about the general biochemical processes occurring in microbial communities, we are often unable to decipher key functions of individual microorganisms within the environment in part owing to the inability to cultivate or study them in isolation. Here, we circumvent this shortcoming through the use of single-cell genome sequencing and a novel low-input metatranscriptomics protocol to reveal the intricate metabolic capabilities and microbial interactions of an alkane-degrading methanogenic community. This methanogenic consortium oxidizes saturated hydrocarbons under anoxic conditions through a thus-far-uncharacterized biochemical process. The genome sequence of a dominant bacterial member of this community, belonging to the genus *Smithella*, was sequenced and served as the basis for subsequent analysis through metabolic reconstruction. Metatranscriptomic data generated from less than 500 pg of mRNA highlighted metabolically active genes during anaerobic alkane oxidation in comparison with growth on fatty acids. These data sets suggest that *Smithella* is not activating hexadecane by fumarate addition. Differential expression assisted in the identification of hypothetical proteins with no known homology that may be involved in hexadecane activation. Additionally, the combination of 16S rDNA sequence and metatranscriptomic data enabled the study of other prevalent organisms within the consortium and their interactions with *Smithella*, thus yielding a comprehensive characterization of individual constituents at the genome scale during methanogenic alkane oxidation.

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Introduction

Smithella, a member of the family Syntrophaceae, has been shown to be a predominant member of microbial communities involved in the anoxic degradation of long-chain alkanes (Zengler *et al.*, 1999; Gray *et al.*, 2011; Siddique *et al.*, 2011; Wang *et al.*, 2011; Scherr *et al.*, 2012; Cheng *et al.*, 2013). The mechanism driving anoxic hexadecane degradation in methanogenic consortia is thus far unknown. However, it is likely that this conversion requires the interaction of syntrophic bacteria, such as members of the Syntrophaceae family, with methanogenic archaea (Zengler *et al.*, 1999). The scope of previous studies has been limited to

identifying Syntrophaceae as key organisms within complex communities facilitating the initial steps of alkane activation (Gieg *et al.*, 2008; Gray *et al.*, 2011; Aitken *et al.*, 2013). This can be attributed to both the difficulty in isolating pure cultures of members of the *Smithella* genus as well as the slow growth and low biomass of the culture, with most experiments taking hundreds of days to complete (Figure 1). To date, only one member of the genus *Smithella*, *Smithella propionica*, has been isolated as an axenic culture (Liu *et al.*, 1999).

Elucidating the genetic basis of mechanisms driving the observed phenotype of unculturable microbial communities is contingent upon the deconvolution of the roles of individual community constituents (Zengler and Palsson, 2012). Owing to recent advances in next-generation sequencing, the study of non-model organisms in microbial communities is becoming increasingly accessible. Whole-genome sequencing of individual species within communities is routinely performed without the

Correspondence: K Zengler, Department of Bioengineering, University of California, San Diego, CA 92093 0412, USA.

E-mail: kzengler@ucsd.edu

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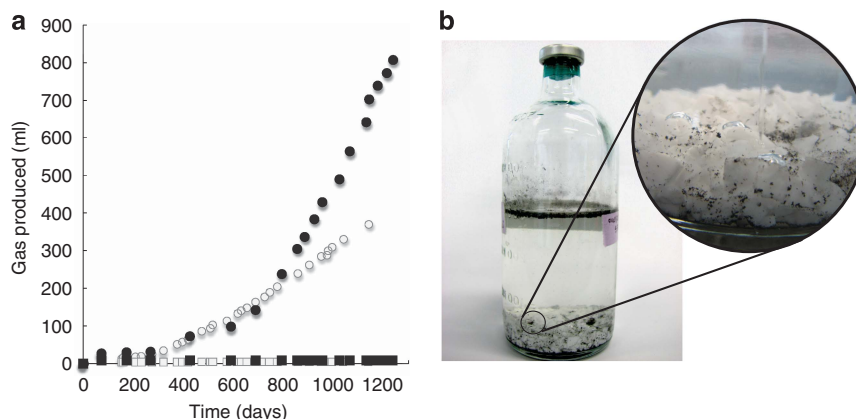


Figure 1 (a) Formation of gas (methane) in an anaerobic enrichment culture growing in 300 ml mineral medium containing hexadecane (●). The methanogenic consortium was propagated in the laboratory and formed a total of 809 ml of gas consisting of 10.9 mm methane (8.81 mmol total formed) after 1141 days of incubation. A control without hexadecane (■) showed no gas formation. The previous enrichment described in Zengler *et al.*, 1999 (open symbols, overlaid for comparison) formed a total of 370 ml of gas after 1051 days of incubation. The community with hexadecane is represented by open circles (○), and the control by open squares (□). (b) The consortium, although highly metabolically active as seen by gas formation (bubbles), grows to very low biomass densities as can be seen in this 3-year-old culture. Teflon boiling stones were coated with hexadecane to increase the contact between cells and the hydrophobic substrate.

need for cultivation (Swan *et al.*, 2011; Yoon *et al.*, 2011; Seth-Smith *et al.*, 2013). Furthermore, unlike hybridization-based approaches, entire transcriptomes can be characterized without the knowledge of existing reference genomes before sequencing (Wang *et al.*, 2009). Here, we study individual unculturable constituents in a low-biomass microbial community degrading hexadecane to methane (Figure 2). We applied a single-cell genome sequencing approach to assemble a working-draft genome of *Smithella*, and subsequently used it to identify potential alkane degradation-related genes and to reconstruct metabolic pathways. We deployed a low-input metatranscriptomic approach to accommodate the low biomass of the culture in order to determine which genes were active during alkane oxidation. After determining the major role of *Smithella* within the community, the metatranscriptomic data sets were extended to characterize the activity of other microbial community members cultured with a variety of substrates, in an effort to elucidate the microbial interactions that mediate community composition fluctuations during growth with different carbon sources.

Materials and methods

Media and cultivation

The original source of the enrichment was sediment from a hydrocarbon-contaminated ditch in Bremen, Germany (Zengler *et al.*, 1999). The consortium was further propagated in the laboratory in an anoxic medium containing 0.3 g NH_4Cl , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g NaHCO_3 , 0.5 g K_2HPO_4 , 0.05 g KBr , 0.02 g H_3BO_3 , 0.02 g KI , 0.003 g $\text{Na}_2\text{WO}_2 \cdot 2\text{H}_2\text{O}$, 0.002 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, trace elements and trace minerals as previously described (Zengler *et al.*, 1999). The medium was sparged with a mixture of N_2/CO_2

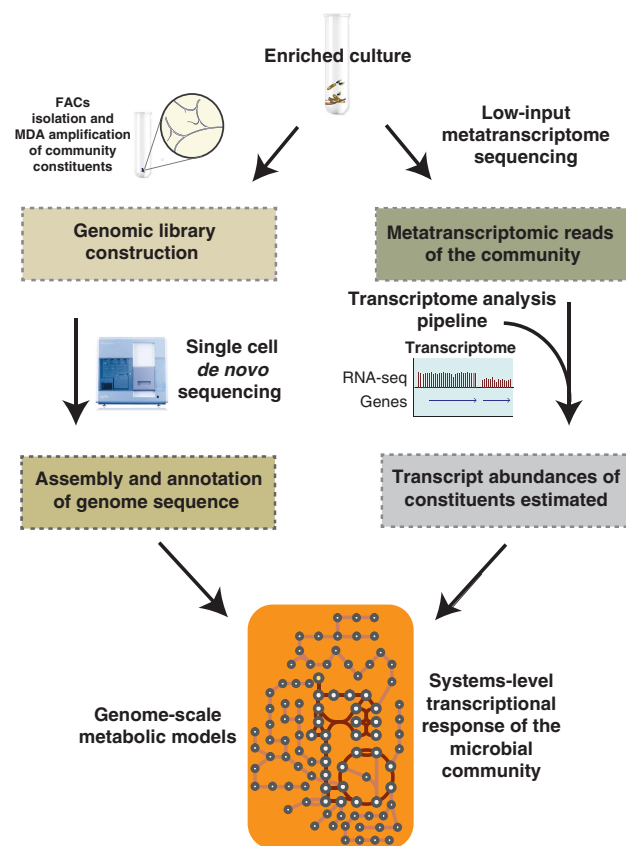


Figure 2 Schematic of the integrated workflow applied to study the methanogenic community. A single-cell genome sequencing approach established a working-draft genome of *Smithella*. Then, low-input metatranscriptomics was used in order to determine which genes were active during alkane degradation. After determining the major role of *Smithella* within the community, the metatranscriptomics data sets were extended to analyze the activity of other microbial community members. A genome-scale metabolic model was used to facilitate the integration of both the genomic and transcriptomic data in order to extract functional information about the organisms.

(80:20 v/v), and the pH was adjusted to 7.0. After autoclaving, anoxic CaCl_2 (final concentration 0.25 g l^{-1}) and filter-sterilized vitamin solution (Zengler *et al.*, 1999) were added. Cells were supplemented with anoxic hexadecane (0.5 ml, equaling 1.7 mmol , on a teflon filter, see Zengler *et al.*, 1999), 5 mM butyric acid or 2 mM caprylic acid and incubated at 30°C in triplicate. Bottles were degassed as necessary to relieve overpressurization. The headspace of a representative active culture was analyzed using gas chromatography (Shimadzu GC 2014, Supelco $30 \text{ m} \times 0.53 \text{ mm}$ Carboxen column, Kyoto, Japan). A thermal conductivity detector was used to identify and quantify the production of methane accompanying cell growth. Ultra pure nitrogen was used as the carrier gas, and the column was run at 35°C for 15 min with a flow rate of 20 ml min^{-1} .

Single-cell sorting, multiple displacement amplification and genome sequencing

Individual cells from a single bottle of the methanogenic alkane-degrading consortium (Figure 1) were obtained by staining the cells with SYTO-9 DNA stain and sorting of single cells using fluorescence-activated cell sorting. Single cells were then lysed as previously described, and the genomic DNA of individual cells was amplified using whole-genome multiple displacement amplification (Swan *et al.*, 2011). Amplified genomic DNA was screened for *Smithella*-specific 16S rDNA gene sequences. Six amplified *Smithella* genomes were selected for next-generation sequencing. The multiple displacement amplified genomes were prepared for Illumina sequencing using the Nextera kit, version 1 (Illumina, San Diego, CA, USA) using the Nextera protocol (ver. June 2010) and a high-molecular-weight buffer. Libraries of these six samples were created and sequenced using an Illumina Genome Analyzer II, generating a total of 174 million paired-end reads using 36 or 58 bp reads with an insert size of 400 (Supplementary Table S1).

Assembly of single-cell genomes

Sequenced reads from *Smithella* were assembled using the *de novo* co-assembler, HyDA (Movahedi *et al.*, 2012, <http://compbio.cs.wayne.edu/software/hyda/>). HyDA is an assembler based on the colored de Bruijn graph. In the beginning, a unique color is assigned to each input data set, which belongs to one species. Here, the *Smithella* genome was co-assembled with the single-cell genomic material from two additional species (*Anaerolina* sp and *Syntrophus* sp) from the culture using three colors. All the colored input data sets were assembled simultaneously in a unified de Bruijn graph. The output of HyDA is a list of contigs and the corresponding average sequencing depth (coverage) for each color/input data set. Zero average coverage indicates the absence of that contig in the

assembled genome of the corresponding data set. Unicolored contigs are pieces of a sequence that are unique to the corresponding data set, and those contigs that have non-zero coverage for multiple data sets are shared sequences. The output of HyDA is the collective list of contigs present in at least one of the input data sets. To run the software, we chose the length of k-mers in the de Bruijn graph to be 25, and the coverage cutoff to trim erroneous branches in the graph was selected to be 100. The contigs were then annotated using RAST (rapid annotations using subsystem technology) (Aziz *et al.*, 2008), and the resulting annotation was used to generate a draft metabolic reconstruction using ModelSEED (Henry *et al.*, 2010). This Whole-Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number AWGX00000000. The version described in this paper is version AWGX01000000.

Low-input metatranscriptomics

To identify metabolically active genes, the transcriptome of the community was sequenced. Twenty milliliters of culture was harvested (from the same bottle used for single-cell genome sequencing) by adding 2 ml stop solution (95% ethanol, 5% TRIzol (Life Technologies, Carlsbad, CA, USA)) to the sample and mixing by inversion. For the fatty acid cultures, cells were harvested after 171 days of incubation (Supplementary Figure S1). Hexadecane samples were harvested 1146 days (3.14 years) after inoculation. The activity of the culture was monitored by measuring the amount of gas and methane produced, as OD_{600} proved to be an inaccurate measure owing to the aggregation of cells within the community. The hexadecane-degrading consortium had produced 180 ml of gas at the time of harvest. Cells were then centrifuged at $10\,000 \text{ g}$ at 4°C for 10 min in 50 ml conical tubes; centrifugation at lower r.p.m. (8000 r.p.m.) resulted in an insufficient recovery of *Smithella* sequences. After the initial pelleting, the supernatant was removed and centrifuged again in 1.5 ml-microcentrifuge tubes at $14\,000 \text{ g}$ at 4°C for an additional 20 min. After centrifugation, the supernatant was decanted and cell pellets were frozen at -80°C until use. Both pellets were combined before RNA isolation.

RNA was isolated, and rRNA was removed using a modified version of the Metabacteria RiboZero rRNA Removal kit (Epicentre, Madison, WI, USA). cDNA was generated using purified mRNA, and sequencing libraries were constructed using a modified version of the Nextera protocol (Illumina). A more detailed version of the protocol is given in the Supplemental Methods.

To benchmark the low-input protocol against standard RNA-seq methods, the standard dUTP method (Parkhomchuk *et al.*, 2009) as well as the low-input method were applied to a *Geobacter sulfurreducens* and *G. metallireducens* coculture (Shrestha *et al.*, 2013).

Mapping of transcriptome reads

The metatranscriptomics libraries generated were sequenced using either a Genome Analyzer IIX or a MiSeq (Illumina). The obtained reads were mapped to the *Smithella* draft genome using the short-read aligner Bowtie (<http://bowtie-bio.sourceforge.net>) (Langmead *et al.*, 2009) with two mismatches allowed per read alignment. To estimate transcript abundances, FPKM values were calculated using the Cufflinks tool (<http://cufflinks.cbc.umd.edu/>) (Trapnell *et al.*, 2010). Differential expression analysis was performed for the community under hexadecane-oxidizing conditions compared with butyric acid- and caprylic acid-oxidizing conditions using the Cuffdiff feature of the cufflinks package. DESeq (Anders and Huber, 2010) was also used to confirm the differential expression observed in the alkane-oxidizing community. The number of reads mapped to each gene was determined using HTSeq (Anders S, <http://www-huber.embl.de/users/anders/HTSeq/>). Differential expression analysis was also calculated for controls. In each case, the library prepared using the standard dUTP method was compared with the library generated by the low-input method described here.

In order to analyze the transcript abundance of genes not present in a specific genome, a *de novo* assembly of the community metatranscriptome under the hexadecane-degrading conditions was constructed using Trinity (Grabherr *et al.*, 2011). The *de novo* metatranscriptome was annotated using RAST (Aziz *et al.*, 2008) and served as a reference for mapping (Langmead *et al.*, 2009). HTSeq (Anders S, <http://www-huber.embl.de/users/anders/HTSeq/>) was used to generate count data for specific genes. Metatranscriptome data analysis can be accessed through GEO, accession no. GSE49830.

16S rDNA sequencing and analysis

DNA was isolated from the community cultured under hexadecane-, butyric acid- and caprylic acid-degrading conditions using Nucleospin XS Tissue columns (Clontech, Mountain View, CA, USA) using the same lysis modifications described in the Supplemental 'Low-Input Metatranscriptomics' section. In place of Superase-In, 1 μ l RNase A (Qiagen, Venlo, Netherlands) was added to cultures, and cells were lysed for 1.5 h. Long primers containing Illumina-compatible adapters were used to amplify bacterial-specific (27F/806R) and archaeal-specific (21F/806R) V1 and V4 regions of the 16S rDNA gene. The PCR product was purified using 0.7 \times AMPure beads, and confirmed using a Bioanalyzer DNA High Sensitivity chip (Agilent, Santa Clara, CA, USA). Regions V1-V2 (read 1) and V4 (read 2) were sequenced on a Miseq (Illumina) using 250 bp on each end. Reads one and two were processed as replicates using QIIME. Standard parameters were used for operational taxonomic unit selection, and

taxonomy was assigned using the most recent version (ver. 12_10) of Greengenes. Operational taxonomic units with only one read were filtered from the results before analysis.

Results

Single-cell genome sequencing and assembly

The *Smithella de novo* assembly generated with HydA resulted in 7697 contigs totalling to 3 213 255 bp (Table 1). The N50 was 6198 bp, and the largest contig was 56 011 bp. These contigs were used as a draft genome for *Smithella* for all downstream analysis. To estimate the completeness of this draft genome, annotated proteins within the genome were compared with clusters of orthologous groups (COGs), categories identified for *Syntrophus aciditrophicus* (strain SB, accession no. CP000252). As to date no members of the genus *Smithella* have been sequenced, *S. aciditrophicus*, a close relative of *Smithella* (Supplementary Figure S2) whose genome has been sequenced, was chosen (McInerney *et al.*, 2007). In all, 2250 of the 2863 (79%) annotated *Smithella* genes were functionally categorized into COGs, with most COGs being represented above 80% compared with *S. aciditrophicus* (Supplementary Table S2). The number of genes falling into the category 'lipid transport and metabolism' is twice as large (209%), as expected based on the *S. aciditrophicus* COGs, hinting at the capability of *Smithella* to metabolize fatty acids, which are potential intermediates of anaerobic alkane oxidation (Rojo, 2009).

After annotating the *Smithella* draft genome, ModelSEED was used to generate a draft metabolic reconstruction and to group genes into corresponding metabolic pathways (Henry *et al.*, 2010). In total, 510 genes were grouped into 731 different metabolic reactions. These reactions encompassed 229 different subsystems (Supplementary Figure S3), including a number of potential pathways related to alkane and fatty acid metabolism. Next, the reconstruction of *Smithella* was compared with a ModelSEED reconstruction of *S. aciditrophicus* to benchmark the completeness of the functional *Smithella* genome and to identify potential pathways involved in alkane degradation. Only genes that were part of complete, functional subsystems were included in the comparison. Of the 1152 genes included in the comparison, 136 were found only in *Smithella* whereas 453 were *S. aciditrophicus*-specific (Supplementary Table S3).

Table 1 *Smithella* draft genome assembly statistics.

Assembly size	3 213 255 bp
G + C content	42%
Largest contig	56 011 bp
Number of contigs	7697
Number of genes	2863
N50	6198 bp

Many of the *S. aciditrophicus*-specific genes fell into the categories DNA metabolism (42) and motility and chemotaxis (36), consistent with our COG analysis that suggests that the categories ‘replication, recombination and repair’ and ‘cell motility’ are less prevalent in the *Smithella* draft genome. Seven fatty acid-related genes were identified as *Smithella*-specific, as well as four genes related to cytochrome biosynthesis.

Genes expressed during alkane degradation and comparative transcriptomics of Smithella

In order to determine what functional genes are active under hexadecane-degrading conditions, we evaluated the transcriptome of *Smithella*. Standard protocols for RNA isolation and sequencing proved to be ineffective owing to the very low biomass of the community. We therefore adapted a low-input metatranscriptomics protocol to accommodate the low biomass of the culture (Figure 1b). The validity and applicability of this protocol to other microbial communities were assessed by analyzing mock communities in biological duplicate using both the low-input method presented here as well as a standard dUTP method (Parkhomchuk *et al.*, 2009). The mock community consisted of a bacterial coculture of *G. sulfurreducens* and *G. metallireducens* (Shrestha *et al.*, 2013). Libraries created via the low-input method utilized 500 pg of starting mRNA, whereas those created according to the dUTP method used 100 ng of starting mRNA. Sequenced reads from the *Geobacter* coculture were mapped to both organisms, and differential expression between the low-input and dUTP libraries for each sample was calculated using the Cufflinks package (Trapnell *et al.*, 2010). No significant, differential gene expression was observed between the two library preparation methods. Gene expression values were also correlated to assess overall transcript representation within each library type (Supplementary Figure S4). The Spearman rank correlation of replicates 1 and 2 was 0.9 and 0.86, respectively.

Using this low-input protocol, a metatranscriptomic library of the methanogenic community

grown with hexadecane as the sole electron donor was generated using ~500 pg of mRNA. The library was sequenced on an Illumina GAIIX, generating 53 million reads (5.3 Gb of data), of which 480 Mb mapped to the *Smithella* draft genome. Interspecies sequence diversity of community members combined with high stringency settings during mapping ensured that only reads derived from *Smithella* were included in downstream gene expression analysis. Our mapping strategy only permitted two mismatches as well as alignment suppression if one reportable alignment already existed for a particular read. This allowed us to isolate a *Smithella*-specific transcriptome profile out of the metatranscriptome (Figure 3). The transcriptomic reads covered 94% of all genes in the 3.2-Mb *Smithella* draft genome. A number of putative genes related to alkane degradation, including potential alkane-emulsification genes and fatty acid degradation genes, were identified as highly expressed (>75th percentile) (Supplementary Table S5) while *Smithella* metabolizes hexadecane.

To discriminate between genes related to alkane and fatty acid metabolism, differential expression of the community utilizing hexadecane, butyric or caprylic acid was explored (Supplementary Table S4, Supplementary Figure S1). Metatranscriptomic reads from these two fatty acid communities were then mapped to the *Smithella* draft genome, and from this data set, we identified genes of *Smithella* that were actively expressed only in the presence of hexadecane. Potential alkane-activation genes were of particular interest. A number of radical-activating enzymes that exhibited homology to gene sequences in known, anaerobic hexadecane-degraders including *Desulfatibacillum alkenivorans* (Callaghan *et al.*, 2012) and *Desulfococcus oleovorans* HDX3 (Aeckersberg *et al.*, 1991), were identified as highly expressed (all but one within the upper 25th percentile) (Supplementary Table S7). Because *Smithella* may be degrading hexadecane through an unknown pathway, the homology of hypothetical proteins (68) falling into the top 10% of all expressed genes during hexadecane degradation were also investigated. Hypothetical proteins

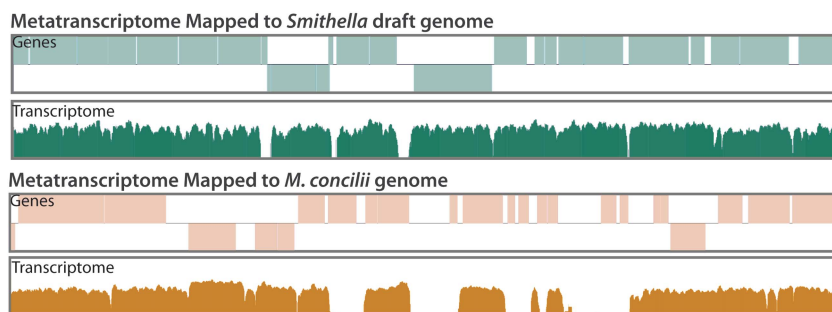


Figure 3 Example of the coverage obtained from the metatranscriptome (hexadecane-degrading community) when mapped to the *Smithella* draft genome (teal tracks) and the published genome of *M. concilii* (orange tracks). This snapshot is representative of the level of coverage observed across the entire genome of these organisms. Ninety-four percent of the genes from *Smithella* were represented by the metatranscriptomic data set.

exhibiting high levels of expression across all three conditions were excluded from the analysis. Twenty one out of 68 candidate proteins were found to have high homology (position-specific iterative basic local alignment score ≥ 200) to hypothetical proteins encoded in the genomes of hexadecane-degraders *D. alkenivorans* and *D. oleovorans* (Supplementary Table S8). The remaining 47 proteins had no homology to known proteins. Because all of these genes were highly expressed, future biochemical analysis of these gene products is necessary to elucidate if these genes are directly involved in hexadecane degradation.

Community composition and interactions during alkane and fatty acid degradation

Elucidating the metabolic activity of *Smithella* subsequently allowed determining potential interspecies interactions within the methanogenic community. Bacterial and archaeal 16S rDNA sequence analysis revealed major shifts in the community composition between growth with hexadecane, butyric acid or caprylic acid (Figure 4b). While *Smithella* comprised 82% of the community during decomposition of hexadecane, its abundance dropped to 17% when butyric or caprylic acid was the energy source. In their stead, members of the families Dethiosulfovibrionaceae (29% and 19%, butyric acid and caprylic acid, respectively) and Desulfobulbaceae (20%) began to dominate the community. Unknown members of the orders Ignivibacteriales (10%) and Bacteroidales (13% and

11%) also became more predominant during growth on fatty acids. Within the archaea portion of the community, *Methanosaeta* was the prevalent methanogen under two conditions. It comprised 93% of the archaeal community when butyric acid was provided, 83% for caprylic acid, but only 37% for hexadecane. Sequences related to *Methanoculleus* (Methanomicrobiales) were found in all conditions at levels below 10%. Under hexadecane-degrading conditions, *Methanocalculus*, another member of the Methanomicrobiales, represented 47% of all archaea (Figure 4b).

Metatranscriptomic data sets were mapped to the genomes of the closest sequenced members identified from the 16S rDNA data to determine metabolically active members of the community. We mapped reads to five Deltaproteobacteria and thirteen different representative methanogens within the orders Methanobacteriales, Methanosarcinales and Methanomicrobiales (Table 2, Supplementary Table S4).

Activity of *Methanosaeta concilii*. Within the hexadecane-degrading culture, 268 Mb mapped to a species related to *M. concilii*, consistent with 16S rDNA data suggesting *Methanosaeta* is an active member of the community. Genes related to energy metabolism and methanogenesis were highly expressed across all conditions. Of particular note was the expression of genes involved in CO₂ fixation (Figure 4a, Supplementary Table S6). Nearly all subunits of the major genes involved in the CO₂ fixation pathway were expressed during hexadecane

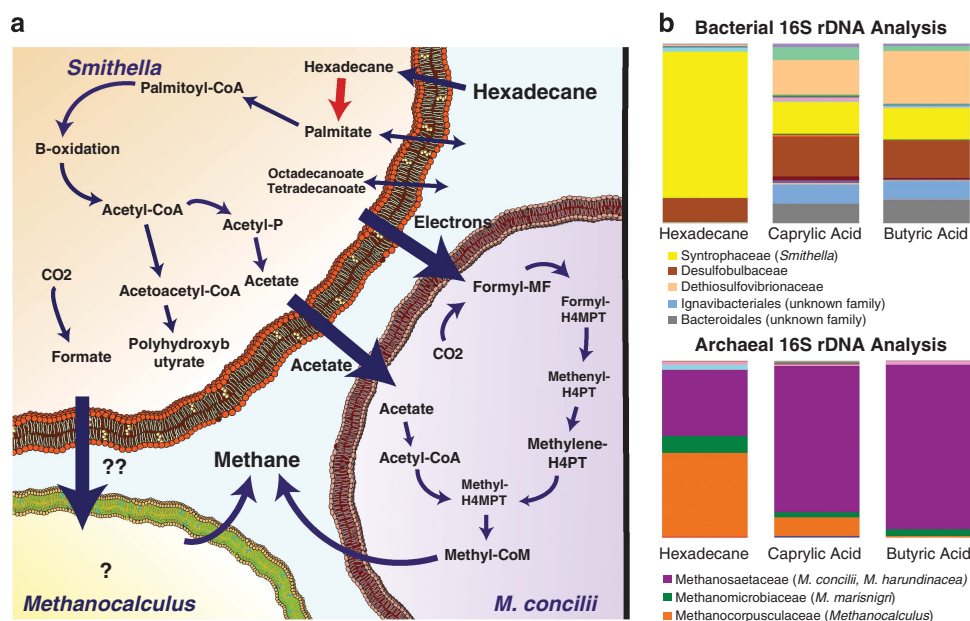


Figure 4 (a) Overview of the metabolism of *Smithella* and two representative methanogens during hexadecane degradation. Active pathways were determined from metatranscriptomic data mapped to the *Smithella* draft genome and the published *M. concilii* genome. The red arrow indicates that the mechanism driving this step is currently unknown. In this paper, we present a list (Supplementary Tables S7 and S8) of highly expressed, hypothetical proteins and radical-activating enzymes that may be facilitating hexadecane activation. (b) 16S rDNA gene analysis of the bacterial and archaeal community under hexadecane-, caprylic acid- and butyric acid-degrading conditions.

Table 2 List of representative genomes used during metatranscriptome mapping.

Species	Order	NC accession	Genome Size (bp)
<i>Desulfobulbus propionicus</i> DSM 2032	Desulfobacterales	NC_014972	3 851 869
<i>Desulfobacterium autotrophicum</i> HRM2	Desulfobacterales	NC_012108	5 589 073
<i>Desulfurivibrio alkaliphilus</i> AHT2	Desulfobacterales	NC_014216	3 097 763
<i>Desulfovibrio vulgaris</i> Hildenborough	Desulfovibrionales	NC_002937	3 570 858
<i>Desulfovibrio desulfuricans</i> ND132	Desulfovibrionales	NC_016803	3 858 580
<i>Methanobacterium</i> sp. AL-21	Methanobacterales	NC_015216	2 583 753
<i>Methanocorpusculum labreanum</i> Z	Methanomicrobiales	NC_008942	1 804 962
<i>Methanoculleus bourgensis</i> MS2	Methanomicrobiales	NC_009051	2 789 773
<i>Methanoculleus marisnigri</i> JR1	Methanomicrobiales	NC_018227	2 478 101
<i>Methanospirillum hungatei</i> JF-1	Methanomicrobiales	NC_007796	3 544 738
<i>Methanohalophilus mahii</i> DSM 5219	Methanosarcinales	NC_014002	2 013 424
<i>Methanobolus psychrophilus</i> R15	Methanosarcinales	NC_018876	3 072 769
<i>Methanosaeta concilii</i> GP6	Methanosarcinales	NC_015416	3 008 626
<i>Methanosaeta harundinacea</i> 6Ac	Methanosarcinales	NC_017527	2 559 043
<i>Methanosaeta thermophila</i> PT	Methanosarcinales	NC_008553	1 879 471
<i>Methanosarcina acetivorans</i> C2A	Methanosarcinales	NC_003552	5 751 492
<i>Methanosarcina barkeri</i> Fusaro	Methanosarcinales	NC_007355	4 837 408
<i>Methanosarcina mazei</i> Gö1	Methanosarcinales	NC_003901	4 096 345

degradation with transcript abundance above the median FPKM (168 for butyric acid comparison and 359 for caprylic acid comparison). Only formyl-MF subunits fmdABCF were expressed below the median FPKM, as well as formyl-MF:tetrahydromethanopterin formyltransferase. Similarly, most subunits of acetyl-CoA synthetase and CO dehydrogenase/acetyl-CoA synthase, the key enzymes facilitating methanogenesis from acetate, were expressed above the median FPKM with only *cdhA* and *cdhE* being expressed below the median FPKM. The acetate transporter, MCON_2287, was expressed below the median FPKM.

Activity of *Methanosaeta harundinacea*. While almost no reads (1.4 Mb) mapped to *M. harundinacea* (another *Methanosaeta* species) during degradation of hexadecane, a significant portion of reads mapped to this species during fatty acid degradation with 58 Mb mapping for butyric acid and 31 Mb mapping for caprylic acid (Supplementary Table S4). Although the reads mapped evenly to the *M. concilii* genome, the mapping to *M. harundinacea* was uneven despite the high overall number of reads aligning to the genome. This discrepancy is likely due to sequence dissimilarities between the published *M. harundinacea* genome and the species of *Methanosaeta* within the culture presented here. Thus, instead of analyzing relative expression levels of genes within a specific condition, count data were used to investigate potentially active genes across all three conditions, as the same regions of the genome should be covered during read mapping. The genes with the highest hits, particularly during caprylic acid degradation, were formate dehydrogenase genes (Mhar_0689: 1678, Mhar_1941: 1151, Mhar_0325: 1084, Mhar_1279: 706 counts) as well as the CO₂ fixation-gene formyl-MF dehydrogenase (Mhar_1287: 697 counts) and the acetate-fermentation genes acetyl-CoA synthetase (Mhar_0749: 967

counts) and acetyl-CoA synthase/decarbonylase (Mhar_2328: 735 counts).

Activity of *Methanoculleus marisnigri*. Reads of cultures growing with butyric or caprylic acid also mapped to *M. marisnigri*, a hydrogenotrophic methanogen. In contrast to *M. concilii*, this organism is incapable of growing with acetate as the sole electron donor (Maestrojuán *et al.*, 1990). A total of 197 Mb mapped to *M. marisnigri* under butyric acid (across 1022 of 2557 genes) and 113 Mb (across 872 of 2557 genes) under caprylic acid-degrading conditions (no reads from hexadecane growth mapped to *M. marisnigri*). Again, genes involved in methanogenesis, such as methyl-coenzyme M reductase and formyl-MF:tetrahydromethanopterin formyltransferase, were highly expressed. However, as a reference genome was used instead of a genome sequence actually obtained from a cell in the community, reads were not efficiently mapped to all genes, likely due to inherent sequence dissimilarities between species as was observed for *M. harundinacea*.

Activity of *Methanocorpusculum labreanum* (*Methanocalculus*). No reads under any condition could be mapped to *M. labreanum*, the only member of the Methanocorpusculaceae that has been sequenced so far. This family represented 47% of all archaea within the hexadecane community, but only less than 10% in the two fatty acid-metabolizing communities, hinting at a specific role of these bacteria related to hexadecane degradation. To try and determine the role of this organism within the community, expression of formate dehydrogenases and the energy conserving hydrogenases (*ech*) was investigated through the use of a *de novo* assembled metatranscriptome. Under hexadecane-degrading conditions, 4069 reads mapped to formate dehydrogenase genes and 579 to *ech* hydrogenase genes,

whereas only 42 and 47 reads mapped to formate dehydrogenase genes and 6 and 318 reads mapped to ech hydrogenase genes in the butyric acid and caprylic acid communities, respectively (Supplementary Table S9). This suggests that both formate dehydrogenases and ech hydrogenases are highly active during hexadecane degradation compared with growth on fatty acids. The lowered transcript counts may also reflect the low *Methanocalculus* abundance in the fatty acid conditions.

Discussion

Obtaining information about the activity of individual members within a microbial community with respect to environmental perturbation, such as availability of different electron donors or acceptors, has long been stymied by the inability to link metatranscriptomic data to specific microorganisms. Metatranscriptomic data were generated for the community growing on hexadecane, butyric acid or caprylic acid using a low-input metatranscriptomics protocol optimized for the very low biomass of the community (Figure 1b). These metatranscriptomes were used in conjunction with the *Smithella* draft genome, obtained from single-cell sequencing and other representative genomes, to investigate both metabolic capabilities and microbial interactions of the community.

In *Smithella*, a number of pathways potentially related to alkane degradation were identified in the genome. Comparison of transcriptomic data obtained from all three carbon source additions explicated the activity of these genes during hexadecane degradation. Emulsification and membrane genes that are known to assist in the degradation of hydrophobic alkanes (Tzintzun-Camacho *et al.*, 2012) were both present and active within *Smithella*. The gene encoding an apolipoprotein N-acyltransferase is of particular note, as previous studies have shown that deletion of lipoprotein synthesis genes can decrease membrane hydrophobicity in bacteria (Okugawa *et al.*, 2012). Expression of this gene during growth with hexadecane may assist *Smithella* to adapt its membrane composition to the presence of the hydrophobic substrate. Transporters for long-chain fatty acids, such as palmitate, octadecanoate and tetradecanoate, were also encoded in the genome and actively expressed during growth with hexadecane only, suggesting that these genes may be assisting in the export of biosurfactants for hexadecane emulsification (Breuil and Kushner, 1980; Liu *et al.*, 2012).

To date, very few enzymes involved in the activation of alkanes without molecular oxygen have been elucidated (So *et al.*, 2003; Zedelius *et al.*, 2011; Callaghan *et al.*, 2012). Although the *Smithella* genome encodes a gene for methylmalonyl-CoA mutase, a protein known to have a role in fumarate-dependent anaerobic hexadecane degradation

(Wilkes *et al.*, 2002; Callaghan *et al.*, 2012), this mode of degradation seems unlikely in this organism. Genes for alkyl-succinate synthase subunits, critical for the fumarate-dependent activation of alkanes, were not identified in the genome. This is consistent with previous studies that suggest a mechanism other than fumarate addition that drives alkane activation under methanogenic conditions (Aitken *et al.*, 2013). Genes highly expressed during growth with hexadecane and not with fatty acids, particularly hypothetical proteins with no annotated function (Supplementary Tables S7 and S8), could provide potential candidates involved in this pathway.

Biological conversion of hexadecane to methane requires the interaction of syntrophic bacteria with methanogenic archaea (Zengler *et al.*, 1999). A number of methanogens, notably the acetoclastic *M. concilii* and *M. harundinacea* as well as the hydrogenotrophic *M. marisnigri* and *Methanocalculus* were found to be highly abundant in our community. On the basis of 16S rDNA data, *Methanosaeta* species were well represented under all three different growth conditions, but dominated during fatty acid degradation (Figure 4b). While *M. concilii* was almost exclusively active during hexadecane degradation based on transcriptomic analysis, both *M. concilii* and *M. harundinacea* were active during oxidation of fatty acids (Supplementary Table S4). Although at least two different species of *Methanosaeta* appeared to be active within the community, they are fulfilling different metabolic roles under each condition. *M. harundinacea* is likely using formate as an electron donor in addition to acetate utilization during fatty acid degradation. The *M. concilii* genome does not encode a complete formate-dehydrogenase complex. Thus, if formate is being used as an electron carrier during hexadecane degradation as was revealed through *de novo* transcriptome analysis, it is not being utilized by a member of *Methanosaeta*.

M. concilii is known for its high affinity to acetate, and is unable to use hydrogen for methanogenesis (Smith and Ingram-Smith, 2007). Despite this, the *M. concilii* genome encodes genes required for CO₂ reduction, which, based on our transcriptome analysis, were all found to be highly transcriptionally active under all three conditions with the majority of the enzymes being expressed within the upper 50th percentile. Although the entire CO₂ fixation branch is active, different subunits of formyl-MF dehydrogenase, the first step in CO₂ reduction (Figure 4a), were expressed at variable levels across conditions. FmdA was the most highly expressed during fatty acid degradation (97th percentile), but did not appear to be expressed during hexadecane degradation (13th percentile). From previous studies, FmdA has been shown to be the catalytic subunit exhibiting amidohydrolase activity (Holm and Sander, 1997), suggesting that *M. concilii* may be utilizing methylamines during fatty acid degradation. Alternatively, FmdC also exhibited

higher levels of expression during fatty acid degradation and is located right after the *FmdA* gene, so these two genes may be co-regulated. *FwdG* was the second most highly expressed (82nd percentile) subunit during hexadecane degradation, whereas *FwdG* was expressed near the median during fatty acid degradation (42nd percentile). *M. concilii* has previously been shown to form conductive aggregates with species of *Geobacter*, suggesting that direct interspecies electron transfer (DIET) can occur between these species (Morita *et al.*, 2011). It has recently been demonstrated by the Lovley group that *Methanosaeta* can reduce CO₂ while accepting electrons by DIET using a transcriptomic and radio-tracer approach (Rotaru *et al.*, 2013). The higher expression of *FwdG* during hexadecane degradation potentially indicates that a source of electrons in addition to acetate is being utilized for methanogenesis, as *FwdG* is predicted to be a potential electron carrier. Although we cannot directly confirm that DIET is occurring in this consortium, it is possible that *Smithella* is transferring electrons to *M. concilii* for CO₂ reduction in addition to acetate during hexadecane degradation.

Methanocalculus, a member of the family Methanocorpusculaceae that can reduce CO₂ with hydrogen or formate as an electron donor (Zellner *et al.*, 1989), was the predominant methanogen under hexadecane-degrading conditions. Members of this family were previously found in methanogenic alkane-degrading communities, composing around 20–30% of the archaeal community (Grabowski *et al.*, 2005; Gray *et al.*, 2011). Although we could not directly confirm the activity of *Methanocalculus*, both formate dehydrogenases and ech hydrogenases were actively transcribed during growth with hexadecane. It has been suggested that methanogenic degradation of long-chain alkanes requires the presence of both acetoclastic as well as hydrogenotrophic methanogens (Zengler *et al.*, 1999; Jones *et al.*, 2008). Thus, it is possible that Methanocorpusculaceae is fulfilling this role in concurrence with *M. concilii* in this community.

The hydrogenotrophic *M. marisnigri*, a member of the Methanomicrobiales, was most abundant during hexadecane degradation (Figure 4b), but only a small percentage of reads (11 762, representing 0.02%) mapped under this condition. This discrepancy likely arises from sequence dissimilarity between sequenced strains available in databases and the actual organism present in the community. Thus, in order to fully assess the activity of specific community members, draft genomes of targeted species must be obtained by a single-cell approach used here or alternatively by a computational approach that bins metagenomic sequences (Chatterji *et al.*, 2008; Dick *et al.*, 2009; Albertsen *et al.*, 2013).

Smithella, a genus that to date has not been sequenced, was targeted for this study. Integration of single-cell genome sequencing and metatranscriptomics

allowed us to identify potential genes involved in anaerobic hexadecane degradation. Extension of the metatranscriptomic data sets to additional representative genomes of community members yielded insight into the nature of potential syntrophic interactions *Smithella* participates in. Furthermore, applying species-specific genome-scale analysis of this community across multiple conditions provided insights into the mechanisms driving major shifts in abundance and activity of key community constituents. Moving beyond purely descriptive, meta-level analyses of communities will eventually allow an understanding of the plasticity of community composition and the capacity of individual members to accommodate and respond to environmental perturbation.

Conflict of Interest

The authors declare no conflict of interest.

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