High-throughput mutation, selection, and phenotype screening of mutant methanogenic archaea

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Abstract

Bacterial and archaeal genomes can contain 30% or more hypothetical genes with no predicted function. Phylogenetically deep-branching microbes, such as methane-producing archaea (methanogens), contain up to 50% genes with unknown function. In order to formulate hypotheses about the function of hypothetical gene functions in the strict anaerobe, *Methanosarcina acetivorans*, we have developed high-throughput anaerobic techniques to UV mutagenize, screen, and select for mutant strains in 96-well plates. Using these approaches we have isolated 10 mutant strains that exhibit a variety of physiological changes including increased or decreased growth rate relative to the parent strain when cells use methanol and/or acetate as carbon and energy sources. This method provides an avenue for the first step in identifying new gene functions: associating a genetic mutation with a reproducible phenotype. Mutations in bona fide methanogenesis genes such as corrinoid methyltransferases and proton-translocating $F_{420}H_2$:methanophenazine oxidoreductase (Fpo) were also generated, opening the door to in vivo functional complementation experiments. Irradiation-based mutagenesis such as from ultraviolet (UV) light, combined with modern genome sequencing, is a useful procedure to discern systems-level gene function in prokaryote taxa that can be axenically cultured but which may be resistant to chemical mutagens.

Keywords

methanogen, *Methanosarcina*, struvite, archaea, methane

Abbreviations

2D, two-dimensional; DMS, dimethylsulfide; DNA, deoxyribonucleic acid; MeOH, methanol; MT, methanethiol; ORFs, open reading frames; TMA, trimethylamine; UV, ultraviolet.

Introduction

Reverse-genetics, the ability to make site-directed mutations in an organism's genome, is the gold-standard for genetic experiments to assess the role of a gene on cell physiology. However, many organisms do not have genetic systems, either because they cannot be cultured easily in the laboratory, because they have robust restriction modification systems that degrade foreign DNA, or because they have formidable structural barriers (cell walls or S-layers) that prevent entry of foreign DNA. Classical forward genetics, using ultraviolet (UV) or chemically induced random mutagenesis and selecting or screening for a desired growth phenotype, has advantages in that the experimenter does not already need a gene identity or a genetic system. Combined with modern advances in genome sequencing technology, UV mutagenesis and phenotype selection/screens may be the technical approach that unlocks the metabolic secrets of microbes which lack genetic systems (1-5).

We have developed several techniques to facilitate high-throughput genetic selections and screens in the strict anaerobe, *Methanosarcina acetivorans*. *M. acetivorans* is a methane-producing archaeon (methanogen). It grows by reducing methanol, methylamines, methylsulfides, carbon monoxide, or acetate to methane gas (6). *M. acetivorans* has the largest methanogen genome sequenced to date, and the chromosome contains a high percentage of unknown genes (7). Because methanogens are distantly related to other model organisms like *E. coli*, up to 53% of the predicted open reading frames (ORFs) in *M. acetivorans* have unknown function (8).

M. acetivorans can use several carbon sources to grow. Therefore, it is possible to mutate genes needed to grow on one carbon source but not on the others. *M. acetivorans* is also fully prototrophic, although supplementation of the medium with defined vitamins increases the growth rate and biomass yield of cultures (data not shown). On the other hand, addition of yeast extract to culture medium can inhibit growth for unknown reasons. The ability to generate and screen large numbers of mutant strains would be valuable for identifying other genes that play a role in methanogenesis, and in defining novel vitamin transport, scavenging, and toxicity pathways. We investigated whether we could develop a UV mutagenesis and high-throughput method to detect previously uncharacterized genes that are involved in methanogenesis from either acetate or methanol.

Though *M. acetivorans* has a reasonably well developed genetic system, the unbiased nature of UV mutagenesis and a classical phenotype selection or screen is invaluable because it will allow us to 1) define gene functions of the many unknown and hypothetical genes, 2) identify transcriptional or post-

translational regulators which may themselves be constitutively expressed, 3) isolate point mutations that can inform about enzyme mechanism and/or structure, and 4) identify genes which may be involved in a given biochemical pathway that we would not have predicted (9).

Using UV mutagenesis and classical phenotype screening requires high numbers of colonies on the order of several thousand for saturation mutagenesis. The high numbers of colonies that must be screened to find mutants with a desired phenotype represents a technical hurdle when studying relatively slow-growing, strict anaerobes like *M. acetivorans*. For instance, classical screens in *E. coli* require several hundred petri dishes, which will take up significant bench and incubator space. However, strict anaerobes must be grown under oxygen-free conditions, and all experimental steps, including incubation of petri plates, and growth curve measurements, must be kept anaerobic (10). Our techniques for high-throughput genetic screening of *M. acetivorans* use a 96-well plate format (**Figure 1**).

Figure 1. Workflow for high-throughput UV mutagenesis and phenotype screening of methanogens.

Although a recent paper describes the clever use of canning jars fitted with rubber gaskets to grow *Methanosarcina* in 96-well plates, specialized anaerobic equipment is necessary to grow hundreds of petri dishes and 96-well plates necessary for high-throughput genetic screening (11). We have put two Wolfe incubators in our anaerobic chamber for growth of up to 306 petri dishes or 96-well plates. The Wolfe incubators are controlled by a gas manifold, allowing the growth of *Methanosarcina* under controlled atmosphere using premixed gases. Assembly of the anaerobic chamber and Wolfe incubators has been previously described (10). Here, we detail our current methods using this equipment for UV

mutagenizing *M. acetivorans* and phenotype screening on agar and in liquid medium in 96-well plates. We also describe the growth phenotypes of 10 mutant strains generated using these procedures.

Materials and Methods

Growth of *M. acetivorans*. *Methanosarcina* strains (Table 1) were grown under strictly anaerobic conditions in HS mineral salts medium (12, 13). For growth on solid medium, cells were plated on HS medium containing 1.4% agar (w/v) with the appropriate carbon source and additions as previously described (10). The following anaerobic additions were added when appropriate: methanol (MeOH, 125 mM), acetate (120 mM), methanol + acetate (125 mM MeOH + 40 mM acetate), trimethylamine (TMA, 50 mM), puromycin (2 µg/ml).

Table 1. Strains described in this study

NB#	Phenotype	Reference
34	Parent ($\Delta hpt::\phi C31$ <i>int</i> , <i>attP</i>)	(13)
89	Slow growth on methanol or methanol + acetate. Struvite crystals on acetate.	This study
90	Slow growth on methanol. Struvite crystals on acetate.	This study
91	Fast growth on methanol or methanol + acetate. Struvite crystals on acetate.	This study
92	Slow growth on methanol. Struvite crystals on acetate. Struvite crystals on acetate.	This study
93	Slow growth on methanol, methanol + acetate, and acetate.	This study
178	Slow growth on methanol.	This study
181	Slow growth on methanol.	This study
193	Slow growth on methanol.	This study
194	Slow growth on methanol or methanol + acetate.	This study
195	Slow growth on methanol.	This study

Genome resequencing. Genomic DNA from 1 ml stationary-phase culture was extracted using the Qiagen Blood genomic DNA extraction kit (Qiagen, USA). Sequencing libraries were constructed using the Nextera XT DNA Library Prep Kit (Illumina, Inc.) according to the manufacturer's protocols. Bridge amplification and sequencing with the MiSeq Reagent Kit v3 (Illumina, Inc.) were performed as described by the manufacturer. The resulting sequences were demultiplexed in BaseSapce. Raw data is available under the BioProject accession number PRJNA335646. Adapter and transposon associated sequences and the last 25 bp of the forward read, and the last 100 bp of the reverse read were removed using cutadapt (version 1.8.1).(14) The 'fastq_filter' command in USEARCH (v8.0.1623) was used to remove sequences containing any ambiguous bases, an expected maximum error rate greater than 0.01,

and sequences shorter than 100 bp.(15) Only reads where both the forward and reverse read were retained after quality control were used in downstream analysis. The Burrows-Wheeler Alignment tool (BWA) (version 0.7.12) was used to align reads against the reference genome, *Methanosarcina acetivorans* str. C2A (NC_003552.1).(7, 16) For each mutant strain, a combination of SAMtools, Picard Tools, and BEDTools were used to mark duplicate reads and summarize the read coverage across the reference genome.(17-19) SNPs and indels were identified using the 'mpileup' command (default parameters) in SAMtools.(17)

Microscopy. Crystals from acetate-grown cultures were harvested, placed on 0.2 µm filters, and split into three treatment groups; washed twice with 100 µL distilled deionized H₂O, washed twice with 70% ethanol, or washed twice with 0.4 M NaCl (iso-osmotic with the methanogen growth medium). Crystals were dried at 37 °C for 48 hours. Crystals were fixed to glass slides with glutaraldehyde, sputter-coated, and observed using a Hitachi S-3000 Variable-Pressure Scanning Electron Microscope.

Powder X-ray Diffraction. Crystals were harvested, washed with 400µL 0.4M NaCl, and dried as described above. Dried crystals (0.0155 g) were ground to powder using a glass pestle in a microcentrifuge tube. The unknown powder was analyzed using a Bruker-AXS D8 Discover High-Resolution Diffractometer with a General Area Detector Diffraction System (GADDS) at the Nebraska Center for Materials and Nanoscience core facility (NCMN). A highly collimated beam of Cu K α radiation delivered by a combination of Goebel Mirror and 0.5 mm monocapillary tube was used for the experiment. The powder sample spread on a glass slide was continuously spun to minimize preferred orientation effects in the diffraction intensities. 2D diffraction data (Debye-rings) was collected, while powder sample and the 2D detector were rotated with respect to the incident beam, in 4 frames. With step-wise increment of 25 degrees, 2D diffraction data was obtained in the angular range of 2θ ~ 0 – 95° and then the data was integrated along the diffraction cone to obtain the 2θ vs. Intensity pattern as shown in **Figure 5E**. Culture medium atom composition (Na, Mg, Ca, K, P, O, C, N, Cl) was used as a filter when matching the unknown sample data using the ICDD PDF-4+ database. A close match was observed for the entry corresponding to that of the mineral Ammonium Magnesium Phosphate Hydrate, (NH₄) Mg (PO₄) (H₂O)₆, also known as struvite. Furthermore, the structure was confirmed and refined by a detailed powder x-ray diffraction profile analysis with a starting model based on ICDD PDF card 04-010-2533. Struvite has orthorhombic crystal structure with a spacegroup of Pmn21. **Figure 5E** illustrates the experimental data, simulation, and the Bragg reflection positions of the refined structure.

The refined lattice parameters are $a = 6.934 \text{ \AA}$, $b = 6.129 \text{ \AA}$ and $c = 11.198 \text{ \AA}$. Traces of no other chemical phases were detected in the x-ray diffraction.

Results

UV mutagenesis to isolate methanogenesis mutants

UV irradiation is ideal to induce mutations because it is not susceptible to hot-spot mutation bias, as can be observed with transposon or insertion element-induced mutations (20-22). UV light induces thymine dimer formation in DNA, which must be repaired by site excision before chromosomal replication can commence (23). Because different *Methanosarcinales* species or strains may produce varying amounts of heteropolysaccharide on the outside of the cell, and may have differences in the S-layer permeability, we wanted to avoid using a chemical mutagen (24-35). However, a drawback with UV irradiation is that UV lamp intensities and exposure can vary. To optimize the UV-mutagenesis we generated a UV killing standard curve method to determine the optimal UV exposure time in our experiments (**Figure 2A**).

Figure 2. Viability counts and growth curves of UV-mutagenized strains. A, UV killing of *Methanosarcina* in Balch tubes. Exponentially growing cultures were exposed to UV irradiation and diluted to extinction in 96-well plates to determine a killing curve. B and C, Cultures grown on methanol (B) or acetate (C) as energy source.

Before UV exposure, 0.5 ml of a recent stationary phase culture was inoculated into fresh Balch tubes containing 10 ml methanol + acetate medium and allowed to grow at 35 °C overnight to approximately mid-exponential phase. The next morning, the cultures were laid on a UV transilluminator, covered in

foil, and exposed to UV irradiation for up to 30 minutes. Cultures were then serially 10-fold diluted to extinction in a 96-well plate in the anaerobic incubator into fresh methanol + acetate medium. The 96-well plates were placed in the Wolfe incubator at 35 °C for 10 days to grow. Plates were scored to determine the number of viable cells per ml after UV exposure. We determined that 10-15 minutes exposure reliably resulted in approximately 2-log killing.

Isolation of mutants with increased or decreased growth

Once the optimal UV mutagenesis conditions were determined, we mutagenized methanol + acetate cultures and plated 100 µl onto high mineral salt (HS) methanol + acetate agar plates to obtain isolated colonies. Plates were incubated for 14 days to allow visible colonies to develop. Next, 96-well agar plates were filled to 200 µl per well with molten agar dispensed from a sterile reagent trough using a serial pipettor. After the agar solidified, individual colonies were picked using sterile sticks and transferred to individual wells, sequentially on a methanol + acetate plate first, onto a methanol plate, and then onto an acetate plate, taking care to keep the same plate coordinates for each colony. Racked, sterile pipette tips were used as a frog to replicate colonies from one 96-well plate to another. Once wells were inoculated, they were placed in the Wolfe incubator to grow at 35 °C. After 2 weeks, plates were compared to identify colonies that appeared to have poor or no growth on one carbon source but not on the other. Cells were then picked using sterile sticks and inoculated into methanol + acetate liquid medium in 96-well plates for secondary screening. Using this method we screened 960 colonies in two weeks. We identified 11 with decreased growth on both acetate and on methanol. 108 mutants had less growth on acetate but wild-type growth on methanol, and 14 mutants had decreased growth on methanol but wild-type growth on acetate (**Table 2**).

Table 2. Mutant screening results

Screen	Format	Number of strains screened	Wild-type growth phenotype	Reduced growth on acetate	Reduced growth on methanol	Reduced growth on methanol + acetate
Primary screen	96-well	960	827	14	108	11
Secondary screen	96-well	35	25	2	6	2
Tertiary screen	Balch tubes	11	1	1	9	10

Growth curves in 96-well plate format

Secondary screening in liquid medium in 96-well plate format is advantageous because manual growth curves using a spec20 spectrophotometer is laborious. We identified a large number of primary hits using high-throughput screening on agar plates, and a high-throughput method to screen mutant growth in liquid cultures was desirable. To do this, a 96-well plate reader and computer were set up within the anaerobic chamber. Care should be taken to keep the anaerobic chamber very dry while sensitive electronics are housed in the chamber under a CO₂ atmosphere. We have been able to keep the plate reader and computer running for at least two years with no discernible effects on performance in our anaerobic chamber.

To screen mutant strains in liquid medium, half the 96-well plate was filled with 180 µl methanol medium, and the other half was filled with 180 µl acetate medium. Mutant cultures (20 µl) were inoculated into wells of both media in the desired number of biological replicates. Growth in each well was measured automatically using the plate reader at 35 °C with high shaking intensity at 2 hour intervals for 5 days (growth on methylotrophic substrates) or two weeks (growth on acetate). Using plate reader software, growth curve data was exported to Excel format for analysis.

Using this method we tested a subset of 35 mutant strains that had decreased growth on methanol or acetate. Of the 35 mutants tested, 10 were confirmed to have reproducible growth phenotypes. The mutants fell into two phenotypic classes. Class I were isolates that had decreased growth on methanol, and Class II were those that had decreased growth on acetate.

Tertiary screening in sealed glass Balch culture tubes

Methanogen medium is often supplemented with sulfide to lower the redox potential (36). Because hydrogen sulfide is volatile, there is a possibility that growth phenotypes may be different when cultures are grown in 96-well plates versus sealed glass Balch tubes. For instance, deletion of the operon encoding CoM-S-S-CoB heterodisulfide reductase, *hdrABC*, causes the cell to produce methanethiol (MT) or dimethylsulfide (DMS), which diffuses into the headspace (37). In a Balch tube, the MT and DMS eventually reaches a threshold partial pressure that induces expression of dimethylsulfide methyltransferases, and the cell consumes DMS. However, the slow-growth phenotype of the Δ *hdrABC* mutant is exacerbated in a 96-well plate, presumably because the MT and DMS can easily diffuse out of the plate into the bulk gas of the anaerobic chamber (data not shown).

The 10 strains that passed secondary screening were grown in Balch tubes to determine if their growth phenotypes were specific to the 96-well format. To eliminate experimental variability, the mutants and parental control were grown in biological replicates on the same batch of culture medium. Of the 10 strains, nine had a reproducible phenotype (**Table 3**). One strain, NB93, had reduced growth rate under all medium conditions tested (growth on methanol, methanol + acetate, and acetate alone). Eight strains had wild-type growth rates on acetate, but decreased growth rate on medium containing methanol or methanol + acetate. One of these strains, NB89, had a severe methanol-specific growth defect that resulted in a 59% slower growth rate and a 10% decrease in biomass on methanol but a 20% decrease in biomass on methanol + acetate. Finally, one strain, NB91, had wild-type growth on acetate, but a 13% increase in growth rate on methanol and methanol + acetate (**Figure 2**).

Table 3. Growth rates and maximum culture density in Balch tubes.

Carbon source	Strain	Generation time (h)	Std dev	p value vs parent ¹
Methanol	Parent	10.4	0.13	1
	NB89	16.5	0.27	0.0000016
	NB90	10.7	0.13	0.034
	NB91	9.2	0.15	0.000040
	NB92	10.2	0.05	0.045
	NB93	11.1	0.17	0.0021
	NB178	11.1	0.37	0.041
	NB181	11.2	0.11	0.00017
	NB193	12.1	0.22	0.000099
	NB194	12.0	0.17	0.000022
	NB195	11.7	0.19	0.00012
Methanol + Acetate	Parent	10.5	0.12	1
	NB89	20.9	3.08	0.0099
	NB90	11.4	1.07	NS
	NB91	9.25	0.20	0.0049
	NB92	10.2	0.03	NS
	NB93	11.8	0.58	0.023
	NB178	12.4	1.53	NS
	NB181	12.0	0.94	NS
	NB193	12.9	2.03	NS
	NB194	13.5	1.52	0.040
	NB195	12.1	1.08	NS
Acetate	Parent	55.0	2.50	1
	NB89	53.4	1.57	NS
	NB90	61.9	4.38	NS
	NB91	57.1	3.89	NS
	NB92	54.9	3.14	NS
	NB93	69.1	3.11	0.0010
	NB178	60.9	5.14	NS

NB181	56.5	5.73	NS
NB193	58.9	4.66	NS
NB194	57.6	3.40	NS
NB195	53.3	3.38	NS

Calculations are based on growth phenotypes from quadruplicate biological replicates.

¹*p* value determined by Student's two-tailed T test ($p < 0.05$).

NS: not significant ($p > 0.05$).

Identification of candidate mutations associated with growth defects

To determine the effectiveness of UV-induced mutagenesis in *M. acetivorans*, genomic DNA from the parent strain (NB34) and nine mutants was resequenced and compared to the reference genome (**Table S1**). (8) As expected, mutations were recovered from all over the chromosome (**Figure 3A**). Two regions of the chromosome, from 2.8-3.0 Mb and 5.5-5.7 Mb accumulated mutations faster than the expected average frequency, and correlated with thymine-rich repeat sequences (**Figure 3B**). The mean number of mutations per strain was 10 (8 SNPs and 2 Indels) with a median number of 5 mutations (**Table S2**). On average, 80% of the mutations were SNPs while 20% were Indels. The UV mutagenesis method described in this manuscript resulted in a frequency of 1.8×10^{-6} mutations per base (**Table S3**).

Figure 3. Mutations generated by UV irradiation. A, Mutations recovered from the parent strain and eight mutant strains are plotted on the circular genome. B, When the cumulative mutations per site (red) is compared to the mean average cumulative mutations (gray), two mutation hotspots are identified at 2.8-3 Mb and 5.5-5.7 Mb.

To identify mutations which may be responsible for the observed phenotypes, the resequenced genomes of the NB34 parent and each mutant were compared to the reference *M. acetivorans* genome

(**Figure 4**). Sixteen mutations (of 306 total) were found to be in common between all strains. Ten mutations were found in the parent and one or more mutant strains, but seemed to have reverted back to the reference sequence in the remaining strains. Of these mutations, all but one were intergenic regions or genes with hypothetical or unknown function. One mutation, a deletion of a GC at position 5079505, was in the coding region of *hypF*, the gene encoding the hydrogenase maturation protein. *M. acetivorans* C2A and the parent strain, NB34, do not synthesize functional hydrogenases and cannot use hydrogen as an electron donor (6). Due to lack of functional hydrogenase expression in the C2A strain, this deletion can be reasonably expected to have no additional effect on the physiology of NB34 or the mutant strains (38).

Figure 4. Identification of unique mutations in resequenced genomes. The reference *M. acetivorans* genome was compared to the resequenced parent strain and the isolated mutant strains. Most mutations were unique (red circles), but a few were shared by two or more sequenced strains.

Strain NB89 had the most severe methanol-specific growth defect. Not only was the growth rate slower than the parent strain on methylotrophic substrates, but the final culture density was also affected, suggesting an energetic or a toxicity effect (**Table 4**).

Table 4. Biomass Measurements

Carbon source	Strain	Max OD ₆₀₀	Std dev	<i>p</i> value vs parent ¹
Methanol	Parent	1.05	0.008	1
	NB89	0.94	0.005	0.0000040
Methanol + Acetate	Parent	1.03	0.030	1
	NB89	0.82	0.050	0.0018
Acetate	Parent	0.67	0.01	1
	NB89	0.68	0.01	NS

Calculations are based on growth phenotypes from quadruplicate biological replicates.

¹*p* value determined by Student's two-tailed T test (*p*<0.05).

NS: not significant (*p*>0.05).

The NB89 genome contained five mutations not found in the parent or in the reference genome (**Table 5**).

Table 5. Mutations identified in the NB89 genome

Genome position	Mutation	Effect	Locus	Predicted function
308383	C to T	Δ160-504	MA_RS01355	AAA ⁺ ATPase
1146094	T to C	N199D	MA_RS24380	PGF transpeptidase
3205790	A to G	intergenic	(tnp)	none
3753590	ΔCTCTGTTGTTAACCCCCTGA	intergenic	(Na ⁺ /H ⁺ antiporter)	none
5645793	ΔTGATAGATGACGTTCCCTGTATCTTCTC	in-frame ΔEKIQGTS	MA_RS23910	formyl transferase (H ₄ PT or tMet)

Three mutations were single base changes, while two were deletions of thymine-rich sequences. Two of the five mutations were in intergenic regions, while three mutations were in predicted protein-coding regions. Based on the genome position and predicted effect, the mutations most likely to contribute to the methanol growth defect are a base substitution at 1146094, an intergenic deletion at position 3753590, and a deletion at position 5645793. The mutation at position 1146094 results in a missense mutation (N199D) in a PGF transpeptidase of unknown function. The mutation at 3753590 results in a deletion 5' upstream of the *mrpA* Na⁺/H⁺ antiporter. MrpA has been shown to contribute to growth on

methanol under low sodium conditions (39). It would seem possible that the 3753590 deletion mutation may have disrupted regulation of *mrpA* gene expression, somehow affecting growth on methanol but not on acetate. However, the mutagenesis and screening was conducted in medium containing 0.4 M NaCl. Under these conditions (substrate and sodium ion concentrations), the $\Delta mrpA$ mutant displayed wild-type growth (39). Therefore the mutation at 3753590 in strain NB89 is unlikely to be the cause of the slow growth phenotype on methanol. Finally, the 5645793 mutation causes an in-frame deletion of residues 74-80 (EKIQGTS) in a predicted formyl transferase of unknown function (locus MA_RS23910). Overall the predicted polypeptide is 87 amino acid residues in length and 10.1 kDa. The deleted residues in the NB89 mutants strain lie just outside the formyltransferase core domain residues 2-68. Nearby on the genome are two methionyl tRNAs, MA_RS23925 and MA_RS23930, suggesting that MA_RS23910 may function as a methionyl-tRNA formyltransferase. A crippled methionyl-tRNA formyltransferase would seem to fit the NB89 phenotype if reduced enzyme activity was enough to satisfy the growth rate on acetate, but was not fast enough to satisfy a faster growth rate on methanol. To directly determine which of these mutations causes the growth defect of the NB89 strain it will be necessary to recreate the mutations and rescue the mutant phenotype by complementation.

Several mutations in bona fide methanogenesis genes or promoters were recovered in the mutant strains. Specifically NB93 has mutations in the methyl-coenzyme M *mcr* promoter and in the acetyl-CoA synthase/decarbonylase synthase promoter, NB181 has a frame shift in F₄₂₀H₂:methanophenazine oxidoreductase *fpoL* subunit, NB193 has a mutation in the *mtsF* methyltransferase promoter, and NB194 has a mutation that results in truncation in the *mtbC1* corrinoid protein. The mutations found in each of the sequenced strains are shown in **Figures S1-S8** and summarized in **Table S4**.

Many of the mutations detected were found in TT-rich regions such as intergenic terminators and promoters, and repeat regions within coding sequences. This is consistent with the mechanism of UV-induced mutagenesis (pyrimidine dimers) and repair by the non-homologous end-joining (NHEJ) DNA repair pathway (40). Therefore the amount and duration of DNA exposure affects the types of mutations recovered and must be experimentally titrated. For instance, excess UV irradiation would be expected to result in numerous severe indel mutations, such as we observed in strain NB195. Strain NB195 had 84 bases inserted at position 273884 (MA_RS11545), four insertions of the same 39 base sequence and six missense mutations in MA_RS1575, and three other deletions at positions 3958294, 4562039, and 5643060. In contrast, strain NB178 had only four point mutations and one insertion at an intergenic repeat region at position 3345530.

Struvite formation on acetate

When collecting tertiary screening data in Balch tubes, NB89 and three other strains were observed to produce crystals during growth on acetate. Crystals were large, between 2-6 mm in length, and rhombic (**Figure 5A**). Small crystals are occasionally observed in parent strain culture tubes when settled, stationary-phase cultures are left on the benchtop for weeks (as might occur after an experiment is completed but before Balch tubes are cleaned). The crystals we observed in the mutant strains were different, however, because of the size, ordered structure, and speed of crystal formation. Crystals began to form as mutant cultures entered late exponential phase, and were reproducible. The acetate crystal phenotype appeared to be heritable when cells were passaged to fresh acetate medium, and when they were switched to methanol medium and back to acetate medium. Crystals were not observed in all mutants, nor were they observed in parent cultures.

Figure 5. Acetate-dependent struvite formation. A, Balch tube showing large rhombic struvite crystals (black arrows). B, Scanning electron microscopy of struvite crystals (bar = 10 μm). C and D, Biological material suggestive of a biofilm (C) or adhered cells (D) were observed on crystal surfaces (bar = 1 μm). E, X-ray powder diffraction spectra of crystals.

We collected the crystals and tested their ability to dissolve in acid. The crystals were air-stable, and dissolved slowly in cold 0.1M HCl, indicating they were not composed of carbonate. We observed crystal morphology by scanning electron microscopy (**Figure 5B-D**). Though cells, or perhaps biofilm, were

observed on the surface of the crystals, the crystal faces were generally smooth, and it did not appear as if cells were trapped within the crystals. Crystal composition was identified using powder x-ray diffraction. The XRD spectra was characteristic of the mineral $(\text{NH}_4)\text{Mg}(\text{PO}_4)(\text{H}_2\text{O})_6$, otherwise known as struvite (**Figure 5E**). The microscopy and XRD data therefore suggest the mutant strains catalyze struvite mineralization by increasing the pH of the medium beyond the bicarbonate/ KPO_4 buffering capacity.

Discussion

Method evaluation

A forward genetics approach has the potential to identify every gene that is involved in expression of a phenotype. In the method described, UV mutagenesis was selected as an inexpensive, universally applicable mutagenesis method. Other forward genetics methods employ chemical mutagens or transposable elements, which have their own strengths and weaknesses (13, 41-45). A particular organism may or may not be sensitive to a particular chemical mutagen, or the cells may have extracellular structures (capsule, hydrophobic pellicle, cell wall structures, etc.) or biochemical detoxification mechanisms (P_{450} -type enzymes) that may pose a barrier for mutagen uptake or sensitivity. Transposon mutagenesis requires a method for efficient delivery of DNA into cells, either by transformation/transfection, conjugation, or phage/virus infection. In addition, some transposons favor insertion “hot spots”, which may result in over-representation of some recovered mutants and under-representation of others (22, 46, 47).

UV mutagenesis generates point mutations across the entire genome, which can result in nonsense or frame-shift mutations in either the promoter (or other cis-regulatory elements) or in the gene reading frame. The degree of phenotype change is a function of how severe the mutation is, ranging from having a subtle effect on transcription/translation, to complete gene loss due to a translational frame shift. One major difficulty with a forward genetics approach is identifying the mutation on the chromosome.

Platforms such as the Illumina MySeq are ideally suited for detection of single-nucleotide polymorphisms, which are easily mapped against a genome sequence. A read depth of 20,000x coverage is generally sufficient for identifying mutations derived from the initial UV mutagenesis event that have become fixed in the culture population. Forward genetics is more experimentally involved and initially expensive than reverse genetics, however, for organisms that lack an efficient genetic system and/or have a relatively high proportion of unknown genes (such as in phylogenetically deep-branching

microbes), forward genetics makes it possible to begin to link genes and transcripts to systems-level phenotypic functions.

While several methanogen species are genetically tractable, many genes remain uncharacterized, and there are likely many genes of unknown function that play a role in growth and methane production. In *M. acetivorans*, acetoclastic methanogenesis is thought to involve 303 genes (by microarray) or 257 genes (by proteomics), and methylotrophic methanogenesis involves 407 genes (by microarray) and 441 genes (by proteomics), for an overall range of 664 to 774 genes which are specifically involved in either pathway, or roughly 13.8% to 16.1% of genome (48-50). Therefore, the probability of finding a mutation in either acetoclastic or methylotrophic pathways is 4.4×10^{-4} under the conditions we describe.

For full saturation mutagenesis of the 5.75 Mb *M. acetivorans* genome with 4807 genes, we should screen approximately 10,000 colonies (51). Assuming a single mutation per colony (the actual number of mutations per chromosome will follow a Poisson distribution and vary depending on mutagenesis conditions), we sampled up to 20% of the genome. Theoretically, we mutated between 133-154 genes in either the acetoclastic or methylotrophic pathways, and we picked 133 colonies, 13.9%, that appeared to have slow or fast growth from the primary screen, in close agreement with the theoretical number predicted from transcriptomics and proteomics. After secondary and tertiary screening, the number of confirmed mutants dropped to 10, and may have resulted from human error when transferring cells from 96-well plates to Balch tubes, may reflect the change in growth conditions between 96-well plates and Balch tubes, or may be a consequence of selection for suppressor or reversion mutants upon subculturing.

Finally, we note that genome copies can affect the efficiency of forward genetic screens. Prokaryotes, and methanogens in particular, may have more than one copy of the genome. Multiple genome copies can serve as templates for DNA repair by homologous recombination. For instance, when *M. acetivorans* grows on methanol, each cell contains 16 copies of the genome (52, 53). In order to recover stable mutant strains, all copies of the genome must contain the mutation. If a mutation in one of sixteen gene copies causes a decrease in fitness, it is only a matter of a few generations before the genome copies segregate, homologous recombination replaces the defective lesion, and the population genotype is converted back to the most-fit allele. Therefore initial selection of single colonies under permissive conditions is critical for maximizing the chance for obtaining strains that will exhibit mutant phenotypes under an alternative (or stressed) growth condition.

Struvite formation can be stimulated by chromosomal mutations.

We were intrigued that several of the mutants produced struvite crystals. Struvite can precipitate abiotically at alkaline pH when phosphate and ammonium concentrations are high. Our observation that struvite formation was reproducible, heritable, and constrained to acetoclastic growth suggests these mutants have mutations that increase nitrogen fixation, have increased CO₂ fixation, or increased proton transport into the cell. Any alteration in these metabolic activities would be expected to tip the balance of conditions towards favoring struvite formation.

There is no current evidence directly linking methanogens with struvite mineral precipitation. However, struvite precipitation is common in anoxic environments inhabited by methanogens. For instance, struvite formation is used in municipal water treatment facilities to capture phosphate and ammonia (54-56). However, struvite can build up in the sediment within pipes carrying anoxic, untreated waste, eventually clogging them. Clearing struvite requires removal and replacement of the obstructed sections, at considerable cost.

Struvite can also form in biological tissues, forming kidney stones, gall bladder stones, and encrusting catheters. Oxalate-producing pathogenic bacteria are known to be associated with kidney and urinary endoliths comprised of oxalate, but there is evidence that some patients may present non-oxalate struvite endoliths that are not associated with bacterial infection (57). Struvite endoliths also cause significant morbidity in some breeds of canines and equines, where they are more commonly found in the colon (58). In the human colon, struvite (non-oxalate) endoliths are associated with Crohn's disease and diverticulitis, diseases which are also linked to perturbations in the methanogen population of the gut microbiome (58-62). Further research is needed to determine if and how methanogens may contribute to altering production of organic acids, CO₂ and/or ammonia by the microbiome to contribute to struvite endolith formation in vivo.

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