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Attempts to cultivate bacteria from deep subsurface aquifers and mountaintop plant communities

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Abstract
In the late 1990s, the limits of life were pushed even further when microorganisms were discovered thriving 2.5 km below the surface of the Earth in deep South African gold mines. These very simple communities were dominated by a single species of bacteria from within the phylum, Firmicutes. *Desulforudis audaxviator* remains unique to a sizeable portion of the South African deep subsurface. At depths below 2.5km, it comprises well over 99% of all organisms present, which presents a unique circumstance in which the environment has provided a natural pure culture. From this naturally occurring pure culture, environmental genomics was applied to obtain the complete *D. audaxviator*genome and thus it's biological functions were established. This presents a unique opportunity to now attempt to grow a previously uncultured organism using its genome as a road map to design a specific cultivation approach for *D. audaxviator*. The genome combined with precise chemical analysis of its native environment has yielded invaluable insights such as the organism's ability to form spores, to reduce sulfate, to fix nitrogen and use ammonia, along with many other unique traits all of which will lead to successful cultivation. Here we describe the genome-enabled cultivation of this to date uncultured microorganism.

Attempts to Cultivate Bacteria from Deep Subsurface Aquifers and Mountaintop Plant Communities

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INTRODUCTION

In the late 1990s, the limits of life were pushed even further when microorganisms were discovered thriving 2.5 km below the surface of the Earth in deep South African gold mines (4). These very simple communities were dominated by a single species of bacteria from within the phylum, Firmicutes. *Desulforudis audaxviator* remains unique to a sizeable portion of the South African deep subsurface. At depths below 2.5 km, it comprises well over 99% of all organisms present: essentially a natural pure culture. An environmental genomic approach was applied by collaborators to obtain the complete *D. audaxviator* genome (Figure 1)(1) and thus it's biological functions (Figure 2)(1) were established. The genome, combined with chemical analysis of its native environment, has yielded valuable insights informing potential cultivation strategies. These include the ability to form spores, reduce sulfate, fix nitrogen, and utilize formate or CO_2 as carbon and energy sources. Here we describe an attempt to perform the genome-enabled cultivation of this to date uncultured microorganism.

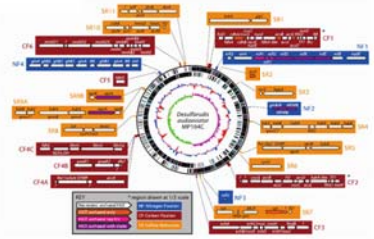

Figure 1: Genome of *D. audaxviator* provided by metagenomics (1) .

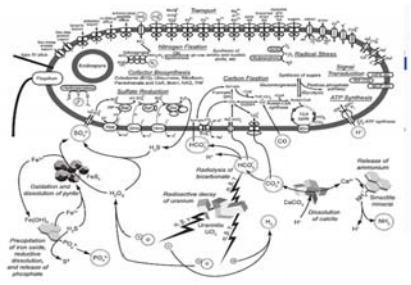

Figure 2: Representation of *D. audaxviator's* internal machinery according to the metagenomic analysis (1).

MATERIALS & METHODS

Basal Media Preparation

Mean concentrations of major ions (2) from boreholes where *D. audaxviator* was previously identified (1) were utilized to develop a basal medium. Six variations were prepared: three with $FeSO_4$ as an H_2S indicator, and three without. Formate and acetate or casamino acids were utilized as potential C sources or spore germinants (Table 1). L-cysteine HCl and resazurin were added and the pH adjusted to 8.6. Media were dispensed into Balch tubes and autoclaved. The tubes were then immediately placed into a Coy Type B anaerobic chamber maintained with N_2, CO_2, H_2 (70/20/10), and sealed with butyl rubber stoppers.

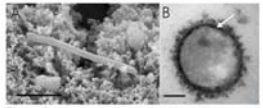

Figure 3: A) *D. audaxviator* cell. B) *D. audaxviator* spore (Chivian, D., E.J. Alm, E.L. Brodie, et al. In Press.)

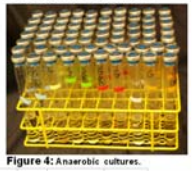

Figure 4: Anaerobic cultures.

Table 3: Basal media variations and gas mixtures

	$FeSO_4 \cdot 7H_2O$	Formate + Acetate	Casamino Acids	H_2, N_2, CO_2	55% H_2
RED	x	x		x	
	x	x		x	x
ORANGE	x		x	x	
	x		x	x	x
YELLOW	x			x	
BLUE		x		x	
		x		x	x
GREEN			x	x	
			x	x	x

Inoculation

Six separate samples of African mine borehole water, previously confirmed to contain *D. audaxviator* spores and cells, were chosen as inocula for each variation of the basal medium. Aliquots of each sample were heat shocked at 80 °C for 10 min before inoculation. Four additional series of inoculations were included with 55% H_2 in the headspace (Table 1). The inoculated tubes were incubated in the dark at 50°C.

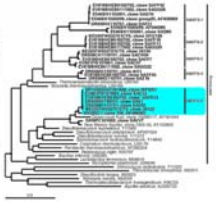

Figure 5: Phylogenetic dendogram with the *D. audaxviator* clones selected to culture (3).

RESULTS

- No definitive growth as of yet.

Table 2: *D. audaxviator* Clones

Name	Sample	**Growth
A	MP104	neg.
B	*MP104-MK	neg.
C	EV818EB6	neg.
D	EV818EB6-MK	neg.
E	DR938H3	neg.
F	W638SC1	neg.
G	CONTROL	neg.

* Tangential flow filtration concentrate.

** As judged by visible turbidity

DISCUSSION / FUTURE WORK

- High Risk Project.
- *D. Audaxviator* may have a doubling time of ~400 years.
- *D. Audaxviator* may be an obligate barophile.
- More variations on media could be developed.
- Culturing of this organism would enable experiments relevant to emerging fields such as astrobiology.

MOUNTAIN *METHYLOBACTERIA*

INTRODUCTION

Methylobacterium are a genus of facultative methylotrophs capable of surviving off the methanol emitted from the stomata on the surface of leaves. These bacteria are commonly referred to as PPFM's (pink-pigmented facultative methylotrophs) due to the distinct pink pigment they produce (1). Endemic plants from Bonanza Peak (10,397 feet, 3169 m) in the spring mountains of NV were collected to determine if specific plant species had unique *Methylobacterium* populations.

Figure 6: *Methylobacterium* plates.

MATERIALS & METHODS

Leaves taken from Bonanza Peak plants were masticated using a sterile tissue grinder and a 1X phosphate buffer. Homogenates were streaked for isolation on solid, defined media with 0.375% methanol added as sole C source. After incubation at room temperature for approximately 1 week, conspicuous pink colonies were further purified by transfer to fresh plates.

Colony PCR from bacterial primers was followed by DNA sequencing to obtain the partial 16S rRNA gene sequence from each. Phylogenetic analysis was performed using MEGA IV and comparative sequences obtained by BLAST search (NCBI)

Figure 7: Bonanza Peak

Figure 8: Amplified DNA

ACKNOWLEDGEMENTS

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RESULTS

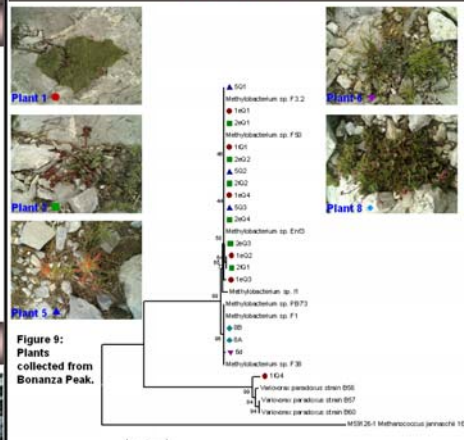

Figure 9: Plants collected from Bonanza Peak.

Analysis of the data would indicate that there is little to no variance of the methylotrophic populations from one plant species to another.

DISCUSSION / FUTURE WORK

- Possible elevation trends in methylotroph abundance.
- Possibly related to UV exposure selecting for PPFM's.

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