Quantitative PCR assay development for monitoring genes suspected of contributing to PFAS degradation by *Gordonia* sp. strain NB4-1Y

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Introduction

- Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are a large group of synthetic compounds found in food packaging, non-stick cookware, water-repellent clothing, and firefighting foams (AFFFs).
- PFAS are characterized by long fluorinated carbon chains, making them incredibly stable and able to easily accumulate in the environment.
- *Gordonia* sp. strain NB4-1Y is a gram-negative bacterium, originally isolated from vermicompost, that has been previously reported to degrade PFAS into numerous breakdown products (Shaw et al., 2019).
- Here six genes of interest and one reference gene are selected as targets for reverse transcriptase quantitative digital polymerase chain reaction (dPCR) assay development.
- Target gene selections are based on previous transcriptome (Bottos et al., 2020) and proteome (McAmmond et al., unpublished) data.

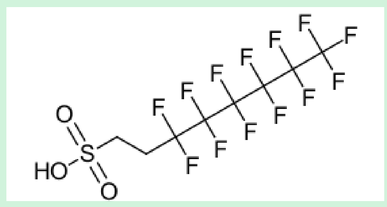

Figure 1. Chemical structure of 6:2 fluorotelomer sulfonate (6:2 FTSA), a sulfonated PFAS.

Hypotheses

- NB4-1Y can use 6:2 FTSA as a sulfur source for growth. Proteins that may be involved in this metabolism have been identified and, if they are involved, the genes coding for them should be expressed more when NB4-1Y is exposed to 6:2 FTSA compared to $MgSO_4$.
- Dimethylsulfone monooxygenase (*sfnG*) gene may code for a 6:2 FTSA desulfinase.
- Four LLM class flavin-dependent oxidoreductase genes code for versatile enzymes known to catalyze a variety of reactions including hydroxylations, epoxidations, and Baeyer–Villiger rearrangements (Maier et al., 2015).
- Acyl-CoA dehydrogenase family proteins potentially involved in formation of acetyl-CoA adducts during PFAS metabolism (Mothersole et al., 2024).
- Specific primers and probes can be designed to quantify the expression of these genes using quantitative polymerase chain reaction (qPCR) and chip digital PCR (dPCR).

Methods

Primer and Probe Design and Validation:

- Used Geneious Prime (Dotmatics, 2024) and Primer Express (Thermo Fisher Scientific, 2019) software along with most complete version of NB4-1Y genome (Ony et al. 2024, GenBank: CP 132196.1) to design forward and reverse primer pairs and PCR probes for six genes of interest and one reference gene.
- Three primer pairs were tested and validated using a combination of end-point PCR to optimize PCR conditions and Sanger sequencing to confirm PCR amplicon identity.

Time Course Experiment:

- NB4-1Y starter cultures used to inoculate sulfur-free acetate (SFA) media 1% (v/v).
- Fluoride readings were taken using a Thermo Scientific Orion Fluoride Ion Selective Electrode. OD readings were measured at 600 nm by VARIOSKAN LUX.
- 10 readings were taken over 16 days.

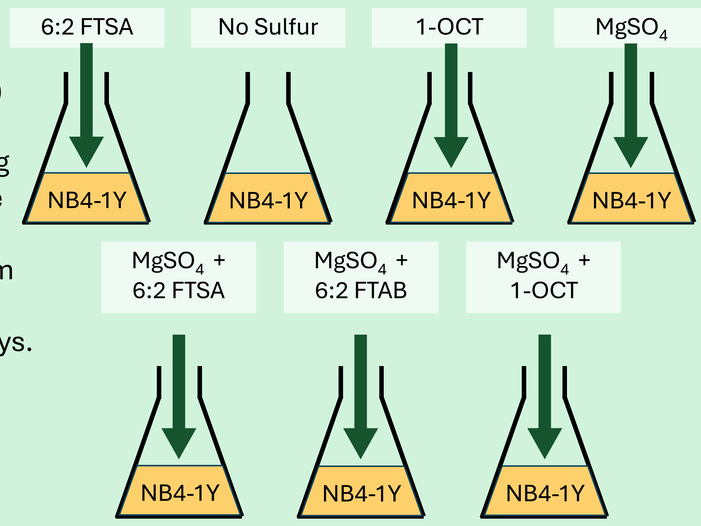

Figure 2. Schematic showing different sulfur conditions used in time course experiment. All treatments in triplicate.

RNA Extraction:

- Followed RNA extraction protocol outlined in Bottos et al. (2020) including two DNAse digestion steps to minimize DNA contamination.
- Confirmed absence of DNA contamination using Qubit Fluorometer High Sensitivity DNA kit, end-point PCR, and gel electrophoresis.

cDNA Synthesis with Reverse Transcriptase:

- Used SuperScript IV VILO Master Mix with ezDNase enzyme and followed user guide protocol. Quantified cDNA using Qubit Fluorometer High Sensitivity DNA kit and visualized using gel electrophoresis.

Quantitative PCR (qPCR):

- Performed multiple qPCR experiments to test individual and multiplexed assays using TaqMan Fast Advanced Master Mix.
- qPCR thermocycling conditions: (1) polymerase activation 95 °C for 20 s, (2) 40 cycles of denaturation 95 °C for 1 s and annealing 60 °C for 20s.

Digital PCR (dPCR):

- Performed dPCR experiment with cDNA and genomic DNA samples using QuantStudio Absolute Q DNA Digital PCR Master Mix.
- dPCR thermocycling conditions: (1) PCR preheat 96 °C for 10 min, (2) 40 cycles of denaturation 96 °C for 5 s and annealing 60 °C for 15 s.

Results

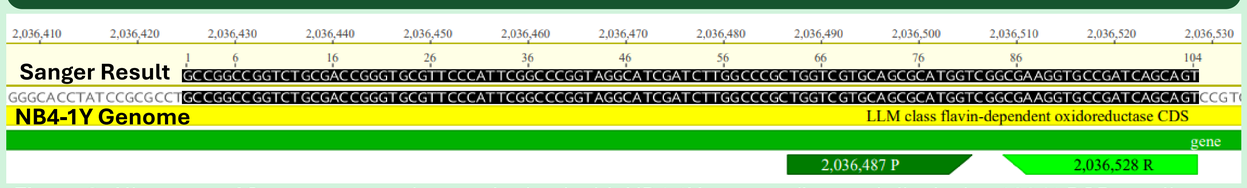

Figure 3. Alignment of Sanger sequencing results (top) with NB4-1Y genome (bottom) displaying 100 % PCR amplicon identity, indicating successful amplification of target gene coding for LLM class flavin-dependent oxidoreductase, Q9K23_RS08675 (Dotmatics, 2024).

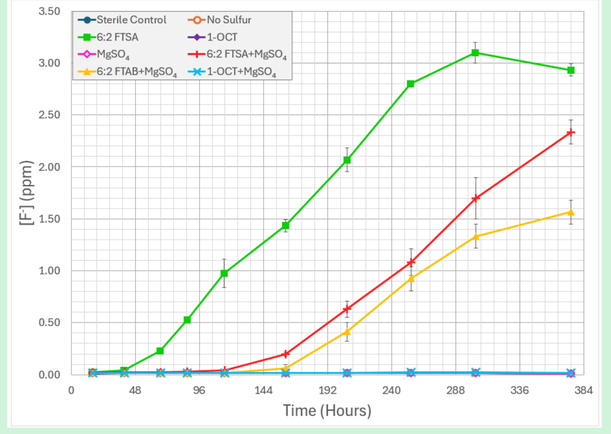

Figure 4. Fluoride release in NB4-1Y cultures provided with various sulfur sources (n = 3; error bars represent standard deviation).

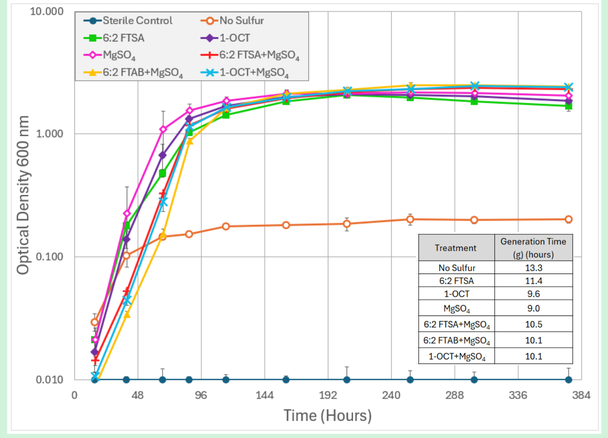

Figure 5. OD 600 nm readings, of NB4-1Y growth provided with various sulfur sources. Plotted using log scale (n = 3; error bars represent standard deviation).

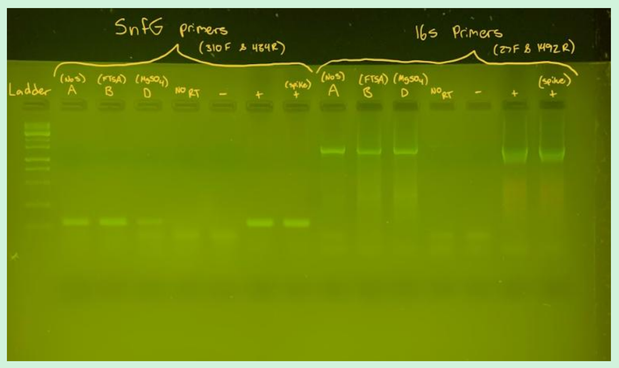

Figure 6: Amplification of *snfG* and 16S rRNA gene fragments from NB4-1Y cDNA using PCR visualized using 2% agarose gel electrophoresis. Shows successful synthesis of cDNA from extracted RNA samples and correct amplicon size.

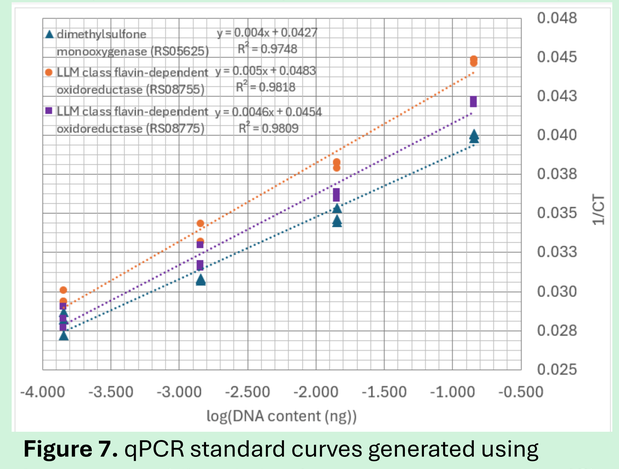

Figure 7. qPCR standard curves generated using independent (dimethylsulfone monooxygenase (*snfG*) and duplexed (LLM class flavin-dependent oxidoreductases) assays displaying 1/CT values as log(DNA content in ng) of NB4-1Y genomic DNA increases.

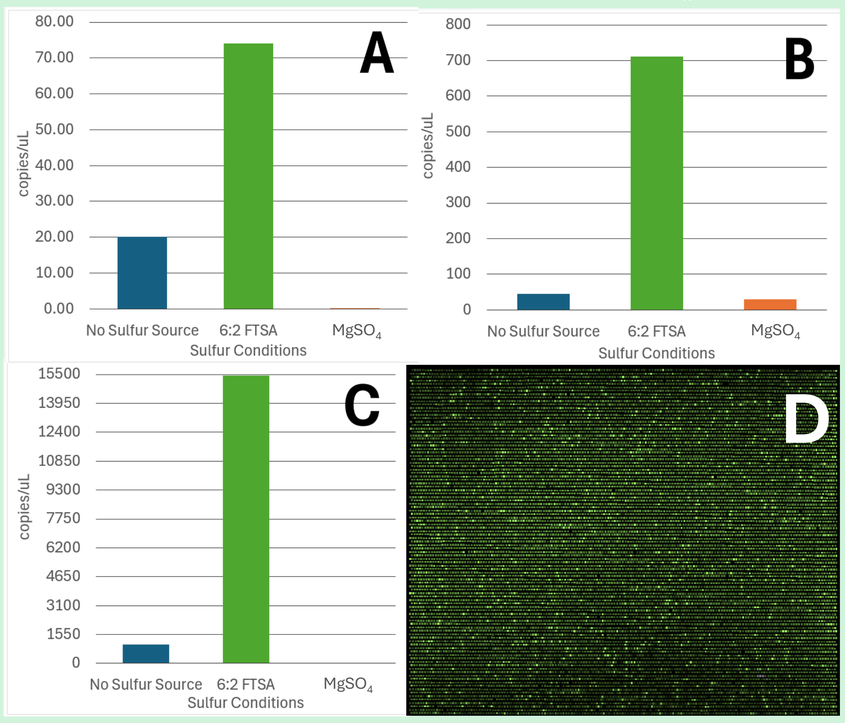

Figure 8. Quantification of target genes (A) dimethylsulfone monooxygenase (*snfG*), (B) LLM class flavin-dependent oxidoreductase (RS08755), and (C) LLM class flavindependent oxidoreductase (RS08775) from cDNA generated from NB4-1Y mRNA using chip digital PCR. (D) dPCR reaction unit with 20,480 fixed array microchambers with a total volume of 9 µL. Green spots indicate presence of target gene.

Conclusion

- Primers and probes were designed and successfully amplified gene regions of interest as confirmed by end-point PCR and Sanger sequencing.
- Initial qPCR and dPCR results showed expression of genes of interest in NB4-1Y samples grown in 6:2 FTSA was higher compared to $MgSO_4$ and no sulfur source controls, thus supporting the stated hypothesis.

Future Work

- Validated primers and probes will be used to quantify mRNA levels over time when NB4-1Y is provided with 6:2 FTSA, $MgSO_4$, or octanesulfonate.
- Data will be compared using multivariate analysis of variance (MANOVA).
- Results will be compared to transcriptome and proteome data, and recommendations will be made to guide future protein purification and characterization experiments.

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