RECENT PROGRESS

The Athabasca oil sands region of Northern Alberta contains one of the largest bitumen deposits and is the largest mining project in the world. Using surface mining, bitumen is extracted from the sands using a hot, alkaline water extraction (Clark process) (1). After settling, bitumen is removed from the surface with the help of diluents, and the remaining 'tailings' are removed to settle in tailings ponds (1). The water extracts all water-soluble components of the sands and clays, which include organic compounds, heavy metals, and salts. Among the organic compounds remaining, naphthenic acids (NAs) are the most abundant (5-120 mg/L) and ***are considered the primary contributor to aquatic toxicity***. NAs are a complex mixture of monocyclic, polycyclic, acyclic, alkyl-substituted carboxylic acids (2) that have demonstrated toxicity against microbes, plankton, plants, fish and mammals (3, 4). For these reasons, NAs are ***the primary contaminant of concern in oil sands process-affected water*** (OSPW), which requires treatment to reduce water toxicity, prior its return to the environment and subsequent land remediation (1).

In my previous NSERC DG ($160,000; 2019-24), my team developed bacterial biosensors that can detect individual NA compounds and diverse NA mixtures. RNA-seq was used to identify NA-induced genes, whose promoters were synthesized and cloned as transcriptional *lux* fusions on a reporter plasmid (5). The transcriptome and gene expression experiments were performed in a *Pseudomonas* sp OST1909 that was derived from OPSW and can degrade NA (Fig 1). We cloned 63 promoter-lux transcriptional fusions and numerous design iterations were performed to optimize the biosensor constructs and NA detection (5). We identified 3 highly induced and dose-dependent promoters with unique NA specificities and limits of detection as low as 1.5-15 mg/L (5). We validated the biosensors with pure compounds, or NA mixtures diluted to ranges found in tailings ponds (5-120 mg/L), and detected NA in raw OSPW samples (5). The genes induced by NA are generally bioremediation, antibiotic resistance, or metabolic genes required to degrade or defend against NA. There is a manuscript in preparation that describes the NA sensing mechanisms of metabolite sensing repressors (AtuR/MarR) that control expression of NA-induced genes, similar to the TetR repressor that controls tet resistance genes when the antibiotic is present. We developed high throughput methods to detect NA in 0.1ml of raw OSPW, or in 0.1 ml of culture supernatants from 24 well microplate cultures (Fig 1, 2). Degradation of NA in the culture supernatants is determined by comparing to sterile positive controls. ***This biosensor tool accelerates the bottleneck in bioremediation research,*** which is using conventional but slow, complex and low throughput analytical chemistry methods to confirm degradation**.** To demonstrate the high throughput nature of this method, we identified 5/4,800 colonies from an *E. coli* metagenomic library, expressing 35kb of DNA from oil sands soil (6), that degrade 9xNA (Fig 2). The 35 kb cosmids from these strains were sequenced to identify genes involved in NA degradation, but targeted or random mutagenesis will need to be performed to identify the genes involved. ***Using transcriptome and metagenomic approaches, we identified potential NA degradation genes, but definitive proof requires mutagenesis and confirmation***. There are no genes that have been identified as NA degradation genes using Koch's molecular postulates. However, this DG proposal aims to fill this gap and to identify all possible NA degradation pathways in oil sands microbial communities.

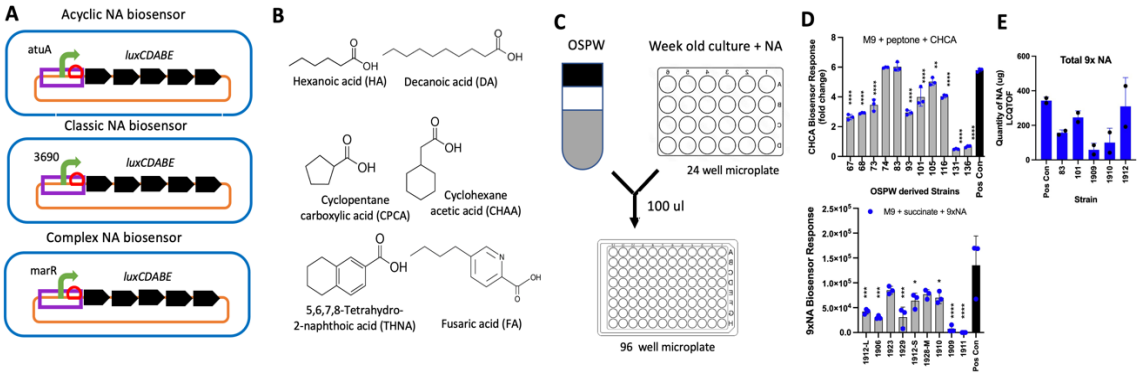

Fig 1. Biosensors[A] that detect acyclic, classic or complex NA compounds[B] and can be used to monitor raw OSPW or small volume microplate cultures[C] for CHCA or 9xNA degradation abilities[D]. LC-QTOF-MS confirmation of 9xNA degradation[E].

OBJECTIVES

For our ***short-term goals***, we have identified four specific aims, which are intended to ultimately identify mechanisms of NA degradation in diverse bacterial species and using diverse NA compounds and mixtures:

1. Conduct systematic culturing of diverse bacterial species from OSPW.
2. High throughput screening of NA degradation phenotypes with biosensors.
3. Identify defined, minimal communities of NA degraders.
4. Genome comparison and genetic screening for NA degradation genes.

The ***long-term goal of our research program*** is to assess the genomic view of NA degradation by combining the information from all published NA degradation studies (gene expression, transcriptome, proteome) along with our genetic and bioinformatic approaches, to further our understanding of how bacteria degrade these specific hydrocarbons. Working collaboratively with the oil sands industry, we will thoroughly assess the feasibility of the bioaugmentation approach as applied to constructed wetlands, in order to optimize bioremediation in situ during water treatment.

LITERATURE REVIEW

***Bioremediation is a feasible and scalable solution for cost-effective treatment of OSPW*.** Although there are other possible methods to treat NA, such as ozonation, photocatalysis, coagulation, adsorption; bioremediation is a probable process to be employed in constructed wetlands located on mining sites (7, 8, 1). Here, the OSPW is transported to and filtered through wetlands during a 2–3-week process, where the organic compounds are degraded by microbes present in OSPW and supported by plant enhanced NA degradation (3,4). I am part of a collaborative GROW genomics project, which is testing mesocosms (10) and constructed wetlands on an industry mining site (8), to assess the microbial communities, bacterial gene expression profiles (metatranscriptomes), and their ability to treat and remove NA from OSPW. This is considered an inexpensive, passive, rapid, and effective way to treat NA (wpsites.ucalgary.ca/grow/research/).

Mechanisms of NA degradation of naphthenic acids remain poorly understood. Many studies have demonstrated NA bioremediation by single or mixed bacterial species cultures, under aerobic and anaerobic conditions, typically in small scale culture or bioreactor experiments (11, 3, 12, 13). Most NA degradation experiments involve mixed cultures that were enriched from OSPW as the inoculum, in media using NA as a sole carbon source. The majority of studies use simple, model NA compounds or commercially available NA mixtures, where the preferred experiments are done with NA that are extracted from OSPW. While NA can be degraded, it is thought that NA structures containing highly branched alkyl chains, or highly cyclized ring structures (eg. diamondoid) are more difficult to degrade (3, 14). We and others have used transcriptomics (15, 16, 5) and proteomics (17) to identify NA-induced genes and proteins, which identify potential bioremediation genes. Bioinformatic predictions have also identified potential enzymes for degradation (18, 15). **All these studies are suggestive of degradation function but they require a genetic confirmation using mutant studies**. To degrade cyclic NA, there needs to be ring opening enzymes, resulting in long chain fatty acid compounds, which require multiple cycles of the beta-oxidation pathway, generating acetyl-CoA that funnels into the TCA cycle. Multiple groups have implicated in beta-oxidation genes in fatty acids/acyclic NAs (18, 15–17, 8, 5). However, there is tremendous diversity beta-oxidation pathways and they can involve many auxiliary enzymes to deal with saturated and unsaturated fatty acids (19). Fewer gene candidates have been identified that are NA ring-cleaving enzymes for cyclic NA compounds. In our OSPW derived *Pseudomonas* isolates, we can find many homologs (10-25) of most genes identified by these predictions, revealing the lack a precision and the limitations of bioinformatic predictions. In the GROW project, metatranscriptome analysis of mesocosms supplemented with OSPW has been performed. This approach can give insights into gene expression of native OSPW communities, and potential NA remediation genes, it is still not definitive for

identifying the NA degradation genes. Given the gene redundancy and absence of direct proof of NA degradation, ***we propose a direct and high throughput genetic screening approach to identify specific NA degradation genes*** from the isolates recovered in this study.

METHODOLOGY

Aim 1. Systematic culturing of diverse bacterial species from OSPW: Culture-independent 16S rRNA sequencing experiments have confirmed diverse communities surviving in tailings ponds (13). In the literature, and in the current GROW team project, multiple labs have performed basic microbiological culturing of isolates from OSPW, using selective (NA as sole carbon source) and non-selective conditions. Collectively, only 50-100 strains have been cultured by GROW researchers, but very few have been demonstrated to degrade NA. (unpublished data). ***We propose to carry out a systematic microbiological culturing of as many isolates as possible from OSPW*** (provided by our ongoing collaborations with CNR or Imperial Oil). Culture-dependent methods are generally more successful when culturing bacteria from humans, such as the case of culturing 90% of bacterial families from the CF lung infections that are detected by metagenomics (20). Graduate student #1 project: We will use a similar approach of culturing bacteria from OSPW by: ***1)*** using a combination of selective and non-selective growth media; ***2)*** using varying growth parameters (rich and mostly minimal media, time, temperature, aerobic/microaerophilic); ***3)*** using mainly agar plates (or other gelling agents), which allow for cross-feeding between microbes; and, ***4)*** enriching for growth with NA as a substrate, or with the addition of growth limiting nutrients (trace metals, alternative electron acceptors e.g. nitrate, vitamins). We are confident that progress can be made in isolating and working with a large collection of isolates from OSPW. In addition, we will mine the literature to identify any new approaches to improving the recovery of environmental microbes (21–23). We considered devices such as the iCHIP (24), others have had limited success (pers communication, R Levesque). Metagenomic community analysis (16S rRNA sequencing) has been used extensively and will be employed to compare and estimate the success of microbial culture efforts.

Aim 2 High throughput screening of NA degradation phenotypes with biosensors:. To accelerate our ability to identify NA degraders from isolates recovered from OSPW, we developed 24 well microplate methods of culturing microbes with NA for days to weeks. Using a small collection of ~20 strains from our lab, and others, we have identified bacterial species that can degrade single model NA compounds (CHCA; cyclohexane carboxylic acids) and isolates that can degrade simple mixes of nine commercially available NA compounds (Fig 1). Using 96 well microplates, we add our NA detecting biosensors in co-culture with potential NA degraders from an oil sands metagenomic library (6), which allows the biosensor to produce a luminescent response proportional to the remaining NA concentration (Fig 2).

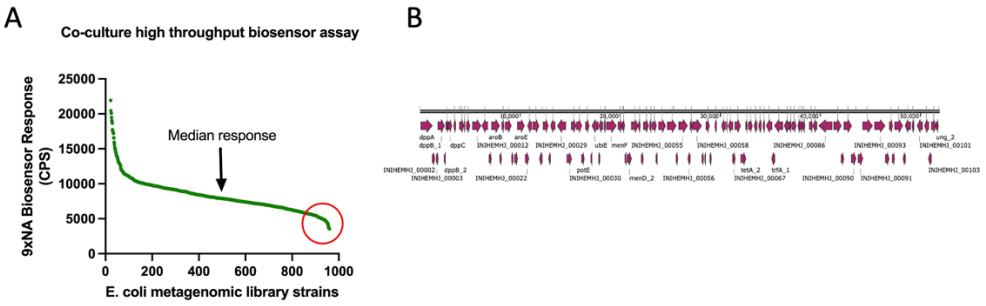

Fig 2. 1000 colonies from an *E. coli* metagenomic library were grown for 3 days with 200 mg/L of a 9xNA mix, and the *p3690-lux* biosensor was added in co-culture with fresh media and antibiotic to limit *E. coli* growth[A]. Colonies with a 50% reduced biosensor response (red circle) were retested and sequenced[B].

Our collaborator Vogon Laboratories performs LCQTOF-MS (25), ***which has confirmed NA degradation in strains identified by our biosensor methods*** **(**Fig 1E**)**. Graduate student #1 project: All bacterial species isolated in Aim 1 will be tested in high throughput microplate cultures using NA biosensor screening. Using our three unique biosensors (Fig 1) that can cumulatively test for bacterial degradation of at least 11 individual compounds (4 acyclic NA, 7 cyclic NA), as well as simple 9xNA mixes, the commercially available acyclic NA, or the complex extracts of NAFCs (naphthenic acid fraction compounds) from

OSPW (26, 2) (provided by collaborator Dr John Headley (Environment, Canada). The goal is to identify a large panel of strains that can degrade a wide range of NA structures and mixtures.

Aim 3. Identifying defined, minimal communities of NA degraders: Extending the Aim 2 approach, we can also test small combinations of individual bacterial isolates in community, for the ability to completely degrade NA in short time frames. For example, there are 1023 possible combinations of 10 unique bacterial species. After monitoring NA degradation of large numbers of communities, which allow for metabolic synergies, molecular methods (eg. qPCR) will be used to confirm relative abundance of each microbe in each community, as not all members may grow equally. We will also perform high throughput growth condition testing to identify supplements to minimal media (defined or dilute1/32 LB) that can improve growth and remediation. We have a CFI proposal in preparation to purchase an OpenTron liquid handling station to assist with high throughput, accurate pipetting to establish a large range of conditions, by using serial dilutions of media components. The culturing studies in Aim 1 may inform the identification of growth conditions that can be explored in greater detail in this aim. Together, this aim could identify conditions and communities for optimal NA degradation, which may support the concept of 'bioaugmentation' in real world water treatment systems.

Aim 4. Genome comparison and genetic screening for NA degradation genes: Several reviews on NA degradation have discussed ***potential NA degrading pathways and genes (3, 8)***, ***but there is no direct evidence to support their degradation function.*** Grad student #2 project: We propose to make mini-Tn5-lux mutant libraries (27) in NA degrading strains from Aims 1-2 and use our biosensors for high throughput screening to identify mutants that do not degrade NA. One possible mutant screening strategy is to use the *pmarR-lux* biosensor (5) to screen for mutants with defects in degradation when exposed to 2 ring model compounds (5,6,7,8-Tetrahydro-2-naphthoic acid, THNA) or the complex NA extracts from OPSW (provided by J Headley), or use the *p3690-lux* biosensor (5) to screen for degradation phenotypes against classic NA model compounds (cyclopentane carboxylic acid, CPCA; or cyclohexane acetic acid, CHAA), or the *patuA-lux* biosensor (5) to screen for defects in acyclic fatty acid degradation using the Sigma-Aldrich NA mixtures, or single compounds (citronellic acid, stearic acid). Growth defect phenotypes may also be measured, although growth is limited when NA are used as sole carbon sources in some of our oil sands derived strains (OST1909), but there is still evidence of degradation by low biomass. Mutants identified with a defect in degrading one NA compound can be assayed to determine their role in degrading other single compounds or mixes. The site of Tn insertions will be mapped with arbitrary or inverse PCR. We have extensive experience working with mutant libraries and mapping Tn insertions (27), and have already prepared, screened and identified NA-induced Tn insertions in an environmental *Pseudomonas* isolate from OSPW(5).

As alternative approaches, we will test existing knockout mutants available in our *Pseudomonas aeruginosa* mini-Tn5-lux mutant library (27) for NA degradation phenotypes, such as fadD1, fadD2, fadB, PA0508, PA0506, among others. Wild type P. *aeruginosa* PAO1 has been shown to degrade acyclic NAs (28), and the 9xNA mix (unpublished data). Targeted mutagenesis can also be attempted in OSPW-derived strains, if transposon mutant screens fail to identify degradation mutants. Allelic exchange methods for *P. aeruginosa* could work in other species. Finally, we have sequenced 5 cosmids from the *E. coli* strains expressing cosmids with environmental DNA (Fig 2D). Annotation has identified candidate degradation genes, but we will use EZ::Tn mutagenesis kit (Lucigen) to mutagenize the cosmids in vitro, and then re-introduce the mutant plasmids back into wild type *E. coli*, and biosensor screen the resulting transformants for colonies that lose the ability to degrade NA. Collectively, we will investigate numerous OSPW derived bacterial isolates, a common laboratory strain (PAO1), and the metagenomic library strains, with the goal of identifying diverse NA degradation pathways. In addition, the genomes of all prioritized NA degrading isolates will be sequenced to facilitate genomic comparison of degraders and non-degraders, in addition to those already sequenced (29–32) with collaborator Dr Roger Levesque (Laval University).

IMPACT. There is a need to understand the molecular mechanisms of naphthenic acid and fatty degradation in bacteria. The approach described here involves a traditional microbiological attempt to grow native bacterial isolates from the tailings ponds, an environment that contains microbes originating from the oil sands. These strains have been selected for their ability to survive and degrade the petroleum hydrocarbons found in the oil sands and have survived the bitumen extraction process. The NA biosensors represent an innovative high throughput screening tool that will be employed to screen many wild type isolates, as well as mutant libraries in select strains, for NA degradation abilities. For this study, we can use both model NA compounds and actual NAs extracted from tailings ponds. There is great potential to increase our knowledge of bacterial degradation of naphthenic acids and fatty acids as carbon and energy sources. This knowledge could ultimately inform possible bioremediation approaches of using native OSPW microbes to treat and remove the priority contaminants in the large volumes of wastewater generated by oil sands mining. Bioaugmentation has not been explored in the area of naphthenic acids bioremediation, and there may be an acceptance of applying this concept to constructed wetlands. This research could also identify genetic modification strategies to enhance degradation of native strains, but this approach has very little social acceptance in practice. This research describes a novel application of the NA biosensors, in addition to their proposed application in environmental monitoring of water for NA, both of which would support human and animal health in the proximity of the oil sands region.