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The Symbiosis between *Frankia alni* and Alder Shrubs Results in a Tolerance of the Environmental Stress Associated With Tailings from the Canadian Oil Sands Industry

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Abstract

Alders are well recognized for their ability to colonize the harsh environments created by either natural processes or human activity. They establish symbiosis with the *actinomycete Frankia alni* which supplies 70 to 100 % of the plant's nitrogen requirements. An important challenge facing the oil sands industry in Alberta, Canada, is management of the toxicity of Tailing Sands (TS) that are alkaline, saline and contain Naphthenic Acids (NA).In order to begin to understand how alders, *Frankia alni* and their symbiosis perform and adapt to these challenging environmental conditions, the tolerance of the microsymbiont (*Frankia alni*), the host plants (alders) and their symbiosis to specific compounds found in TS was studied. In addition, the metabolic response of *Frankia alni* ACN14a to the presence of NA was characterized. The *Frankia alni* strains tested tolerated both high pH and NA levels in addition to salts near or at the concentrations found in TS. Furthermore, actinorhizal symbiosis can establish itself under these conditions. NaCl was observed to exert the greatest stress on the establishment of symbiosis, decreasing the efficiency of the actinorhizal symbiosis. Inoculation of the alder plants with Frankia alni induced a significant increase in aerial biomass allocation. Finally, intracellular proteins in *Frankia alni alni* ACN14a whose expression level were influenced by naphthenic acids were identified. Together, these results demonstrate that actinorhizal alders show significant promise for use in the revegetation of lands affected by the mine tailings of the Canadian petroleum industry.

Keywords: Actinorhizal symbiosis; Petroleum industry; Naphthenic acids; Proteome; *Alnus viridis* ssp; *crispa*, *Alnus incana* ssp. *rugosa*

Abbreviations: Arbuscular Mycorrhizae (AM); Ectomycorrhizal fungi (ECM); Flame Ionization Detector (FID); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium, inner salt)(MTS); Naphtenic Acids (NA); Phenazine Methosulfate (PMS);Tailing Sands (TS)

Introduction

Frankia alni is a filamentous N_2-fixing soil bacterium that establishes a root nodule-forming symbiosis (the actinorhizal symbiosis) with over 220 angiosperm species belonging to 8 families and 25 genera [1,2]. *Frankia alni* is not an obligate symbiont, instead it can colonize two distinct ecological niches: the roots of actinorhizal plants and soil. *Frankia alni* is able to adopt four morphological structures: hyphae, vesicles, sporangia and reproductive tolurose hyphae [3]. *Frankia alni* vesicles contain the nitrogenase enzyme that catalyzes nitrogen fixation, which may be used to fix atmospheric nitrogen either symbiotically or saprophytically [4,5]. During the symbiosis, *Frankia alni* provides 70 to 100% of the plant's nitrogen requirements. In exchange, it receives photosynthates. One of the common host of Frankia alnie, alders, are well-recognized for their ability to colonize the harsh environments created by either natural processes (volcanic eruption, slides, and forest fires), or by human actions (hydroelectric dam construction, mining and oil sands excavation) [6]. Their global importance is evident, as the genus *Alnus* comprises 34 species that possess a wide geographic distribution in temperate regions due to their great tolerance to cold and to their ability to colonize varied environments [7,8]. The actinorhizal symbiosis in alders produces high annual nitrogen fixation rates in spite of the short 3 month growing seasons found in the majority of the temperate regions. Symbiotic alders contribution to nitrogen soil enrichment can reach 300 kg $N \cdot ha^{-1} \cdot year^{-1}$, which is comparable to legume symbiosis [1,8,9]. Alders are also able to establish a tetrapartite symbiosis (alder, Ectomycorhizal Fungi (ECM), Arbuscular Mycorrhizae (AM), *Frankia alni*) that facilitates their uptake of other nutrients (e.g. phosphorus) and water in soil [6-8].

Aside from alders, which are mainly temperate trees and shrubs, tropical and subtropical actinorhizal species like *Casuarina* were used in diverse revegetation trials and often beneficiated from their symbiotic association with *Frankia alni* to survive harsh conditions. *Casuarina* species were effective windbreakers for crops in Egypt, China and Tunisia, reduced the volume of saline drain water in California and stabilized sand dunes in Senegal and China [10, 11]. *Casuarina* species also showed great success in afforestation of clay and sand reclaimed sites [10], rehabilitation of limestone spoil from a cement factory [11], improvement of soil physical and biochemical properties of a reclaimed mine site [12] and growth in bauxite mine spoil [13]. Finally, *Casuarinaequisetifolia* showed tolerance to a combination of 1% NaCl and 10 g diesel fuel per kg soil in a pot trial demonstrating its potential in petroleum polluted soils rehabilitation [14].

The bitumen reserve in Canada is estimated at 173 billion barrels, ranking the country as possessing the second largest petroleum reserve behind that of Saudi Arabia. The major oil sand reserves are located in the Athabasca, Peace River and Cold Lake regions of Alberta and

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represent a total area of 77,000 km^2 [15]. The land area currently being excavated covers 420 km^2, a figure that is expected to climb to 15,000 km^2 in 45 years [16,17]. The Clarke hot caustic soda method is employed to extract the bitumen from the sand [18]. Once the extraction is complete the solid fraction, known as the tailing sands, is alkaline (pH 8.5), saline (40 mM NaCl and 2 mM Na_2SO_4) and contains Naphthenic Acids (NA) in addition to napthenate salts [18-21]. High salinity generally causes three major problems for living organisms: hyper osmotic shock, deregulation of $K^+$and Ca^{2+} ion transportation across membranes and the toxic effects of specific ions on both protein and nucleic acid metabolism, membrane integrity and enzymatic activities [22-24]. One of the major challenges facing the oil sands industry is the management of the toxicity of the extraction process' residues, which are termed Tailing Sands (TS). Their toxicity is attributed to their high levels of NA [18,19], a complex mixture of carboxylic acids in which the alkyl group can be substituted by either acyclic and/or cyclic aliphatic chains [25]. The toxicity of NA stems mainly from their surfactant properties which allow them to cross cellular membranes relatively easily [18].

Currently, alders used in TS revegetation trials are planted in TS either mixed or layered with natural soils and overburden [26]. The goal of this study was to investigate the limits of this phytotechnology and to evaluate precisely how the interactions between the microsymbiont and the plant are affected when they are directly exposed to TS stress. The tolerance of the microsymbiont (*Frankia alni*), the plant (alders) and their symbiosis to both the organic and inorganic compounds found in TS was evaluated under controlled conditions. In addition, the metabolic response of the *Frankia alni alni* ACN14a strain in the presence of NA was characterized.

Material and Methods

Frankia alni strains and growth media

The *Frankia alni* strains used in this study were isolated from both alder and casuarina shrubs [27]. *Frankia alni* sp. strains ACN10a (ULQ010201001) and AvcI1 (DDB01020110), as well as *Frankia alni alni* ACN14a (ULQ010201401) were isolated from green alder shrubs (*Alnusviridis* ssp. *crispa*) growing in Canada. *Frankia alni* sp. strain Cj1-82 (ORS 021001) was isolated from a *Casuarina junghuhniana* and *C. equisetifolia* hybrid growing in Thailand. The *Frankia alni* strains ACN10a, ACN14a and Cj1-82 were kindly provided by Université Laval (Centre d'Étude de la Forêt), while strain AvcI1 was obtained from the ATCC (Manassas, US, #33255). All *Frankia alni* strains were grown at 30°C in the dark using defined BAP medium with succinate (BAPS) containing the following final concentrations : Na-propionate, 5 mM; Na-succinate, 18.5 mM; $MgSO_4 \cdot 7H_2O$, 0.2025 mM; $CaCl_2 \cdot 2H_2O$, 0.135 mM; NH_4Cl, 5 mM; KH_2PO_4, 9.993 mM; $FeSO_4 \cdot 7H_2O$, 0.01755 mM and $Na_2EDTA \cdot 2H_2O$, 0.01789 mM. The medium was supplemented with 1 mL per liter of medium with the following micronutrient stock solution : H_3BO_3, 2.86 $g \cdot L^{-1}$;$MnCl_2 \cdot 4H_2O$, 1.81 $g \cdot L^{-1}$;$ZnSO_4 \cdot 7H_2O$, 0.22 $g \cdot L^{-1}$;$CuSO_4 \cdot 5H_2O$, 0.08 $g \cdot L^{-1}$;$Na_2MoO_4 \cdot 2H_2O$, 0.025 $g \cdot L^{-1}$ and $CoSO_4 \cdot 7H_2O$, 0.001 $g \cdot L^{-1}$; as well as 0.1 mL per liter of medium with the following vitamin stock solution : thiamine· HCl, 0.1 $g \cdot L^{-1}$; pyridoxine· HCl, 0.5 $g \cdot L^{-1}$; nicotinic acid, 0.5 $g \cdot L^{-1}$; D-biotin, 0.225 $g \cdot L^{-1}$; folic acid, 0.1 $g \cdot L^{-1}$; D-pantothenic acid, 0.1 $g \cdot L^{-1}$ and riboflavin, 0.1 $g \cdot L^{-1}$. Lastly, the pH was adjusted to 6.7 prior to sterilisation by autoclaving.

Microbial tolerance screening

The *Frankia alni* cultures were grown for one week at 30°C in 1 L Erlenmeyer flasks containing 300 ml of BAPS. The cells were harvested by centrifugation (3440×g). Washed and resuspended in BAPS. At this point, the wells of a 96-well plate (Falcon, Whitby, ON) containing BAPS media in the first column (as positive controls), and BAPS media containing increasingly strong TS agents (left to right columns) were inoculated (except the two last down rows which were the reference wells) with *Frankia alni* sp. at an equivalent of 15 µg/ml of mycelial protein in a total volume of 200 µl/well. The plates were incubated for two weeks at 30°C. The TS stressor agents used were: alkalinity up to pH 9.5 (modified with NaOH), naphthenate salts up to 400 ppm (Acros Organics, Geel, Belgium), NaCl up to 500 mM, Na_2SO_4 up to 300 mM, NA up to 750 ppm (Acros Organics, Geel, Belgium) and a mixture (termed amalgam in this study) of the latter three chemical compounds up to 80 mM, 4 mM and 120 ppm, respectively. Tailing sands typically possess a pH of 8.5 and contain stressor levels of: 40 mM NaCl, 2 mM Na_2SO_4 and 60 ppm NA. These concentrations were therefore defined as "1x" in the "amalgam" series. Following the two weeks incubation, the reduction potentials (microbial respiration) of the cultures were measured using the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium, inner salt) (Promega, Madison, WI) and PMS (phenazinemethosulfate) (Sigma, St-Louis, MO) as described previously [28]. After 4 h of incubation at 30°C with the MTS-PMS reagent, the 96-well plates were centrifuged, the supernatants transferred to new plates and the absorbance at 490 nm determined. The relative MTS reduction was established using the positive control (*Frankia alni* sp. without stressor agents) and was fixed at 100 %. In all of the results shown herein, the 490 nm absorbance of the positive control was at least three times higher than that of the reference wells (i.e. the background level). *Frankia alni* tolerance was defined as a minimum of 25% or more of relative MTS reduction (compared to control).

Plant and symbiosis tolerance screening

The alders used in this study were *Alnus incana* (L.) Moench ssp. *rugosa* (speckled alder; seed lot 8431680.0) and *Alnus viridis* (Chaix) DC. ssp. *crispa* (green alder; seed lot 8360545.0). These species are indigenous to the Alberta oil sands region, and their seeds were provided by the National Tree Seed Centre of Canada, (Fredericton, NB). The seeds were surface sterilized twice with hydrogen peroxide 30 % for 20 min before being rinsed three times with sterile distilled water for 10 min and incubated for 24 h at 4°C in the dark for stratification [29]. The following day, the seeds were again sterilized using the same procedure and put on Petri dishes containing germination medium (Murashige and Skoog basal salt mixture (Sigma, St-Louis, MO), 4.3 $g \cdot L^{-1}$; sucrose, 25 $g \cdot L^{-1}$;Gelzan™ CM (Sigma, St-Louis, MO), 3 $g \cdot L^{-1}$ and 1 ml of Gamborg's vitamins solution at pH 5.7 (Sigma, St-Louis, MO)). The seeds were incubated for 3 weeks in order to allow for germination to occur. Following germination, 15 plantlets per treatment were transferred to 5 CYG germination pouches (Mega International, St-Paul, MN) (3 plantlets per pouch). The pouches were kept upright using custom-made metal racks which were placed in modified polycarbonate containers (with lids) and autoclaved. Each container had two 2.5 cm holes that were covered with 0.22 µm membranes (Millipore Corporation, Bedford, MA) in order to permit gas exchange. Plants were grown in a planetarium with a 16 hour/8 hour light/dark regime at 22°C and 18°C, respectively. The light intensity was 500-600 µmol photons m^{-2} s^{-1}. The plants were watered aseptically on a weekly basis with 15 ml of 0.1X Hoagland's solution [30] containing 5 ppm of nitrogen, in the form of KNO_3, for about 15 weeks. Two weeks after the transfer of plants to growth pouches, plants slated for inoculation were inoculated with*Frankia alni alni* ACN14a (15 µl packed cell volume per pouch) and the TS stressors agents were added. Since NA requires ethanol for dissolution, control pouches (with plants) treated

with ethanol (200 µl ethanol/pouch) was included in the experiment. The plants were harvested and fresh weight data were collected for the following parameters: total weight, aerial parts weight, root weight and nodule weight. The number of nodules per plant was also determined, and the nitrogen fixation rate of the nodules measured using the acetylene reduction assay. In all, 93% and 89% of the pouches in these experiments (for speckled and green alder, respectively) were harvested, as some growth pouches were affected by fungal contamination and had to be eliminated during the experiment.

Nitrogenase assay (acetylene reduction assay)

All of the nodules excised from a pouch were transferred to a single 10 ml vial (Chromatographic Specialties Inc., Rockwood, TN) capped with a 20 mm septum stopper (Bellco Glass Inc., Vineland, NJ) and an aluminum ring seal (Chromatographic Specialties Inc., Rockwood, TN). The vials were incubated for 24 h in a 10% acetylene atmosphere in the planetarium. Following this incubation, 5 ml gas samples were transferred to 3 ml Exetainer (Labco, Wycombe, UK) storage vials before being analyzed as follows. Gas samples (2 ml) were taken from the Exetainer vials and injected into the 250µl loop of a CP-3800 gas chromatograph (Varian, Walnut Creek, CA). A Hayesep N 80-100 column was used to separate analytes, and the detection of ethylene production was performed by Flame Ionization Detector (FID). The Varian star 6 chromatography workstation version 6.20 program was used to analyze the chromatograms

Proteomic study of *Frankia alni alni* ACN14a

Growth conditions: *Frankia alni* ACN14a was grown in BAPS medium (5L) in a BioFlo 110 bioreactor (New Brunswick Scientific, Edison, NJ). The culture was harvested while it was in exponential phase, determined by protein quantification assay with the Bradford method (Biorad, Hercules, CA) after lysis of the *Frankia alni* cells. Cell lysis was achieved using 0.6-0.8 mm zirconium/yttrium stabilised beads (Sigmund Lindner, Warmensteinach, Germany) with 6 × 15 sec treatments, at an intensity of 6.5 m/s on a FastPrep-24 (MP Biomedicals, Solon, OH) that were separated by 2 min incubations on ice. The culture was centrifuged at 7000×*g* and the resulting cell pellet was washed once in $KH_2PO_4$10 mM, pH 6.7 prior to re-suspending the cells in fresh BAPS. For the proteomic experiment, two bioreactors were simultaneously inoculated at a concentration of 15 µg/ml of mycelial protein in 2.2 L of BAPS. The cultures were incubated at 25ºC, 150 rpm agitation, at constant pH (6.7) and with constant dissolved O_2 level of 100%. When the cultures reached the mid-exponential phase, between 75 and 120 µg/ml of mycelial protein, 60 ppm of Naphthenic Acids (NA) were added to one of the two bioreactors and the incubations were continued for 48 h. At this point, both cultures (control and test) were harvested for intracellular proteins purification.

Purification of total intracellular proteins

The culture samples were centrifuged at 3440×*g* for 10 min at 4°C and the resulting cell pellet were washed three times with sonication buffer (10 mM Tris-HCl, 1 mM PMSF, 2 mM EDTA, pH 7) prior to being resuspended in 4 ml of the same buffer. The *Frankia alni* cells were sonicated on ice with a Vibra Cell model VCX 130PB sonicator (Sonics and Materials Inc., Newtown, CT) 4×1 min at 50% amplitude. The sonicated samples were centrifuged at 3440×*g* and the resulting supernatants were recovered and added to 5 supernatant volumes of acetone in order to precipitate the proteins. The precipitation reactions were incubated at -20ºC for 3 h. The proteins were resolubilized in sample rehydration buffer (8 M urea, 2% (v/v)) Pharmalyte pH 4-6.5 (Amersham Biosciences Corp., Piscataway, NJ), 260 µl of Triton X-100, 1.25 ml of 10% NP-40, 70 mg DTT, 50µl of bromophenol blue 1%, fill at 25 ml with dH_2O). Protein quantification in the purified extracts was performed using the 2D quant-kit as described by the manufacturer (Amersham Biosciences Corp., Piscataway, NJ).

Proteome analysis

The rehydration of the 11 cm immobiline dry strips pH 4-7 (GE healthcare, Uppsala, Sweden) was carried out in a reswelling tray with rehydration buffer containing 200 µg of the purified intracellular proteins. A multiphor 2 electrophoresis unit (Pharmacia LKB, Uppsala, Sweden) equipped with a cooling bath (20ºC) was used to perform the isoelectro focalization. A three-step programmed voltage ramping was used (300 V – 6 h, 300 to 3500 V – 1.5 h and 3500 V – 9 h). The isoelectrically focused dry strips were incubated twice for 15 min in 10 ml of equilibration buffer. DTT (10 mg/µl) and iodoacetamide (25 mg/ml) were respectively added for the first and the second equilibration steps as described in 2-D Electrophoresis, Principles and Methods (GE Healthcare). SDS-PAGE was performed with a 12% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel [31]. The gels were electrophoresed at 150 V for 1.5 h, followed by 300 V for 5 h. The molecular weight standard ladder used was the Amersham low molecular weight calibration kit for SDS electrophoresis (GE Healthcare, Buckinghamshire, UK) and the gels were stained with colloidal Coomassie blue G-250. For proteomic studies three sets of bioreactor trials (consisting of the "control" and "test" bioreactors) were performed yielding a total of 12 protein extracts which were analyzed and quantified using the Phoretix 2D version 2004 program (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Proteins were selected if they were either always up-regulated in each individual trial, or were always down-regulated in the "test" sample as compared to the control. Selected proteins were excised from the polyacrylamide gel and sent to the Centre de génomique de Québec (Québec, Canada) for the sequencing and identification steps described below. Student t-tests were performed on selected proteins in order to establish the significance of differences observed in their accumulation levels between the test and control samples.

In-gel digestion, mass spectrometry and protein identification

Excised polyacrylamide gel spots were transferred to individual wells in 96-well plates and washed with water. Tryptic digestion was performed using a MassPrep liquid handling robot (Waters, Milford, USA) according to the manufacturer's specifications and the protocol of [32] with the modifications suggested by [33]. Briefly, the proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using a final concentration of 105 mM of modified porcine trypsin (Sequencing grade, Promega, Madison, WI) at 58°C for 1 h. The digestion products were extracted using a 1% formic acid, 2% acetonitrile solution followed by a 1% formic acid, 50% acetonitrile solution. The recovered extracts were pooled, vacuum centrifuge dried and dissolved in 8 µl of 0.1% formic acid solution. The resulting samples (4 µl) were analyzed by mass spectrometry. Peptide samples were separated by online Reversed-Phase (RP) nanoscale capillary liquid chromatography (nanoLC) and were analyzed by electrospray mass spectrometry (ES MS/MS). The experiments were performed using a Thermo Surveyor MS pump connected to a LTQ linear ion trap mass spectrometer (ThermoFisher, San Jose, CA) equipped with a Nano electrospray ion source (ThermoFisher, San Jose, CA). Peptide separation was performed on a PicoFrit column BioBasic C18, 10 cm×0.075 mm internal diameter, (New Objective, Woburn, MA) with a linear gradient from 2-50% of acetonitrile, 0.1% formic acid solution for 30 min at flow rate of 200 nL/min (obtained

by flow-splitting). Mass spectra were acquired using a data dependent acquisition mode using Xcalibur software version 2.0. Each full scan mass spectrum (400 to 2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion (30 sec exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.0). Mascot was set up to search the uniref100_15_3_Frankia alni_1854 database (unknown version, 18819 entries, November 2009) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification and the oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed. Scaffold_2_06_00 (Proteome Software Inc., Portland, OR) was used to validate the MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [34]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [35]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Statistical analysis

A two-way ANOVA (analysis of variance) with Bonferroni post-tests was performed on plant total weight, on plant aerial to root weight ratios and on inoculated to non-inoculated plant total weight. A one-way ANOVA with Tukey's post-tests was performed on both plant nodule numbers and nodule acetylene reduction assays (results not shown).

Results

Microbial tolerance

In order to identify the tolerance threshold of *Frankia alni* ssp. to environmental stresses, four *Frankia alni* sp. strains were studied: ACN10a, AvcI1, ACN14a and Cj1-82. All strains exhibited tolerance to the stressors levels found in tailing sands (40 mM NaCl, 2 mM Na_2SO_4, and 60 ppm NA), as can be observed in the "1x" condition

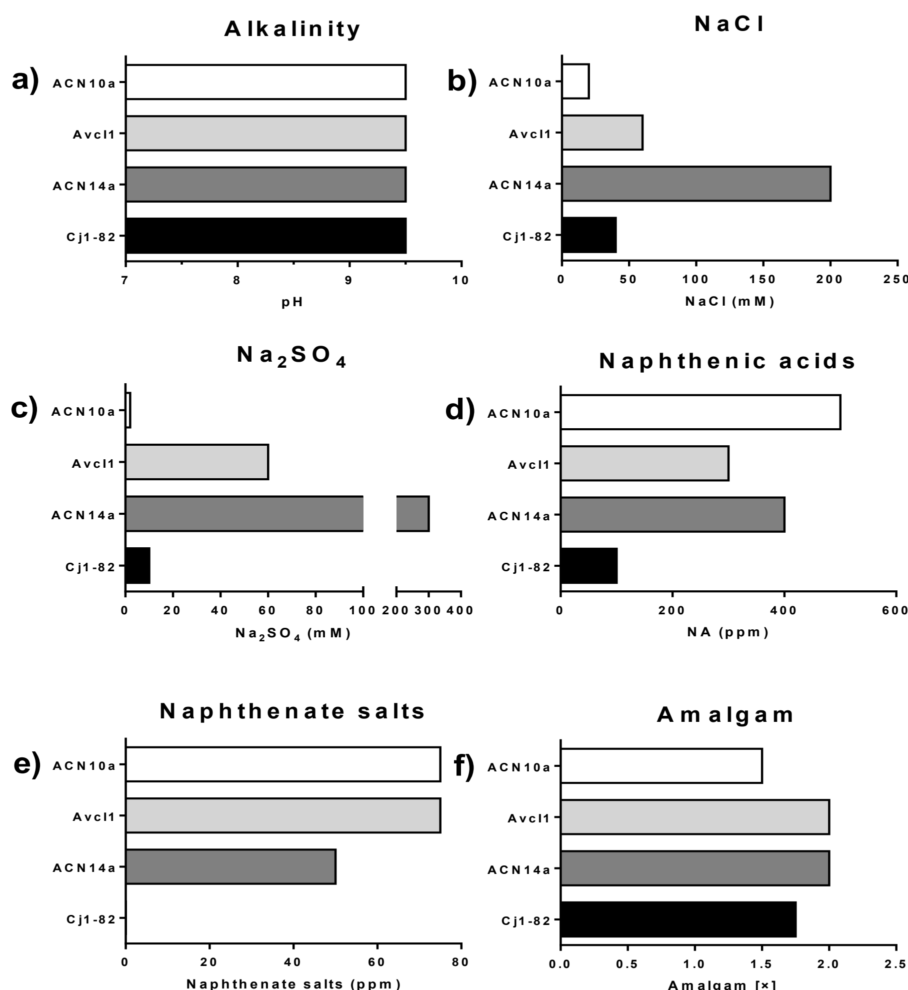

Figure 1: Tolerance of *Frankia alni* spp. strains to tailing sands stressors. (a) alkalinity, (b) NaCl, (c) Na_2SO_4, (d) napthenic acids, (e) naphtenate salts, (f) amalgam (NaCl, Na_2SO_4 and naphtenic acids). In f) the [x] indicates the multiple of the concentrations found on site. Each bar represents the strains tolerance; meaning the maximal concentration/value of stressor that allows the strains to produce at least 25% of MTS reduction signal compared to control condition (without stressor).

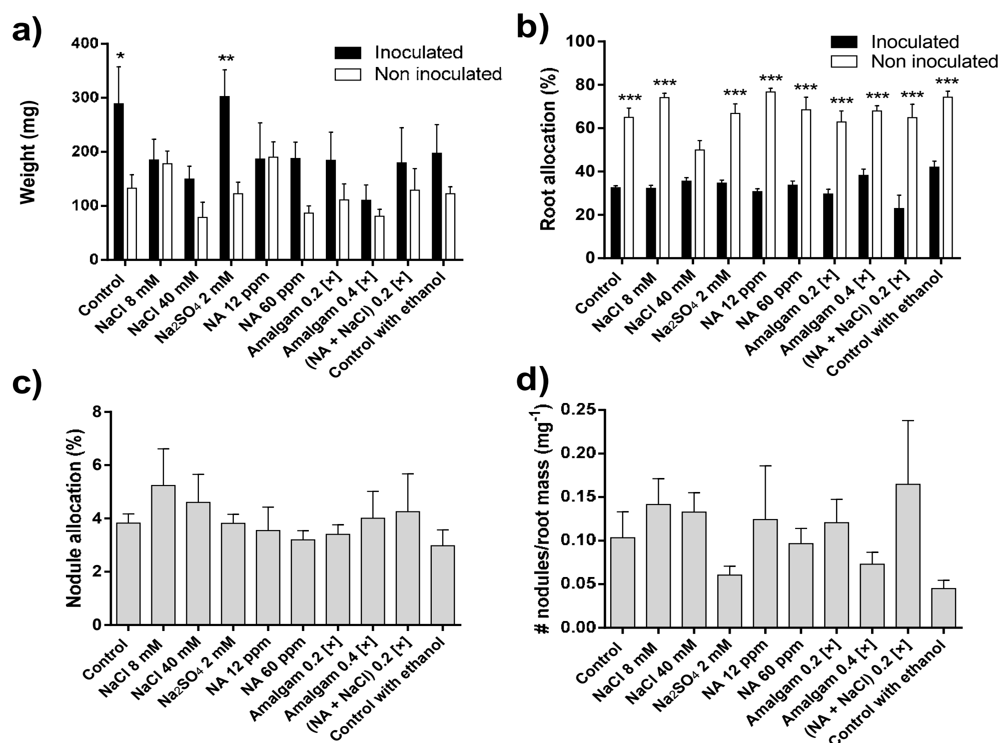

Figure 2: *Alnus rugosa – Frankia alni* sp. ACN14a symbiosis tolerance to tailing sand stressor agents. The amalgam consists of a mixture of NaCl, Na_2SO_4 and naphtenic acids where 1[x]=the concentrations found in tailing sands. Black bars: inoculated alders, hatched bars : non-inoculated alders. (a) Plant total weight; (b) Allocation of biomass to roots; (c) Nodules' mass as a percentage of the total mass; and, (d) Specific Nodule Number (SNN). Each bar represents the average value obtained from alders exposed to the same treatment. SNN=number of nodule per mass of root. A two-way ANOVA with Bonferroni post-tests was performed on (a) and (b), and a one-way ANOVA with Tukey post-tests was performed on (c) and (d). Error bars represent the SEM. The significant P values are indicated as follows: *, P<0.05; **, P<0.001; and, ***, P<0.001.

of the amalgam series (Figure 1f). All strains also showed a high level of tolerance to alkalinity, surviving up to pH 9.5 (Figure 1a). ACN14a showed the highest tolerance to NaCl, followed by AvcI1, while ACN10a was the most sensitive (Figure1b). Sodium sulfate (Na_2SO_4) was found to be detrimental to strains ACN10a and Cj1-82, while it was tolerated by AvcI1 and, to a higher degree, by ACN14a (Figure 1c). Strain Cj1-82, a casuarina isolate, was the most sensitive to NA and naphthenate salts, whereas these organic compounds were tolerated by the strains ACN10a, ACN14a and AvcI1 (Figure 1d and 1e).

Tolerance of the alder-*Frankia alni* symbiosis to environmental stress

The tolerance of the speckled alder-*Frankia alni* symbiosis was measured in the presence of TS stressor agents (Figure 2). The inoculation of speckled alder in the presence of stressors only increased the total alder biomass significantly in the presence of 2 mM Na_2SO_4, as was observed for the alders receiving only nutrient solution (control) (Figure 2a). Inoculation, in all treatments except for 40 mM NaCl, decreased alders' allocation of tissue to roots (Figure 2b). No significant fluctuation in the nodule biomass allocation, or in the specific nodule number, was observed under any of the treatments (Figure 2c and 2d). Equivalent level of nitrogen fixation, as measured by the ARA assay, were observed by the alder nodules produced under all treatments, with the exception of the 40 mM NaCl condition for speckled alder under which little acetylene reduction was detected (results not shown). Statistical analysis however revealed no significant differences in nitrogen fixation activities across treatments, nor could confirm null nitrogen fixation in alders exposed to 40 mM NaCl.

In the green alder-*Frankia alni* symbiosis trial, regardless of the treatments, inoculation did not significantly increase the plants' total weight (Figure 3a). In addition, the green alder's total weights were comparable to those of the speckled alders (Figure 2a). However, under many treatments the inoculation with *Frankia alni alni* ACN14a significantly increased the plants' aerial allocation versus root allocation (Figure 3b). As was the case for speckled alder, nodule allocation did not fluctuate significantly across the treatments (Figure 3c). However, the number of nodules per plant root biomass did vary (Figure 3d). Overall, the results suggest that some treatments were stressful, but that none of these treatments (e.g. NA 60 ppm) yielded significant differences when compared to those of the control. In effect, NA 12 ppm increased the average number of nodules produced by alders (Figure 3d).

For both green and speckled alder, no stressor treatment significantly decreased the total plant biomass; however, the correlations related to total biomass were observed to be highly significant (p 0,0001). Total biomass deposition by green alders correlated more strongly with root mass development in the absence of *Frankia alni* inoculation (r=0.994 vs 0.792). In the presence of *Frankia alni*, green alder total biomass correlated more strongly with nodule mass (r=0,999). Speckled alder total biomass was strongly correlated with root biomass in both the presence and the absence of *Frankia alni* (r=0,969, 0,993, respectively). Its total biomass correlated less with nodule biomass (r=0,865) than was the case for green alder (r=0,999).

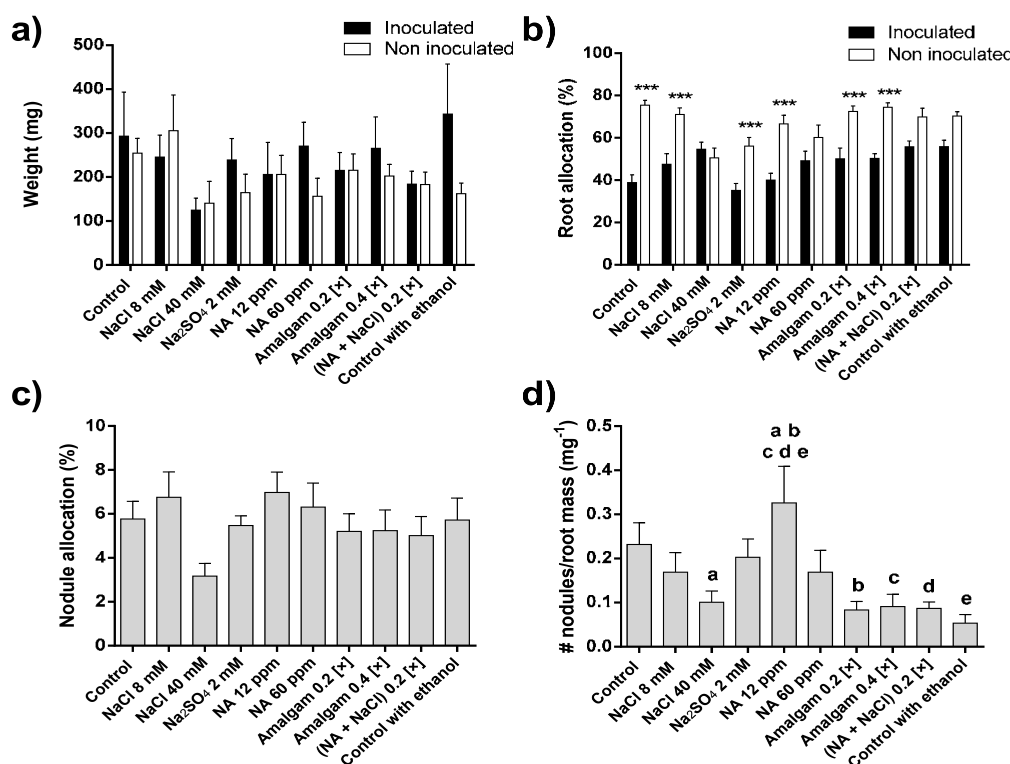

Figure 3: *Alnus crispa – Frankia alni* sp. ACN14a symbiosis tolerance to tailing sand stressor agents. The amalgam consists of a mixture of NaCl, Na_2SO_4 and naphtenic acids where 1[x]=the concentrations found in tailing sands. Black bars: inoculated alders, hatched bars : non-inoculated alders. (a) Plant total weight; (b) Allocation of biomass to roots; (c) Nodules' mass as a percentage of the total mass; and, (d) Specific Nodule Number (SNN). Each bar represents the average value obtained from alders exposed to the same treatment. SNN=number of nodules per mass of root. A two-way ANOVA with Bonferroni post-tests was performed on (a) and (b), and a one-way ANOVA with Tukey post-tests was performed on (c) and (d). Error bars represented the SEM. The significant P values are denoted as follows: ***, P<0.001; letters, P<0.05.

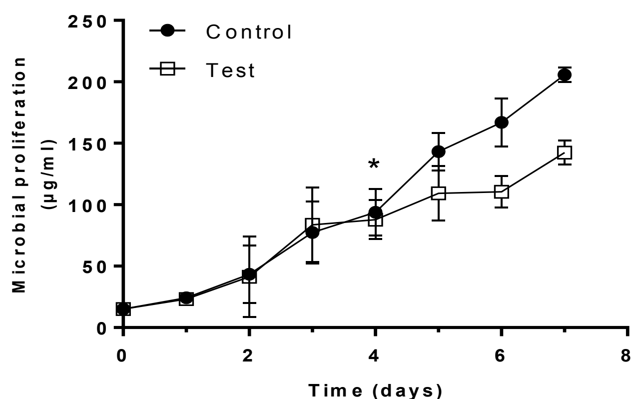

Figure 4: Growth of *Frankia alni alni* ACN14a in fermentors. The curves represent the average protein concentration for 3 biological replicates. * indicates the day of the 60 ppm NA spike.

Proteomic study

Unlike the static *Frankia alni* sp. Erlenmeyer culture method [1,4,36], the culture method employed here in the bioreactor permitted reproducible growth patterns of dispersed mycelium compatible with subsequent metabolic investigations. In fact, the growth of *Frankia alni alni* ACN14a was reproducible for all three culture replicates under all conditions (control and test; Figure 4). Moreover, the growth of *Frankia alni alni* ACN14a under both conditions were similar until day 4, at which point the test bioreactor was spiked with NA at a final concentration of 60 ppm. From day 4 on, the growth in the test bioreactor slowed down as compared to that in the control bioreactor (Figure 5). On day 6, culture samples from both bioreactors were harvested for both the intracellular proteomic study and the analysis of the proteomes. Based on the criteria described in the material and method section, five protein spots were selected for further analysis by nanoLC-MS/MS protein sequencing. The selected proteins are shown on the two-dimension gel electrophoresis in Figure 5. Of the five, one appeared to be down-regulated and four up-regulated in the presence of NA (Table 1). The down-regulated proteins, Q0RTT7, are involved in transcription regulation and correspond to nusG. Two of the up-regulated proteins, Q0RTR6 and Q0RG14, are involved in fatty acid and lipid metabolism. Moreover, Q0RG14 as well as Q0RMH3 are involved in the metabolism and degradation of complex organic compounds which molecular structure resembles NA. Q0RMH3 is also part of many metabolic pathways of carbohydrates, lipids and amino acids. The remaining up-regulated protein Q0RMH1 is insufficiently characterized for its function to be identified.

Discussion

Microbial tolerance screening

The results presented here demonstrate that all of the *Frankia alni* strains tested largely tolerate the environmental stress associated with

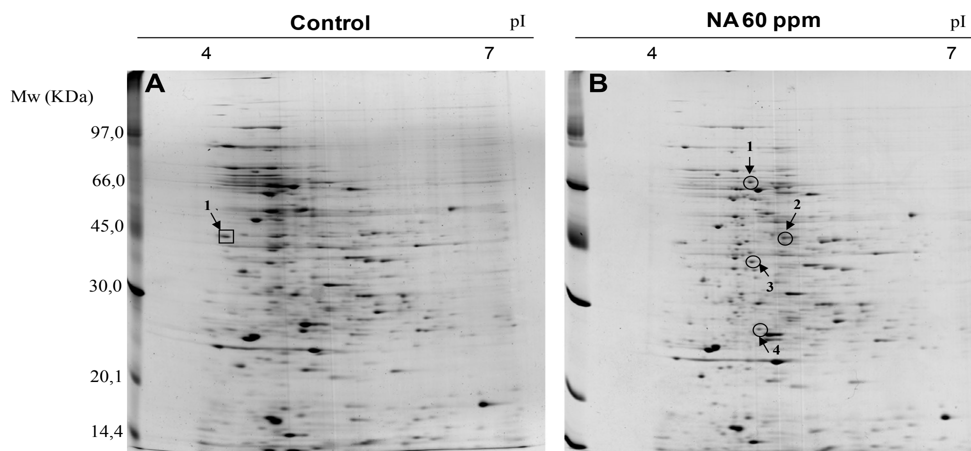

Figure 5: Two-dimensional electrophoresis of intracellular protein extracts from *Frankia alni* sp. strain ACN14a exposed to NA. (a) Image of 2-DE of intracellular proteins extract from *Frankia alni* sp. strain ACN14a grown in BAPS medium. (b) Image of 2-DE of intracellular proteins extract from *Frankia alni* sp. strain ACN14a exposed to NA 60 ppm. Square and arrow with corresponding number from Table 1 indicate proteins down-regulated by the addition of NA 60 ppm, and circles and arrows with corresponding number from Table 1 indicate proteins up-regulated by the addition of NA 60 ppm. Intracellular protein extracts (200 µg) were separated on pH 4 - 7 linear IPG strips, followed by 12.5% SDS–PAGE electrophoresis.

Name	Fold variation	Gene locus	Protein accession no.	Putative function	No. of matching peptides	Sequence coverage (%)
Down-regulated protein						
1. nusG	N/A	FRAAL1064	Q0RRT7	Transciption termination and antitermination, interacts with termination factor rho and RNA polymerase	17	38
Up-regulated protein						
1. Aldehyde dehydrogenase	6.4*	FRAAL2632	Q0RMH3	Carbohydrates, lipids and amino acids metabolism, terpenoids and polyketides metabolism, xenobiotics biodegradation	27	48
2. fadA (thiolase)	5.0*	FRAAL4934	Q0RG14	Fatty acid, lipids and amino acids metabolism, terpenoids and polyketides metabolism, xenobiotics biodegradation	18	55
3. Uncharacterized protein	4.4*	FRAAL2634	Q0RMH1	Unknown	10	30
4. Dehydrogenase	N/A	FRAAL0356	Q0RTR6	Lipid transport and metabolism	13	38

*Indicates a significant increase in protein accumulation ($p<0.05$)

Table 1: Intracellular proteins down- or up-regulated in *Frankia alni alni* ACN14a exposed to 60 ppm NA.

TS (pH 8.5, 40 mM NaCl, 2 mM Na_2SO_4, and 60 ppm NA). In the context of the Albertan oil sands exploitation, the TS that are produced are not homogenous and therefore differ from site to site. There may also be some revegetation areas where the concentrations of the TS stressor agents may not be proportional, and/or may be higher than the typical concentrations [18,20]. In this context, the most promising strains among those tested are AvcI1 and ACN14a as they possessed the highest tolerances to each of the individual stresses (salinity, NA, and naphthenate salts), and they exhibited the highest relative MTS reduction at 2× amalgam. Since the genome of *Frankia alni alni* ACN14a has been sequenced and annotated, this strain was chosen to pursue the symbiosis and proteomics experiments in this study.

Plant and symbiosis tolerance screening and nitrogen fixation

It was observed that both green and speckled alders, in the majority of treatments, develop more aerial biomass when inoculated than when non-inoculated. This observation is particularly important since nitrogen deposition through leaf fall is the main mechanism through which alder's condition soil, thereby promoting plant succession [37]. The decrease in the inoculated plant root allocations, as compared to those of the non-inoculated plants, confirms that these symbiotic plants adapt their morphology to photosynthetically support the higher energy requirements of the symbiosis. In addition, it corroborates similar earlier observations made with black alder (*Alnusglutinosa*) in hydropony [38]. The relative biomass allocated to the nodules, by both green alder and speckled alder, was not significantly different across the various treatments. This, and the fact that the total biomass for both species was also relatively constant, but always higher for inoculated alders, suggests that alders have the capability to establish and sustain the actinorhizal symbiosis in the experimental conditions used here.

Although *Frankia alni* sp. ACN14a was able to keep its infectivity in the 40 mM NaCl treatment when in the presence of both species of alder; the symbiosis itself may have loss its effectiveness. The modest increase or decrease in the aerial tissues allocation may have caused a deficiency in photosynthates for the microsymbiont, thereby reducing nitrogen fixation. It is know that in young alders, nitrogenase activity is highly dependent on newly synthesized photosynthetates transported from the leaves to the root nodule [1,39]. It remains to be verified whether or not nitrogenase was produced, inhibited or lacked an energy supply. The 40 mM NaCl treatment significantly decreased

the establishment of the actinorhizal symbiosis in green alder species. These results corroborate previous findings that showed a decrease in actinorhizal plant growth, nodulation and nitrogen fixation in the presence of salinity [2, 37, 40-42].

In the context of revegetation based on biomass production, the results presented here suggest that the use of actinorhizal alders to revegetate lands affected by TS is possible. Aside from leaf loss, alders may contribute root exudates to the rhizosphere which can promote an increase of 10 to 100 times in the normal microbial populations [7,43]. In the hydroponics-based symbiosis tolerance experiments, it was impossible to use the amalgam of stressor agents at the concentrations found on site (1x) due to their lethality to both plant species. This apparent discrepancy can be explained by the higher bioavaibility of the stressor agents in the hydroponic growth pouches as alder growth was shown in a pot trial using TS [44].

As was previously observed in both actinorhizal and legume symbiosis [41,45], the results presented here suggest that *Frankia alni* is more tolerant to both salinity and NA than both of its host plants; this means that alder is the most sensitive symbiotic partner in this association. The effect on aerial biomass deposition of the inoculation of alders with *Frankia alni alni* ACN14a was positive. At the concentrations found in TS, the NaCl stress is more acute on symbiosis than that of either NA or Na_2SO_4. In both of the symbiosis tolerance experiments, the results show great variability likely due to the plant's genetic variability. However, using seeds rather than alder clones was paramount in order to be able to describe the response (including variability) of these alder species.

Proteomic study

The growth rate of *Frankia alni alni* ACN14a was observed to be reduced but not inhibited in the presence of NA. This is not surprising given that cyclic hydrocarbons are known to slow down the growth of both bacteria and fungi [46]. NA, due to its surfactant properties can pass more easily through the cell wall and affects membrane integrity. The primary functions of the membrane are to maintain the anion gradients that drive various endergonic processes, to maintain intracellular ions concentrations at levels that are compatible with enzymatic activities and to form a matrix for membrane-embedded enzymes [18,46]. Two of the proteins that were found to be up-regulated in the presence of NA are involved in fatty acid or lipid metabolism, suggesting a rearrangement of the composition and structure of the membrane. These two proteins were a dehydrogenase, for which the up-regulation was not statistically significant, and fadA up-regulated 5X ($p<0.05$). The dehydrogenase possessed both a conserved 3-ketoacyl-(acyl-carrier-protein) reductase and a NAD (P) (+)-binding domain. The first domain is involved in fatty acid elongation and its activity is NADPH-dependent [47]. fadA is part of the 3-ketoacyl-CoA thiolases (thiolase 1) which are primarily implicated in the degradation of the beta-oxidative fatty acids, and possess a broad chain-length specificity [48]. Modifications such as changes in the degree of saturation of the phospholipids' acyl chains that restabilize membrane fluidity [46], have been observed in the presence of lipophilic compounds in microorganisms. If NA caused membrane disruption, osmotic stress could have caused a toxicity response. It should be noted, however, that this is in contradiction with the growth of *Frankia alni alni* ACN14a observed in the presence of 60 ppm NA [18]. In addition, since many bacteria adapt to osmotic stress by accumulating osmolytes derived from intermediary metabolites such as amino acids [45,49-51], the significant up-regulation of the proteins Q0RMH3 and Q0RG14 involved in their production tends to confirm that hypothesis. To date the results suggest that *Frankia alni alni* ACN14a was able to maintain its membrane integrity in the presence of NA. This is of interest since TS contain a high level of salinity. The last up-regulated protein was a putative uncharacterized protein (4.4 fold increase). It could be involved in many metabolic pathways, and has a 71% amino acid sequence identity with amidohydrolase 2 of *Frankia alni alni* sp.CN3.

A transcription regulator, transcription anti termination protein nusG, appeared to be down-regulated in presence of NA. In *Escherichia coli*, this proteininteracts with the termination factor rho to increase the efficiency of transcriptional termination [52]. The involvement of bacterial transcription termination control in physiological processes, especially in stress responses, is currently poorly understood. Clearly, this finding warrants further investigation [53].

This study has shown that *Frankia alni alni* spp.'s tolerance to the environmental stresses associated with the mine tailings produced by the Canadian petroleum industry varies amongst the strains. The tolerance level of alder-isolated Frankie is sufficient to permit their survival, their proliferation and the preservation of their capability to infect both green and speckled alders. Although alders may be more sensitive to the TS stressors, the results presented here demonstrate that actinorhizal symbiosis (i.e. inoculation with *Frankia alni alni* sp.) can improve aerial biomass development and further deposition. The study of the proteome of *Frankia alni alni* ACN14a exposed to NA revealed the existence of two up-regulated proteins involved in fatty acid metabolism; which can explain its survival and/or proliferation in the presence of NA.

Finally, the selection of compatible as well as stress tolerant microorganisms and host plants must be performed in order to obtain a well-developed actinorhizal symbiosis. Further understanding of the tolerance of the alder-*Frankia alni alni* sp. symbiosis to anthropogenic stress will be required in order to apply actinorhizal revegetation and ecosystem rehabilitation technologies with any consistent success. Testing the symbiosis performance under stress, under the controlled conditions developed here, is an important first step towards such understanding.

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