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Original Article

Molecular response to nitrogen starvation by *Frankia alni* ACN14a revealed by transcriptomics and functional analysis with a fosmid library in *Escherichia coli*

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ABSTRACT

The transcriptome of *Frankia alni* strain ACN14a was compared between in vitro ammonium-replete (N-replete) and ammonium-free dinitrogen-fixing (N-fixing) conditions using DNA arrays. A Welch-test ($p < 0.05$) revealed significant upregulation of 252 genes under N-fixing vs. N-replete (fold-change (FC) ≥ 2), as well as significant downregulation of 48 other genes (FC ≤ 0.5). Interestingly, there were 104 *Frankia* genes upregulated in vitro that were also significantly upregulated in symbiosis with *Alnus glutinosa*, while the other 148 genes were not, showing that the physiology of in vitro fixation is markedly different from that under symbiotic conditions. In particular,in vitro fixing cells were seen to upregulate genes identified as coding for a nitrite reductase, and amidases that were not upregulated in symbiosis. Confirmatory assays for nitrite reductase showed that *Frankia* indeed reduced nitrite and used it as a nitrogen source. An *Escherichia coli* fosmid clone carrying the *nirB* region was able to grow better in the presence of 5 mM nitrite than without it, confirming the function of the genome region. The physiological pattern that emerges shows that *Frankia* undergoes nitrogen starvation that induces a molecular response different from that seen in symbiosis.

1. Introduction

Frankia alni ACN14a [1] is a filamentous actinobacteria that fixes dinitrogen in symbiotic root nodules of the dicotyledonous hosts *Alnus* and *Myricaceae* [2]. The first *Frankia* strain isolated 40 years ago [3] was shown to form efficient nodules on its host plants and thus fulfill Koch's postulates [4]. Shortly afterward, it was also shown to fix gaseous dinitrogen N_2 in vitro [5], contrary to most *Rhizobium* strains that fix N_2 only in symbiosis. The existing *Frankia* symbiotic strains have been characterized as belonging to three phylogenetic clusters: cluster 1 comprises *Alnus*-infective and *Casuarina*-infective strains; cluster 2, *Rosaceae/Cucurbitales/Ceanothus*-infective strains; and cluster 3, *Elaeagnaceae/Rhamnaceae/Gymnostoma*-infective strains [6]. When it reduces atmospheric dinitrogen in symbiosis or in vitro under aerobic conditions, *Frankia* from clusters 1 and 3 produce specialized cells called vesicles [7] with numerous tightly compacted hopanoid lipid layers that create a barrier to oxygen diffusion [8]. Their porosity is increased by host-derived defensin proteins wherein gene expression is upregulated in symbiosis [8], and bind massively to vesicles and marginally to hyphae [9]. In vitro, *Frankia* preferentially uses nitrogen compounds as the nitrogen source, like NH_4^+, but also glutamine and other amino acids (Asp, His, Ala, Pro, etc) [10]. Up to the present, there is no report of its ability to use other nitrogen sources such as nitrate or nitrite that are widely available in soils [11].

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In a wide range of organisms, the main pathway for ammonium assimilation is glutamine synthetase (GS), glutamine 2-oxoglutarate amino transferase or glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). Amino acids catabolized as glutamate (Asp, Ala, Pro), through aspartate transaminase and similar enzymes, do not release ammonium ions, permitting sustained nitrogen fixing activity [10]. Regulation of nitrogen homeostasis varies in different organisms. In general, the master controller of nitrogen status is the PII protein, a conserved regulator present in plants and bacteria that reacts to the levels of glutamine, 2-oxoglutarate, AMP, ATP and pyrophosphate PPi [12]. PII proteins, also designated as GlnB or GlnK, are ATPases that control the activity of a diverse range of enzymes (GS, N-acetyl-L-glutamate kinase (NagK)), transcription factors and membrane transport proteins such as AmtB [13]. The intracellular 2-oxoglutarate pool is inversely related to the cellular nitrogen status because of the GS/GOGAT cycling, where high levels of 2-oxoglutarate indicate nitrogen deficiency and inhibit PII. Conversely, an active PII, under conditions of high glutamine (low 2-oxoglutarate), is known to bind to the integral membrane ammonia channel protein AmtB, thereby blocking the flux of ammonium into the cell [14]. They constitute a second master regulator, GlnD corresponding to a uridylyltranferase/uridylyl-removing enzyme. GlnD senses the nitrogen status of the cell and uridylylates the product of *glnB*, the PII protein, under nitrogen limitation conditions, or deuridylylates it under nitrogen excess conditions [15], which then activates transcription of nitrogen-regulated genes. A *Sinorhizobium meliloti glnD* mutant was found to have an unusual phenotype: while it is able to fix nitrogen, it apparently does not release ammonium to the plant but an unidentified form of nitrogen that the plant cannot metabolize [16] suggesting that GlnD plays a role larger than simply post-translationally modifying the PII protein [17].

Nitrogen assimilation of *Frankia* operates different enzymes according to its growth conditions of fixation. In-vitro-produced vesicles were shown to contain glutamine synthetase (GS), but not GOGAT nor glutamate dehydrogenase (GDH), two enzymes that produce glutamate, while hyphae had all three enzymes, thus enabling them to complete the GS/GOGAT cycle and subsequent synthesis of other amino acids [18]. The physiology of *Frankia* in symbiosis is less well known, but it is generally assumed that it does not assimilate NH_4^+ as in pure cultures, but rather, exports ammonium that is then assimilated by the plant's GS [19], an enzyme found immunolocalized in infected cells [20], upregulated with an FC of 5 [20] and absent from the isolated microsymbiotic vesicle clusters [21].

Transcriptomics enables following changes occurring upon physiological transition and identifying genes and proteins involved in such processes, especially useful in microbes, for which there is no genetic transformation available. The first transcriptomic study performed in *Frankia* [22] showed that previously identified genes involved in symbiosis (*nif*, *hup*, *shc*, *suf*) were upregulated, but that there were no “smoking gun” upregulated symbiotic clusters. In *Frankia*, CcI3 strain studies on in vitro N-fixing cells showed upregulation of *hsps* (for heat shock proteins) and transposases genes, as well as modest upregulation of *nif* genes, but provided little information on nitrogen assimilation. We undertook the following study so as to understand physiological changes occurring in the *F. alni* ACN14a strain under aerobic N-fixing vs. N-replete conditions. Because transformation has not been achieved [23,24], functional analysis of *Frankia* remains a true challenge in this genus. For these reasons, we developed a comprehensive gain-of-function approach in a heterologous host with a set of fosmids covering the whole *Frankia* genome, and we performed functional analysis of one of the genes shown to be upregulated upon changes in the nitrogen status.

2. Material and methods

2.1. Frankia *growth conditions*

F. alni ACN14a was isolated from *Alnus crispa* [25]. It was grown in BAP medium [26] modified by the addition of the three B vitamins, thiamine (0.1 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}) and pyridoxine (0.1 mg L^{-1}) instead of biotin, and with 5 mM ammonium chloride, NH_4Cl (N-replete condition) or without (N-fixing condition). The growth conditions and RNA extraction from the two cultures were the same as those described previously [22]. Three biological replicates were performed for each assay. Growth of *F. alni* ACN14a was assessed in BAP medium with ammonium (0, 1, 5 mM), potassium nitrite (0, 1, 5 mM) or potassium nitrate (0, 1, 5 mM). Growth assays in BAP were monitored over a 14 day time course and absorbance (or optical density, OD) at 600 nm, respiration assayed through iodonitrotetrazolium reduction activity (IRA) and nitrogen fixation assayed through acetylene reduction activity (ARA) as described previously [9] and Wilcoxon rank sum statistical tests were used to compare effects of all pairwise condition at each day of growth with p-value <0.05 as the significant threshold (using R software version 3.4.2©2017).

2.2. *Transcriptome analysis*

Microarray experiments for in vitro N-fixing cells and data analysis were performed as described previously for symbiotic cells [22]. Briefly, NimbleGen microarrays containing probes designed for 6340 genes out of the 6777 present were manufactured and hybridized by the NimbleGen expression service (Reykjavik, Iceland). After data normalization, GeneSpring gene expression analysis software 7.3 (Agilent Technologies, Massy, France) was used for statistical comparisons and filtering of the data. Genes showing significant differential expression between the two biological conditions were selected based on the three following criteria: a p-value ≤ 0.05 using the Welch t-test with multiple correction of Benjamini and Hochberg [27], a fold-change ≥ 1.8 (or ≤ 0.55) in all nine pairwise comparisons yielded by the three biological replicates of each condition (3×3) and an average fold-change ≥ 2 (or ≤ 0.5).

Quantitative real-time RT-PCR (qRT-PCR) was performed as described previously [22]. Genes and primers used are listed in Supplementary Table S1. Expression values were normalized using *infC* gene expression (FRAAL5216), and T-Student p-values were calculated.

2.3. F. alni *ACN14a fosmid library construction and* Escherichia coli *heterologous expression*

The fosmid bank was built using chromosomal DNA purified by a phenol-chloroform method, passed through a syringe to fragment it, and checked to contain fragments above 40 kb. The ends were repaired so as to be blunt and 5′-phosphorylated, ligated and cloned into vector pCC1FOS (Epicenter, Madison, WI, USA). They were then encapsulated by lambda packaging extract (Epicenter) and infected into *Escherichia coli* strain EPI300-T1R (Epicenter). Ends of the 2000 *Frankia* inserts in fosmid clones were sequenced by Biofidal (www.biofidal.com) and positioned on the *Frankia* ACN14a genome (aligned to NCBI). The different fosmids were extracted by Midiprep (Macherey–Nagel, Hoerdt, France) and transferred to *E. coli* DH10B strain [28] after electrotransformation. The different clones were grown in defined medium (M9 1X) supplemented with thiamine (10 μg/ml), leucine (100 μg/ml), chloramphenicol (12.5 μg/ml) and with or without nitrogen sources. Nitrogen sources used were ammonium chloride or potassium