

Identification by Suppression Subtractive Hybridization of *Frankia* Genes Induced under Nitrogen-Fixing Conditions^{∇†}

Masatoshi Yamaura, Toshiki Uchiumi, Shiro Higashi, Mikiko Abe, and Ken-ichi Kucho*

Graduate School of Science and Engineering, Kagoshima University, Korimoto 1-21-35, Kagoshima 890-0065, Japan

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***Frankia* is an actinobacterium that fixes nitrogen under both symbiotic and free-living conditions. We identified genes upregulated in free-living nitrogen-fixing cells by using suppression subtractive hybridization. They included genes with predicted functions related to nitrogen fixation, as well as with unknown function. Their upregulation was a novel finding in *Frankia*.**

Frankia is a Gram-positive actinobacterium that establishes symbiosis with several angiosperms termed actinorhizal plants and forms nitrogen-fixing nodules on their roots (20). *Frankia* also fixes nitrogen in free-living culture under nitrogen-free conditions (19). Induction of the nitrogen-fixing ability is accompanied by differentiation of vesicles (19). Vesicles are spherical cells specialized to nitrogen fixation and are surrounded by multilayered lipid envelopes by which nitrogenase is protected from oxygen (3). *Frankia* plays an important role in the global nitrogen cycle, yet little is known about the genes involved in the induction of nitrogen-fixing activity. Recently, three *Frankia* genome sequences were determined (15), which facilitates the genetic dissection of *Frankia* biology. In this study, we identified *Frankia* genes induced in nitrogen-fixing cells under free-living conditions by using suppression subtractive hybridization (SSH) (4).

Induction of nitrogen-fixing cells. We cultured *Frankia* sp. strain HFPCcI3 (CcI3) (21) in a modified BAP medium (14) (N⁺) in which 10 mg/liter FeNa-EDTA was replaced with Fe-citrate, 0.001 mg/liter CoSO₄ · 7H₂O was added, biotin was removed, and the pH was adjusted to 6.7, to late log phase at 28°C with a magnetic stirrer bar stirring at 300 rpm. In order to induce nitrogen-fixing ability in CcI3, we washed the cells twice with N⁺ medium without NH₄Cl (N⁻) and resuspended half of them in N⁻ medium. The remaining half was resuspended in N⁺ medium as a control. We measured the nitrogen fixation activity of the cells as acetylene reduction activity (ARA) by following the procedure described in reference 14 and using a GC8-AIF gas chromatograph (Shimadzu, Kyoto, Japan). We extracted protein by heating the cells at 90°C for 15 min in 1 N NaOH (18) and normalized ARA to the amount of protein in the cells. Nitrogen fixation activity in *Frankia* became evident 4 days after transfer (dat) to N⁻ medium and peaked at 6 dat (Fig. 1A, open circles). We detected no nitrogen fixation activity in the cells kept in N⁺ medium (Fig. 1A, closed circles).

At 6 dat in cells grown in N⁻ medium, we observed the presence of vesicles (Fig. 1B). We isolated total RNA from the 6-dat cells by the cetyltrimethylammonium bromide method (12) and analyzed the expression level of the *nifH* gene encoding dinitrogenase reductase by quantitative reverse transcription-PCR (qRT-PCR; see Table S1 in the supplemental material for the primers used) using SYBR *Premix Ex Taq* II (Perfect Real Time; Takara Bio, Shiga, Japan) and a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). We observed drastic upregulation of the *nifH* gene under the N⁻ condition (Fig. 1C). The results indicate that cells of CcI3 were completely shifted to nitrogen-fixing status at 6 dat to N⁻ medium.

SSH using eukaryotic mRNA-like RNA. SSH is a powerful tool that can be used to screen differentially expressed genes (4). SSH uses two cDNA samples—a “tester” and a “driver”—and transcripts contained only in the tester are amplified as transcripts common to both the tester and the driver are suppressed. A problem, however, is that the method was designed for eukaryotes (4). To apply SSH to a prokaryote, *Frankia*, we artificially generated eukaryotic mRNA-like RNA (eml-RNA) from total RNA. We purified mRNA from 20 µg total RNA using a MICROBExpress bacterial mRNA purification kit (Ambion, Austin, TX) according to the manufacturer's protocol, repeating the procedure twice, and polyadenylated 1 µg purified mRNA in 25 µl of a solution containing 40 U of poly(A) polymerase (USB, Cleveland, OH), 1× poly(A) polymerase reaction buffer, and 0.5 mM ATP at 37°C for 2 min. We added the three exogenous control RNAs to the polyadenylation reaction mixture (accession numbers: control 1, AB510589; control 2, AB510590; control 3, AB510588), those which were synthesized by *in vitro* transcription using T3 RNA polymerase (Stratagene, La Jolla, CA). We added two control RNAs to the tester and driver mRNAs in equal amounts (control 1 and control 2) that mimicked common transcripts. We also added a control RNA only to the tester mRNA (control 3) that mimicked a transcript expressed specifically in the tester sample. Weight ratios of control RNAs to mRNA were 0.5% for control 1 and 0.05% for control 2 and control 3. The polyadenylated mRNA was purified by phenol-chloroform extraction, chloroform extraction, and 2-propanol precipitation. We synthesized cDNA from the eml-RNA with a cDNA synthesis kit (Moloney murine leukemia virus version; Takara

* Corresponding author. Mailing address: Department of Chemistry and Bioscience, Graduate School of Science and Engineering, Kagoshima University, Korimoto 1-21-35, Kagoshima 890-0065, Japan. Phone: 81-99-285-8165. Fax: 81-99-285-8163. E-mail: kkucho@sci.kagoshima-u.ac.jp.

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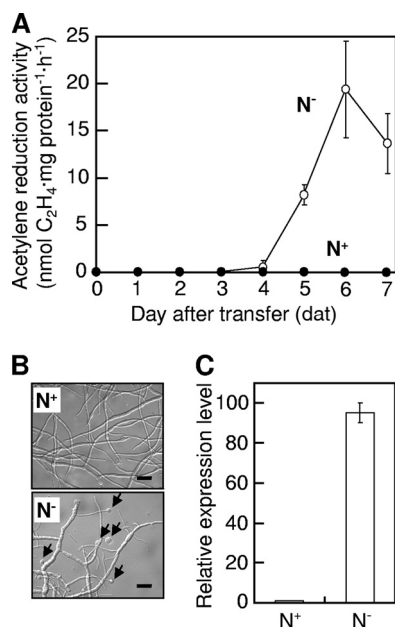


FIG. 1. Acclimation of *Frankia* sp. strain CcI3 cells to nitrogen-free conditions shown by the time course of acetylene reduction activity (A), nitrogen fixation vesicle formation (B), and *nifH* expression (C). (A) Acetylene reduction activity was measured in strain CcI3 grown in BAP medium containing 5 mM NH₄Cl (N⁺, closed circles) or without a nitrogen source (N⁻, open circles). Error bars represent standard deviations ($n = 2$). (B) Photomicrographs of the *Frankia* cells were taken at 6 dat to N⁺ (top) or N⁻ (bottom) conditions. Arrows indicate vesicles. Bars, 1 μm. (C) qRT-PCR of the *nifH* gene was performed using total RNA extracted from the cells at 6 dat to N⁺ or N⁻ conditions. Error bars represent standard deviations ($n = 3$).

Bio) by using an oligo(dT) primer. Electrophoresis of cDNA showed a smear pattern below about 3 kb (data not shown), indicating that eml-RNA was successfully generated. We amplified the cDNA by PCR prior to SSH according to the procedure described in reference 9. This step enabled us to perform SSH with only ~100 ng cDNA, which we obtained from a single synthesis reaction. We performed SSH using eml-RNA from N⁻ cells as the tester and that from N⁺ cells as the driver, essentially by following the procedure described in reference 5. To confirm successful subtraction, Southern blot analysis was performed by using the AlkPhos Direct Labeling and Detection System (GE Healthcare Bio-Sciences, Little Chalfont, United Kingdom). The signal strengths of the control 1 and control 2 cDNAs were drastically reduced in the subtracted cDNA compared with those of the tester and driver cDNAs (Fig. 2). The signal strength of control 3 cDNA, in contrast, increased in the subtracted cDNA pool (Fig. 2). Moreover, the signal strength in the endogenous *Frankia* genes (*nifH*, Francci3_0998 and Francci3_4072), which were specifically expressed in tester RNA, increased similarly (Fig. 2). These results indicate that in the subtracted cDNA pool the transcripts expressed in both the tester and driver samples were suppressed while those expressed only in the tester samples were enriched.

Identification of genes induced in nitrogen-fixing cells. Subtracted cDNA fragments were cloned into a plasmid vector. We randomly selected 96 clones from the subtracted library for

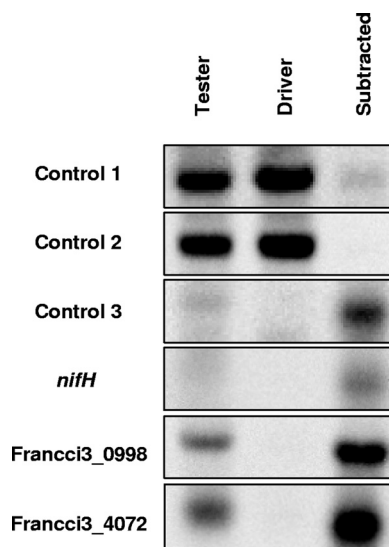


FIG. 2. Southern blot analysis probed by control cDNA fragments, *nifH*, Francci3_0998, and Francci3_4072. Tester-specific induction of the Francci3_0998 and Francci3_4072 genes was found in this study (Table 1).

sequencing and identified genes by a BLAST search (2) against the CcI3 genome (15); however, 63 clones contained the 16S or 23S rRNA gene. A mathematical model of SSH shows that abundant transcripts are prone to be detected as false positives even if they are not differentially expressed (7). Our mRNA contained a significant amount of residual rRNA even after purification (data not shown), which is probably why we identified so many rRNA gene clones. Of the remaining 33 clones, 31 contained a coding sequence of a gene (Table 1) and 2 contained an intergenic sequence. We confirmed the expression levels of the 10 genes under N⁺ and N⁻ conditions by qRT-PCR (see Table S1 in the supplemental material for primers), and 9 (90%) were indeed upregulated in N⁻ cells (Table 1), indicating the reliability of our procedure.

Genes identified by SSH contained those whose annotated function was relevant to nitrogen fixation (Table 1). Francci3_4489 showed homology to *nifV*, which encodes homocitrate synthase in other *Frankia* strains and in nitrogen-fixing cyanobacteria. Homocitrate is a component of nitrogenase FeMo cofactor (8, 10, 13). Francci3_2461 showed homology to an enzyme in *Thermobifida fusca*, nonribosomal peptide synthetase, which catalyzes the synthesis of a siderophore (6). Siderophores mediate the uptake of Fe and Mo ions, which are required by the nitrogenase complex (11). Francci3_2514 exhibited homology to a putative alcohol dehydrogenase in the rhizobium *Bradyrhizobium japonicum*, where the gene is upregulated 54.4 times in nitrogen-fixing root nodules compared with free-living cells (16). We found two genes related to energy metabolism processes such as fatty acid beta oxidation (Francci3_1000) and glycolysis (Francci3_4022), one of which was confirmed to be upregulated under the N⁻ condition by qRT-PCR. Activation of these genes is reasonable since nitrogen fixation is a highly energy-consuming reaction (17). We also found two ribosomal protein genes (Francci3_0586 and Francci3_3603) in the subtracted library, one of which was

TABLE 1. Genes identified by SSH in this study

Gene ^a (no. of clones identified)	Annotation ^b	Relative expression level ^c (N ⁻ /N ⁺ ± SD) (n = 3)
Francci3_0394 (1)	Cupin 4	ND ^d
Francci3_0586 (1)	Ribosomal protein S19	ND
Francci3_0806 (2)	Hypothetical protein	ND
Francci3_0998 (4)	Hypothetical protein	82.9 ± 10.5
Francci3_0999 (1)	Crotonyl coenzyme A reductase	ND
Francci3_1000 (1)	3-Hydroxyacyl coenzyme A dehydrogenase	11.6 ± 0.8
Francci3_1277 (1)	Hypothetical protein	ND
Francci3_1531 (1)	Hypothetical protein	ND
Francci3_1533 (1)	Phosphoenolpyruvate phosphomutase	ND
Francci3_1601 (1)	Fibronectin, type III	ND
Francci3_1761 (1)	DNA topoisomerase II	ND
Francci3_2461 (2)	Nonribosomal peptide synthetase	14.6 ± 1.1
Francci3_2465 (1)	Hypothetical protein	ND
Francci3_2514 (1)	Zinc-binding alcohol dehydrogenase	4.3 ± 0.2
Francci3_2726 (2)	Hypothetical protein	ND
Francci3_2753 (1)	Rhodanese like	121 ± 11
Francci3_2949 (1)	Catalase	0.5 ± 0.1
Francci3_3603 (1)	50S ribosomal protein L32	37.6 ± 8.2
Francci3_4022 (1)	Glyceraldehyde-3-phosphate dehydrogenase	ND
Francci3_4072 (3)	Hypothetical protein	1,536 ± 118
Francci3_4255 (1)	CarD family transcriptional regulator	3.6 ± 0.7
Francci3_4465 (1)	OsmC-like protein	ND
Francci3_4489 (1)	Homocitrate synthase (<i>nifV</i>)	452 ± 21

^a Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/fra_c/fra_c.home.html).

^b National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

^c Determined by qRT-PCR. Normalized by 16S rRNA.

^d ND, not determined.

upregulated 37.6 times in the N⁻ cells. Because the onset of nitrogen fixation requires *de novo* protein synthesis (1), upregulation of translation-related genes would be necessary. In addition, we identified seven hypothetical protein genes with no predicted function. Notably, upregulation of Francci3_4072 was drastic (1,536 times). A homologue of the gene was found only in *Frankia alni* strain ACN14a, suggesting that the gene plays a unique role in *Frankia* nitrogen fixation.

Upregulation of those genes was a novel finding in *Frankia*; none of them were found in the previous proteome analysis (1). For a better understanding of nitrogen fixation, the function of the genes we identified here should be analyzed by disruption by the transformation method currently being developed in our laboratory (12).

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