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Characterization of nitrate and nitrite utilization system in *Rhodococcus jostii* RHA1

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A polychlorinated-biphenyl degrader, *Rhodococcus jostii* RHA1, has the potential to be used in soil for the remediation of environmental contamination. It has been found that RHA1 genes, ro06365 (*narK*) and ro06366, encoding a nitrate/nitrite transporter and nitrite reductase, respectively, were highly upregulated during the growth in sterile soil. In this study, these genes and ro00862, a paralog of ro06366 were characterized to reveal the nitrate and nitrite utilization systems of RHA1. The transcriptional induction of ro06366 (*nirB1*) and ro00862 (*nirB2*) by either nitrate or nitrite was revealed by qRT-PCR. Deletion mutants for each gene exhibited retarded growth on either nitrate or nitrite as a sole nitrogen source. Furthermore, their double mutant, Dnit, grew on and consumed neither nitrate nor nitrite as a sole nitrogen source, suggesting that both *nirB1* and *nirB2* are involved in the utilization of nitrite and nitrate. A *narK* mutant, DnarK, exhibited no growth on nitrate and retarded growth on nitrite as the sole nitrogen source. DnarK showed no consumption of nitrate and reduced consumption of nitrite, suggesting that *narK* is essential for nitrate uptake and is partially involved in nitrite uptake. The induced transcription of *nirB1*, *nirB2*, and *narK* was repressed in the presence of 3 mM ammonium or more. The upregulation of *nirB1* and *narK* in sterilized soil containing ammonium and nitrate suggests that the ammonium concentration of the sterilized soil is equivalent to less than 3 mM. The unique nitrogen metabolism system of RHA1 and its importance for the growth in soil are discussed.

[**Key words:** *Rhodococcus*; Nitrate utilization; Nitrite reductase; Ammonium repression; Actinobacterium]

A strong polychlorinated-biphenyl-degrading actinomycete, *Rhodococcus jostii* RHA1, which was isolated from γ-hexachlorocyclohexane-contaminated soil, degrades a broad range of polychlorinated biphenyls through co-metabolism with biphenyl and utilizes a wide range of aromatic compounds, carbohydrates, and steroids as the sole carbon source (1–7). Hence, RHA1 can be a promising bacterium for the bioremediation of contaminated environments, including soil. DNA microarray analysis performed to address the important genetic traits for its growth in soil revealed 165 soil-specific genes that were upregulated during growth of RHA1 in sterilized soil (8). Among these 165 genes, a gene cluster containing ro06365–ro06368 was highly upregulated in sterilized soil. The results of RT-PCR analysis using RHA1 cells grown in the presence of nitrite or nitrate indicate that the genes ro06365–ro06369 constitute an operon as illustrated in Fig. 1A, and are transcriptionally induced specifically in the presence of nitrite or nitrate. The ro06365, ro06366, ro06367, ro06368, and ro06369 genes are annotated as nitrite/nitrate transporter, nitrite reductase [NAD(P)H] large subunit, nitrite reductase [NAD(P)H] small subunit, possible transcriptional regulator, and hypothetical protein genes, respectively. The inactivation of this gene cluster suggested that it is indispensable for the growth in soil. Furthermore, the gene inactivation of ro06366, which was annotated as a nitrite reductase, indicated the partial involvement of ro06366 in growth in soil.

Because RHA1 cannot grow anaerobically by nitrate respiration and utilizes both nitrate and nitrite as the sole nitrogen sources, this gene cluster is estimated to play a key role in the utilization of nitrate and nitrite. Nitrate can serve as a sole nitrogen source for plants, many fungi, and certain bacteria, and the genetics of nitrate assimilation have been extensively studied in both plants and fungi. In contrast, nitrate assimilation in prokaryotes is less well studied. Nitrate is reduced through nitrite to ammonium, which is integrated into central nitrogen metabolism, and nitrate utilization involves nitrate uptake into the cell, the reduction of nitrate to nitrite by nitrate reductase, and the reduction of nitrite to ammonium by nitrite reductase (9). Although most rhodococcal strains are able to reduce and utilize nitrate, many of them do not use nitrite as a sole nitrogen source, which is likely due to the absence of a nitrite transporter (10).

In this study, nitrite reductase genes, including ro06366 and the nitrate/nitrite transporter gene ro06365 (*narK*) were characterized in detail to reveal the nitrate/nitrite utilization system in the strain RHA1.

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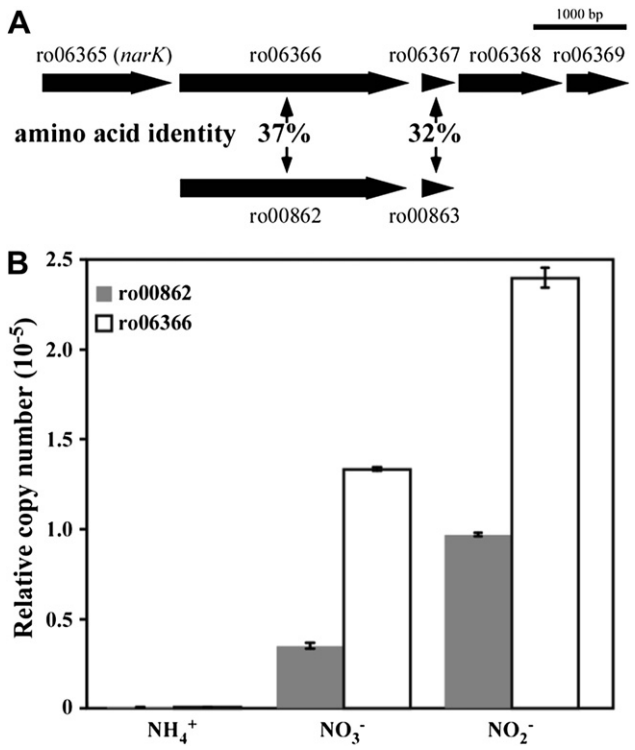

FIG. 1. (A) Gene organizations of ro06365 (*narK*) to ro06369, ro00862, and ro00863. ro06365–ro06369 are annotated as nitrite/nitrate transporter, nitrite reductase [NAD(P)H] large subunit, nitrite reductase [NAD(P)H] small subunit, possible transcriptional regulator, and hypothetical protein genes, respectively. ro00862 and ro00863 are annotated as the large and small subunit genes of nitrite reductase [NAD(P)H], respectively. (B) qRT-PCR analysis of ro00862 (gray bars) and ro06366 (open bars) in minimal salt medium containing different nitrogen sources. Total RNA was isolated from the RHA1 cells grown on ammonium, nitrate or nitrite as a sole nitrogen source using biphenyl as a sole carbon source. Each relative copy number is estimated by dividing the copy number of the target gene transcript by that of the 16S rRNA gene transcript in the same sample. The data are mean values ± standard deviations from three independent experiments.

MATERIALS AND METHODS

Bacterial strains and culture conditions *R. jostii* strain RHA1 and its mutant derivatives were grown at 30°C in one-fifth diluted LB (1/5 LB) or in W minimal salt medium (11). For solid medium, 15 g/L of agar was added. Biphenyl was used as a sole carbon source and supplied as a vapor during the growth on plates. To examine the assimilation of nitrogen sources, RHA1 cells grown in 50 ml of 1/5 LB for 48 h were washed twice with 50 ml of W minimal salt medium without ammonium sulfate and inoculated at an OD_{600} of 0.2 in 100 ml of W minimal salt medium containing 10 mM biphenyl and 1 mM potassium nitrate or 1 mM potassium nitrite as a sole nitrogen source. Reproducible results were obtained using not 10 mM but 1 mM potassium nitrite and 1 mM potassium nitrate.

DNA manipulations The DNA manipulations were performed essentially as described by Ausubel et al. (12) and Sambrook et al. (13). Gene deletion mutants were constructed by allelic exchange using *sacB* counter-selection and confirmed by Southern hybridization using the DIG system (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), according to the manufacturer's protocol, and nucleotide sequencing (14). The nucleotide sequences were determined using the dideoxy termination method with a CEQ 2000XL genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, USA). A Sanger reaction was performed using a CEQ dye terminator cycle sequencing quick start kit (Beckman Coulter, Inc.) (15). Homology searches were performed using Swiss-Prot/TrEMBL with the BLAST program and genomic BLAST. DNA fragments were recovered from agarose gels using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA).

RNA isolation RNA was isolated from the cultured cells as described by Gonçalves et al. (16). To preserve the cells prior to RNA isolation, 1/10 volume of 10% acid phenol (pH 5.0) in ethanol was added to the cultures. The cells were collected by centrifugation at 10,000 ×*g* for 10 min at 4°C, suspended in 1.0 ml of ice-cold Tris–EDTA buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5) plus 2.0 ml RNAprotect bacteria reagent (Qiagen), and incubated for 5 min at room temperature. The cells were then recovered by centrifugation at 13,000 ×*g* for 2 min at room temperature, frozen, and stored at −80°C. Total RNA was isolated by vortexing with glass beads, treatment with hot phenol plus sodium dodecyl sulfate, removal of the debris precipitated with acetate, phenol–chloroform extraction, isopropanol precipitation, DNase treatment, and purification using an RNeasy mini column (Qiagen). The total RNA was stored at −80°C until use.

cDNA synthesis and qRT-PCR Total RNA (6.0 μg) was reverse transcribed using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) with 6-mer random primer (Takara Bio Inc., Otsu, Japan). The synthesized cDNA was subjected to phenol–chloroform extraction and ethanol precipitation, and it was dissolved in 20 μl DNase–free water.

Specific primer pairs for the qRT-PCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 1). The qRT-PCR was performed in a 20-μl reaction mixture containing 1 μl cDNA (0.04 μg), 4 pmol specific primers, and 10 μl Fast SYBR green master mix (Applied Biosystems). The following thermal cycling conditions were used: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A melting-curve analysis was performed at the end of the real-time PCR to verify specific amplification. The 16S rRNA gene was used to normalize the amount of RNA in each sample. Each measurement was achieved in triplicate.

Construction of deletion mutants and complementation plasmids D862, D6366 (8), and DnarK, which have the in-frame deletions of ro00862, ro06366, and *narK* (ro06365) in RHA1, respectively, were constructed by allelic exchange using *sacB* counter-selection, and confirmed by Southern hybridization and nucleotide sequencing. The upstream and downstream fragments of each target gene were cloned into the pK18*mobsacB* to perform allelic exchange. D6366 was used to construct a double mutant, Dnit with the in-frame deletions of ro00862 and ro06366. DNop, which have been described in the previous report (8), has the in-frame deletion of ro06365–ro06368.

For complementation study, the regions containing the ro06365 gene and ro06366–ro06367 were amplified from RHA1 genomic DNA by PCR using the respective primer sets DnarK-com-F plus DnarK-com-R and 66–67-com-F plus 66–67-com-R (Table 1). The amplified fragments of the ro06365 gene and ro06366–ro06367 were independently cloned into pK4 (17) and pFAJ2574 (18), and were designated as pKnarK and pFAJ66–67, respectively, after confirming their nucleotide sequences.

Measurement of nitrogen concentration in liquid medium The nitrogen concentration in the liquid culture was measured using the supernatant of 3-ml aliquots of a 100-ml culture obtained after centrifugation at 10,000 ×*g* for 10 min. The ammonium, nitrate, and nitrite ions in the supernatant were quantified using an RQflex reflectometer (Merck, Darmstadt, Germany).

RESULTS

Transcriptional induction of nitrite reductase genes The ro00862 and ro00863 genes were found to be the paralogs of ro06366 and ro06367, which were annotated as the large and small subunits of a nitrite reductase, respectively (Fig. 1A). The ro00862 and ro00863 genes showed 37% and 32% amino acid sequence identities to ro06366 and ro06367, respectively. To investigate the involvement of ro00862 and ro06366 in the metabolism of nitrate and nitrite in RHA1, quantitative reverse transcriptase real-time PCR (qRT-PCR) was performed for the ro00862 and ro06366 genes. Total RNA was prepared from RHA1 cells grown on biphenyl in minimal salt medium containing ammonium, nitrate, or nitrite as the sole nitrogen source and was subjected to

TABLE 1. Primer sets used in this study.

Target gene(s)	Primer	Sequence (in 5′ → 3′ direction)	Source or reference
Quantitative RT-PCR			
ro00862	qRT ro00862-F	CCTGATGAACCAGCAACTCG	This study
	qRT ro00862-R	GCGGTTCGTAGTACTGGACG	This study
ro06365	qRT ro06365-F	CCGCAACTGGACGGTCTT	8
	qRT ro06365-R	CGAGAATGAACGTCGTGTACGA	8
ro06366	qRT ro06366-F	GCGCAGTACCGCATGAACT	8
	qRT ro06366-R	CATTCCGTGCCCGATGAC	8
16S rRNA gene	16SF	GCAGAAGAAGCACCGGCTAA	11
	16SR	CAACGCTTGCACCCTACGTA	11
Construction of the complementation plasmids			
ro06365	DnarK-com-F	GAATTCGGGACTGTTCTCGTTCATGG	This study
	DnarK-com-R	TCTAGATCACGCCGAAACCGACACCT	This study
ro06366 and ro06367	66–67-com-F	AGATCTGCGTTCGTGTTCGGCTTC	This study
	66–67-com-R	AGATCTGCAGTGTGGCGAACTCTTC	This study