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Transcriptional regulation of the *Rhodococcus rhodochrous* J1 *nitA* gene encoding a nitrilase

(nitrile/promoter/isovaleronitrile/XylS/AraC)

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ABSTRACT **The 1.4-kb downstream region from a nitrilase gene (*nitA*) of an actinomycete *Rhodococcus rhodochrous* J1, which is industrially in use, was found to be required for the isovaleronitrile-dependent induction of nitrilase synthesis in experiments using a *Rhodococcus–Escherichia coli* shuttle vector pK4 in a *Rhodococcus* strain. Sequence analysis of the 1.4-kb region revealed the existence of an open reading frame (*nitR*) of 957 bp, which would encode a protein with a molecular mass of 35,100. Deletion of the central and 3′-terminal portion of *nitR* resulted in the complete loss of nitrilase activity, demonstrating that *nitR* codes for a transcriptional positive regulator in *nitA* expression. The deduced amino acid sequence of *nitR* showed similarity to a positive regulator family including XylS from *Pseudomonas putida* and AraC from *E. coli*. By Northern blot analysis, the 1.4-kb transcripts for *nitA* were detected in *R. rhodochrous* J1 cells cultured in the presence of isovaleronitrile, but not those cultured in the absence of isovaleronitrile. The transcriptional start site for *nitA* was mapped to a C residue located 26 bp upstream of its translational start site. Deletion analysis to define the *nitA* promoter region suggested the possible participation of an inverted repeat sequence, centered on base pair −52, in induction of *nitA* transcription.**

Nitriles, which are generally highly toxic due to their cyano functional group, can be used by some microorganisms as carbon and/or nitrogen sources. Nitrilase catalyzes the direct cleavage of nitriles to the corresponding acids and ammonia (1), whereas nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides (2, 3). Both enzymes are involved in biosynthesis of the plant hormone indole-3-acetic acid (IAA) in plants (4–6) and plant-associated microorganisms (7, 8). Recently, four cDNAs encoding nitrilases that convert indole-3-acetonitrile to IAA have been cloned and characterized from *Arabidopsis thaliana* (4–6). Nitrilase and NHase are also expected to be useful biocatalysts in organic chemical processing (3), because the environmentally friendly bioconversion may provide clean and mild syntheses with high selectivity and yield, which are not easily attained by chemical methods.

Nitriles (e.g., acetonitrile, adiponitrile, and acrylonitrile) are widely manufactured and extensively used by the chemical industry, and nitrile herbicides are also widely applied in agriculture. The release of these artificial nitriles into our environment as industrial waste water and excess agricultural chemicals has threatened the environment; nitrile is an important target in terms of environmental preservation. Stalker *et al.* (9) have cloned the bacterial *Klebsiella ozaenae* gene encoding nitrilase, which degrades a herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile).

Using protein and genetic studies, we have characterized three microbial nitrilases from *Rhodococcus rhodochrous* J1 (10, 11), *R. rhodochrous* K22 (12, 13), and *Alcaligenes faecalis* JM3 (14, 15), which act on aromatic nitriles, aliphatic nitriles, and arylacetonitriles, respectively. These three nitrilases are all strongly induced by the addition of isovaleronitrile to the medium (10, 12, 14); particularly, the induced nitrilase in *R. rhodochrous* J1 corresponds to 35% of all soluble protein, suggesting the existence of a strong promoter (1). This strain also exhibits diverse nitrile metabolism, and it is a "practical fascinating microorganism." *R. rhodochrous* J1 selectively produces not only nitrilase but also two kinds of NHases (H- and L-NHases) depending on each inducer; industrial production of acrylamide from acrylonitrile has been performed using H-NHase (3), and manufacture of a vitamin nicotinamide from 3-cyanopyridine using L-NHase is due to start in next year.

Whereas nitrilase has versatile functions and has received increasingly broad interest in fundamental and applied fields (i.e., IAA biosynthesis, biotransformation, and bioremediation), the mechanisms that regulate nitrilase expression have never been reported in both prokaryotes and eukaryotes. We describe here characterization of the promoter and regulation of the nitrilase gene (*nitA*) in *R. rhodochrous* J1, which exhibits interesting physiological responses in nitrile metabolism.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *R. rhodochrous* J1 was previously isolated from soil (16). *E. coli* JM109 (17) was the host for pUC plasmids. *R. rhodochrous* ATCC12674 was the host for a *Rhodococcus–E. coli* shuttle vector plasmid pK4 (18) and its derivatives, and was used for the expression of the nitrilase gene (*nitA*). *R. rhodochrous* ATCC12674 and the plasmid pK4 were kindly provided by T. Beppu's group (University of Tokyo). The plasmid pNJ10 (11) carrying *nitA* in a 5.4-kb *Pst*I fragment on pUC19 was used for subcloning and sequencing of genes. *E. coli* transformants were grown in Luria–Bertani medium (17). *R. rhodochrous* ATCC12674 transformants were grown in MYP medium (18).

Transformation of *R. rhodochrous* ATCC12674 by Electroporation. DNA manipulation was performed essentially as described by Sambrook *et al.* (17). A mid-exponential culture of *R. rhodochrous* ATCC12674 was centrifuged at 6500 × *g* for 10 min at 4°C and washed three times with demineralized cold water. Cells were then concentrated 20-fold in demineralized cold water and kept on ice. Ice-cold cells (100 μl) were mixed with 1 μg of DNA in 1 μl of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) in a 1-mm gapped electrocuvette (Bio-Rad), and subjected to a 2.0-kV electric pulse from a Gene Pulser (Bio-Rad) connected to a pulse controller (25 μF capacitor;

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Abbreviations: NHase, nitrile hydratase; *nitA*, nitrilase gene; *nitR*, nitrilase regulator gene; ORF, open reading frame.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. D67026).

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external resistance, 400 Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (18) and incubated for 2 h at 26°C. They were then spread on MYP medium containing 75 μg of kanamycin/ml.

Preparation of Cell Extracts and Enzyme Assay. *R. rhodochrous* ATCC12674 transformants were cultured at 28°C for 24 h in MYP medium in the presence or absence of isovaleronitrile (0.1%, vol/vol), harvested by centrifugation at 4000 × *g* at 4°C, and washed with 10 mM potassium phosphate buffer (pH 7.5). The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and 20% (vol/vol) glycerol, disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 × *g* for 10 min at 4°C. The resulting supernatant was assayed for nitrilase by use of benzonitrile as a substrate as described (11). One unit of nitrilase catalyzes the formation of 1 μmol of benzoic acid per min under the above conditions. The protein was determined according to Bradford (19). The specific activity is expressed as units/mg of protein.

RNA Preparation. *R. rhodochrous* J1 collected from an agar slant was inoculated into a test tube containing 5 ml of a medium consisting of 5 g Polypepton (Daigo, Osaka), 5 g meat extract (Mikuni, Tokyo), 0.5 g yeast extract (Oriental Yeast, Tokyo), and 2 g NaCl/liter of tap water (pH 7.0), and incubated at 28°C for 24 h with reciprocal shaking. From this, 1.6 ml was inoculated into a 500-ml shaking flask containing 100 ml of a medium (10 g glycerol/5 g Polypepton/3 g malt extract/3 g yeast extract/liter of tap water, pH 7.2) with or without isovaleronitrile (0.1%, wt/vol), and incubated at 28°C for 48 h with aeration. Cells were collected from 60 ml of such cultures by centrifugation, and total RNA was extracted by the AGPC (acid–guanidium–phenol–chloroform) method (20).

Northern (RNA) Blot Hybridization. For Northern blot hybridization, each RNA sample (30 μg) extracted from *R. rhodochrous* J1 cells as described above was subjected to electrophoresis on a 1% agarose–formaldehyde gel, and transferred to a nitrocellulose membrane filter (Schleicher & Schuell) in 20× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0). Prehybridization and hybridization were carried out at 42°C in a solution consisting of 40% (vol/vol) formamide, 5× SSC, 0.1% (wt/vol) SDS, and 100 μg/ml of sonicated salmon sperm DNA. The DNA fragments used as probes were radiolabeled with a multiprime DNA labeling system (Amersham). Filters were washed twice at room temperature in 40% formamide, 5× SSC, 0.1% SDS, and then washed three times at room temperature in 2× SSC solution with 0.1% SDS.

Primer Extension Analysis. The primer 5′-GAATGTGTTTGTGTATTCGACCATG-3′, complementary to positions 620–644 (see Fig. 3), was synthesized and then labeled with [^{32}P] at the 5′ end by polynucleotide kinase. Reverse transcriptase-mediated primer extension was performed by the method of Sambrook *et al.* (17).

RESULTS

Expression of *nitA* in *R. rhodochrous* ATCC12674. To identify the sequence element required for the expression of *R. rhodochrous* J1 *nitA*, we constructed a set of plasmids containing sub-fragments of a 5.4-kb *Pst*I fragment from pNJ10 (11) inserted at the *Pst*I site of the *Rhodococcus–E. coli* shuttle vector pK4 (Fig. 1). These plasmids were used to transform *R. rhodochrous* ATCC12674 and the resulting transformants were cultured in MYP medium (18) with or without isovaleronitrile (0.1%, vol/vol). Enzyme assays using benzonitrile as a substrate for each cell suspension (Fig. 1) or cell-free extract (Table 1) revealed that, in addition to *nitA* itself, at least a 0.6-kb upstream region (from the *Nhe*I site to the 5′-end terminus of *nitA*) and a 1.4-kb downstream region (from the 3′-end terminus of *nitA* to the *Eco*T22I site) are required for the appearance of nitrilase activity, as in pYHJ40. As we had previously found in *R. rhodochrous* J1 (10, 21), the presence of isovaleronitrile in the culture medium showed salient enhancement of nitrilase activity in the *Rhodococcus–E. coli* host–vector system used in this experiment. The transformant harboring pYHJ20 exhibited the highest activity [0.537 μmol·min^{-1}·(mg protein)$^{-1}$].

Nitrilase formation in the transformants was examined by SDS/PAGE (Fig. 2). The transformants carrying pYHJ10, pYHJ20, or pYHJ40 expressed larger amounts of a protein of 41.5 kDa, in the presence of isovaleronitrile, than the transformants carrying pK4, pYHJ30, or pYHJ50. The protein, which corresponded to about 4% of all soluble protein in the supernatant of cell-free extracts of the pYHJ20-carrying transformant cultured in the presence of isovaleronitrile, were found to be the nitrilase encoded by *nitA* by its N-terminal amino acid sequence on a gas-phase amino acid sequencer (Applied Biosystems 470A) (Fig. 3). When the pK4-derivative plasmids used in this experiment were introduced into *E. coli* JM109, nitrilase activity could not be detected, even after

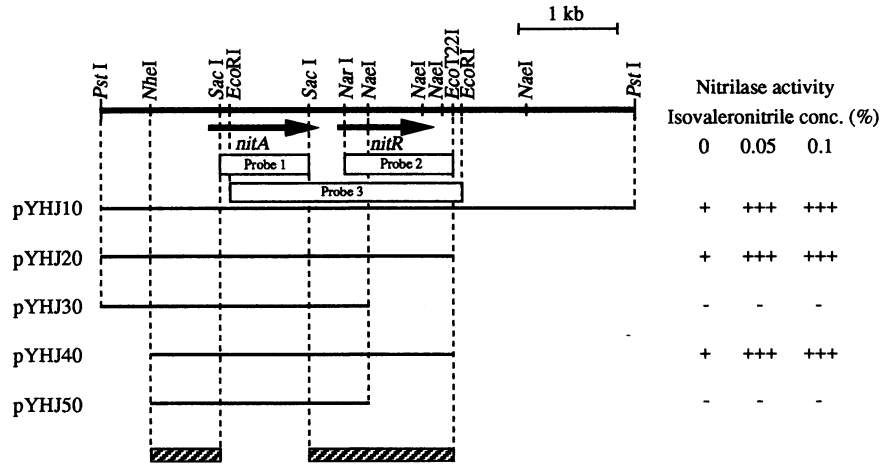

FIG. 1. Construction of a set of plasmids (*Left*) and nitrilase activity of each *R. rhodochrous* ATCC12674 transformant (*Right*). (*Left*) For clarity, only restriction sites discussed in the text are shown. Various deletion plasmids are diagrammed below the restriction map. Plasmids (pYHJ10~50) were constructed by inserting each restriction fragment from pNJ10 into the *Pst*I site or a *Pst*I site that had been filled-in with T4 DNA polymerase of pK4. In the case of pYHJ40 and pYHJ50, the *Nhe*I sites of their inserted fragments were also filled-in with T4 DNA polymerase during the construction. The region sequenced this time is indicated by shaded boxes. The probes used in the Northern blot analysis are shown by open boxes. (*Right*) nitrilase activity of whole cells was detected as described using benzonitrile as a substrate. +++, much; +, trace; −, not detected.

Table 1. Nitrilase activity in cell-free extracts of *R. rhodochrous* ATTCC12674 transformants containing various plasmids

Plasmid	Isovaleronitrile	Specific activity, units/mg
pK4	−	ND
	+	ND
pYHJ10	−	0.003
	+	0.193
pYHJ20	−	0.019
	+	0.537
pYHJ30	−	ND
	+	ND
pYHJ40	−	0.002
	+	0.297
pYHJ50	−	ND
	+	ND

ND, not detected.

culture in the presence of isovaleronitrile (data not shown), suggesting that an *E. coli* RNA polymerase could not recognize the promoter of *nitA* from *R. rhodochrous* J1.

Primary Structure of the Flanking Region of *nitA*. The *Bsm*I–*Pvu*I 1.29-kb fragment containing *nitA* was sequenced previously (11). We further sequenced the *Nhe*I–*Bsm*I upstream region and the *Pvu*I–*Eco*T22I downstream region required for nitrilase production (Fig. 3).

An open reading frame (ORF) (start and stop codons at nucleotides 1970 ATG and 2927 TAG, respectively) downstream of *nitA* was 957 nucleotides long, and encoded a protein of 319 amino acids (35.1 kDa). The ORF was designated *nitR*. The *nitR* gene product (NitR) is significantly similar in amino acid sequence to the positive regulator XylS of a xylene-metabolism in *Pseudomonas putida* (22) and to AraC, the positive regulator of arabinose-metabolism in *E. coli* (23): 20.3% identity of amino acids in 310 overlapping residues between NitR and XylS; 17.1% identity in 282 residues between NitR and AraC (Fig. 4). The similarities were confined to the carboxyl termini of the proteins, where a DNA binding motif (helix–turn–helix) is present in the XylS/AraC family (24). The algorithm of Garnier *et al.* (25) for the corresponding region of NitR revealed that it would be organized as a helix–turn–helix motif (data not shown). The similarity of NitR to XylS and AraC, and the importance of *nitR* for

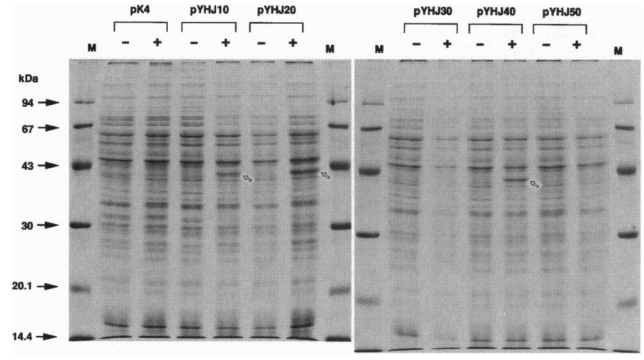

FIG. 2. Coomassie stained SDS/PAGE showing nitrilase formation in *R. rhodochrous* ATCC12674 transformants. Lanes M were loaded with the following molecular mass standards: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Lanes: +, 100 μg of cell-free extracts from *R. rhodochrous* ATCC12674 transformants cultured in the medium supplemented with (0.1%, vol/vol) isovaleronitrile; −, 100 μg of cell-free extracts from *R. rhodochrous* ATCC12674 transformants cultured in the absence of isovaleronitrile. Open arrows indicate the band corresponding to the nitrilase.

nitrilase production, provided strong evidence that NitR would be a positive regulator of *nitA* expression.

Northern Blot Hybridization. The DNA fragments shown in Fig. 1 were used as probes against total RNA from *R. rhodochrous* J1 cultured in the medium in the presence or absence of isovaleronitrile, to determine whether transcription of *nitA* and *nitR* was altered by the culture conditions. Northern blots were probed with labeled fragments (Fig. 1) specific for *nitA* (probe 1), *nitR* (probe 2), or *nitA* plus *nitR* (probe 3). Probes 1 and 3 both hybridized to a 1.4-kb band corresponding to *nitA* and less intensely to bands that lie between 2 and 2.5 kb. Probe 2 hybridized to a band at about 2 kb. These findings were obtained only in induced cultures, indicating that nitrilase formation would be regulated at the transcriptional level (Fig. 5). Considering the presence of a putative rho-independent transcriptional terminator ($\Delta G = -44.6$ kcal/mol) in the noncoding region between *nitA* and *nitR* (see Fig. 3) together with the above findings, it is possible that *nitR* is transcribed by low-level transcriptional read-through from the *nitA* promoter. The divergent transcription of the regulatory and cognate structural genes observed for most members of the *xylS/araC* family (24) was not found for *nitR*.

Mapping of *nitA* Transcript. Because nitrilase is produced at high levels in *R. rhodochrous* J1 (10) and in the appropriate *R. rhodochrous* ATCC12674 transformants after induction (Fig. 2), a strong promoter containing a binding site specific for NitR was expected to be present close to *nitA*. To map the initiation site of *nitA* transcription, we used primer extension analysis with total RNA from *R. rhodochrous* J1 grown in the medium in the presence or absence of isovaleronitrile. A single site of initiation of transcription was identified at nucleotide position 595, only in RNA from the induced culture (Fig. 6). This nucleotide is 26 bp upstream from the ATG initiation codon of *nitA* (Fig. 3). Around 35 bp upstream of the transcriptional start site, possible −35 (TTCATG) and −10 (TACTGT) sequences have been found, which were similar to those of *casA*, a cellulase gene from *Streptomyces* sp. [(TTCACC) for −35 and (TACCGT) for −10] (26).

We tried to do the primer extension analysis using oligonucleotide primers complementary to the 5′-terminal region of *nitR* or its neighboring regions. However, we could not detect the extension products irrespective of the presence of isovaleronitrile (data not shown). This suggests that *nitR* would be transcribed from *nitA* promoter and not from its own promoter, consistent with the findings shown by the Northern blot analysis.

Truncation of the Upstream Sequence of *nitA*. To define more precisely the region of the promoter essential for the isovaleronitrile-inducible expression of *nitA*, deletions were introduced in the upstream region of *nitA*. The *Nhe*I–*Eco*T22I fragment (equal to the inserted fragment of pYHJ40) from pNJ10 was inserted into the *Eco*RV–*Pst*I sites of pBluescript SK(+) (Toyobo, Osaka) after the *Nhe*I end was filled-in with T4 DNA polymerase. The resulting plasmid pNITUP was used to generate deletions from the *Nhe*I/*Eco*RV site. The area deleted from the insert was determined by restriction endonuclease mapping and subsequent DNA sequence analysis of the deletion mutants, as shown in Fig. 3. Each shortened fragment was ligated into pK4, and the resulting plasmids (pYHJ101~pYHJ107) were used to transform *R. rhodochrous* ATCC12674. The transformants were assayed for the ability to produce nitrilase with or without isovaleronitrile using MYP medium. In *R. rhodochrous* ATCC12674, the smallest insert conferring nitrilase activity was pYHJ105 containing an 89-bp sequence upstream from the transcriptional start site. Transformants harboring pYHJ106 containing a 47-bp upstream sequence of the transcriptional start site or pYHJ107 containing a 22-bp upstream sequence of the translational start codon, showed no nitrilase activity (Table 2). An inverted repeat sequence centered at −52 was entirely contained in pYHJ105,

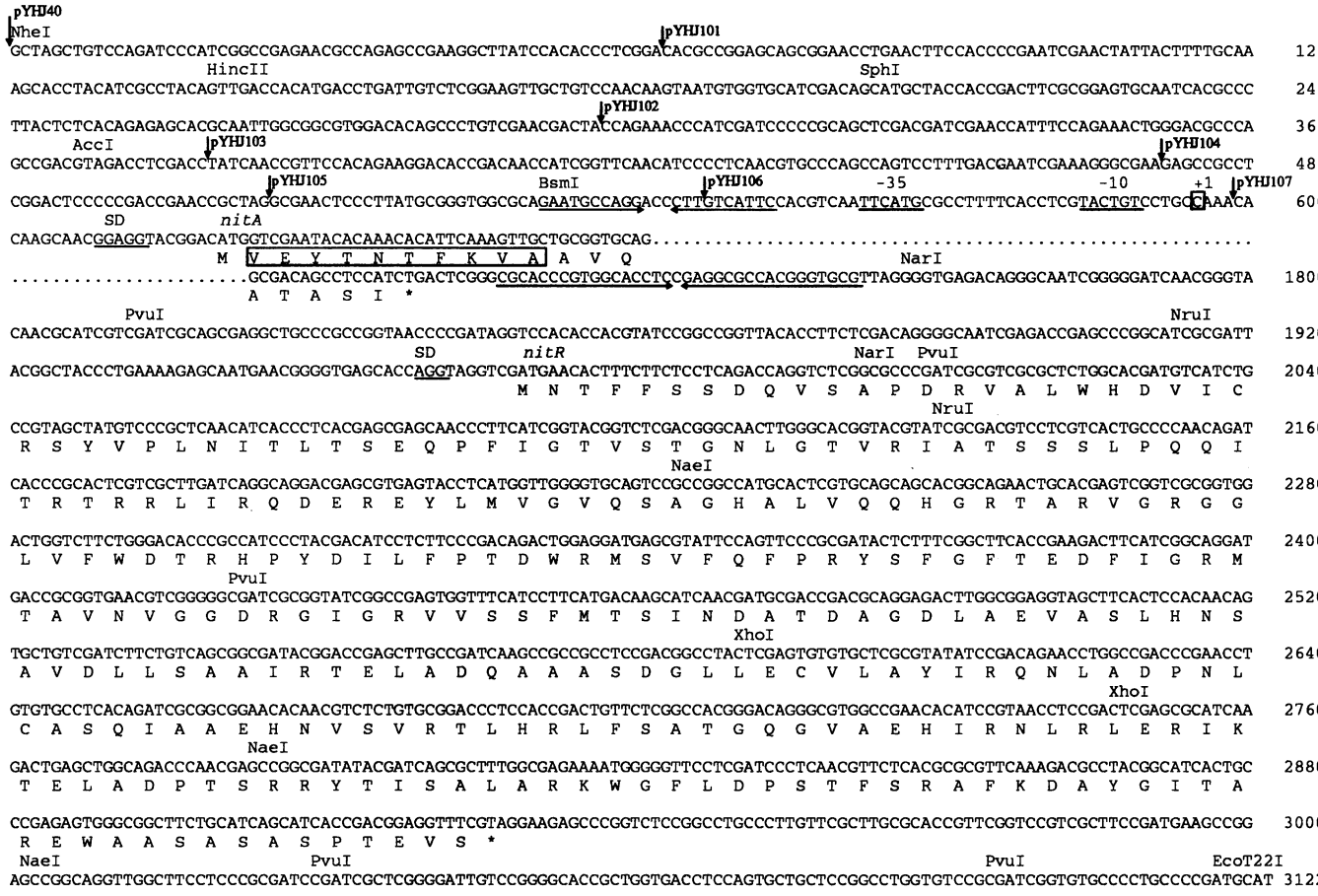

FIG. 3. Nucleotide sequence of the flanking region of *nitA* from *R. rhodochrous* J1. The deduced amino acid sequences using standard one-letter amino acid abbreviations are shown below their respective nucleotide sequences. Boxed amino acid residues indicate the N-terminal sequence of nitrilase determined by Edman degradation. Shine–Dalgarno sequences are underlined. The stop codons are indicated by asterisks. The cytidine residue in a box is the transcriptional start site of *nitA* determined by primer extension analysis. The *nitA* sequence (denoted with gaps) was published earlier (ref. 11, GenBank accession no. D11425). Endpoints of deletions introduced from upstream are marked by vertical arrows. Inverted repeats are shown by converging arrows.

but all of the left-hand half of the repeat and 3 bp of the right-hand half were missing in pYHJ106, suggesting that the inverted repeat could possibly serve as a binding site for NitR.

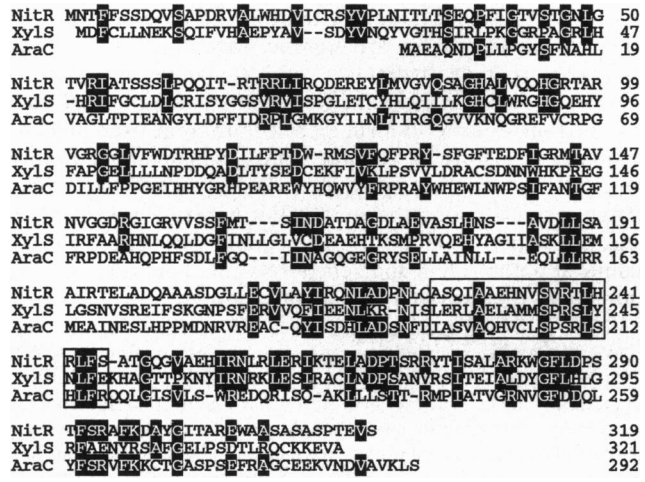

FIG. 4. Comparison of amino acid sequences of NitR from *R. rhodochrous* J1 and homologous proteins. Amino acid sequences of NitR from *R. rhodochrous* J1, XylS from *P. putida* (22), and AraC from *E. coli* (23) were aligned by introducing gaps (hyphens) to achieve maximum homology. Residues in black boxes indicate identical sequences. The helix–turn–helix motif (amino acids 226–245 of NitR) is enclosed by boxes.

DISCUSSION

The genus *Rhodococcus* has recently received much attention in terms of its high ability on biodegradation and biotransformation (27); but its genetic information has been extremely limited, whereas *Rhodococcus* is registered as a member of actinomycetes (28). Here, a *Rhodococcus* host-vector system was used to examine the mechanism involved in the control of *R. rhodochrous* J1 *nitA* expression, using as host *R. rhodochrous* ATCC12674, which exhibited no detectable nitrilase activity (Fig. 1, Table 1). We demonstrated that the transcription of *nitA* is regulated in response to isovaleronitrile added to the culture medium both in the original *nitA*-containing *R. rhodochrous* J1 and in the appropriate transformants of *R. rhodochrous* ATCC12674. Evidence is also presented for the existence of the regulatory gene, *nitR*, the product of which (NitR) is required for the isovaleronitrile-dependent induction of *nitA*. NitR is related to bacterial transcriptional regulators belonging to the XylS/AraC family, although sequence similarity is not so high. This family, members of which are positive regulators involved in metabolism of carbon sources and in pathogenesis, is characterized by the sequence similarity within the carboxyl terminus, which is the region containing a helix–turn–helix DNA binding motif (24, 29). Within this family, for regulators recognizing chemical signals (inducers), the nonconserved N-terminal region is presumed to be responsible for binding the activator molecule. Deletion of the central and 3′-terminal region of *nitR* resulted in the complete loss of *nitA*-encoded nitrilase activity. We have previously reported

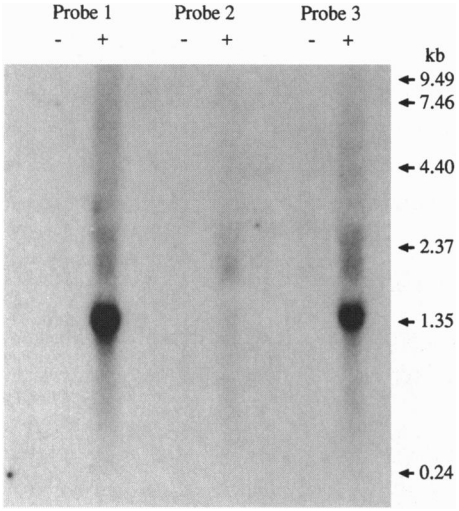

FIG. 5. Northern blot analysis of *R. rhodochrous* J1. An RNA ladder (Bethesda Research Laboratories) was used as a size marker. The probes used in the experiment are shown in Fig. 1. Lanes: +, 30 μg of RNA extracted from *R. rhodochrous* J1 cultured in the medium supplemented with (0.1%, vol/vol) isovaleronitrile; −, 30 μg of RNA extracted from *R. rhodochrous* J1 cultured in the absence of isovaleronitrile.

that *nitA* modified in the sequence upstream of the ATG start codon is expressed under the control of the *lac* promoter to ≈50% of the total soluble protein in *E. coli* JM105, even when *nitR* is absent in the downstream region of *nitA* (11). These findings indicate that *nitR* functions as a transcriptional activator for the formation of NitA. This positive regulatory system by the XylS/AraC family is the first, to our knowledge, reported for an actinomycete.

We describe here the mapping of a transcript in *Rhodococcus* species for the first time to the best of our knowledge.

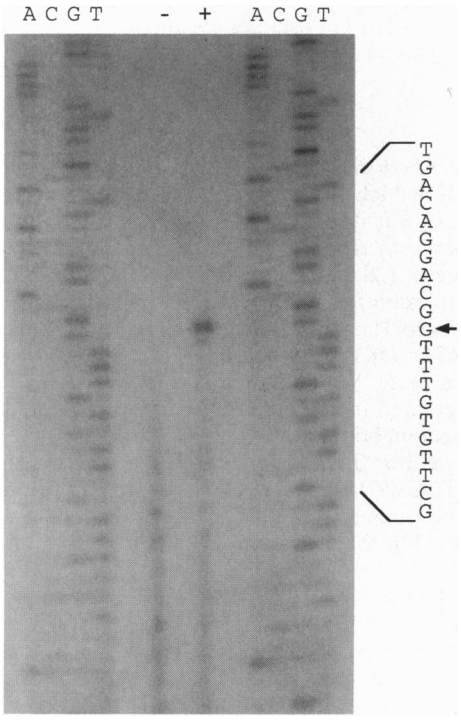

FIG. 6. Mapping of the 5′ end of the *nitA* transcript. Primer extension analysis using total RNA isolated from *R. rhodochrous* J1 cultured in the presence (lane 2) or absence (lane 1) of isovaleronitrile was carried out. Primer extended products were electrophoresed in parallel with sequence ladders generated with the same primer. The position of the transcription start site is marked by an arrow.

Table 2. Nitrilase activity in cell-free extracts of *R. rhodochrous* ATCC12674 transformants containing plasmids in which the upstream sequence of *nitA* was deleted

Plasmid	Isovaleronitrile	Specific activity, units/mg
pYHJ40	−	0.002
	+	0.297
pYHJ101	−	0.008
	+	0.425
pYHJ102	−	0.009
	+	0.433
pYHJ103	−	0.004
	+	0.514
pYHJ104	−	0.009
	+	0.530
pYHJ105	−	0.005
	+	0.476
pYHJ106	−	ND
	+	ND
pYHJ107	−	ND
	+	ND

ND, not detected.

Because the amount of nitrilase produced by *R. rhodochrous* J1 after induction can reach 35% of all soluble protein, *nitA* is expected to have a strong promoter. The −35 and −10 regions for the transcriptional start site of *nitA* were selected by their sequence similarity to the actinomycete, mainly the streptomycete consensus promoter sequence, TTGAC(A/G)-17 bp-TAg(A/G)(A/G)T (30). However, the relatively short (15 bp) distance between these hexamers in *R. rhodochrous* J1 may imply that the putative −35 region does not play an important role, a situation not uncommon among promoters dependent on additional transcriptional activator including those dependent on XylS or AraC. On the other hand, the similarity of the *nitA* promoter sequence to the *E. coli* consensus is relatively low, consistent with the failure of the pK4-derivative plasmids used in this experiment to direct detectable nitrilase production in *E. coli* JM109. Deletion analysis suggested the possible participation of an inverted repeat sequence, centered on bp −52, in induction of *nitA* transcription.

A new family of carbon–nitrogen hydrolases has recently been proposed (31, 32) by several conserved motifs, one of which contains an invariant cysteine as shown in our nitrilases (11, 13, 15). Nitrilases are significantly similar to aliphatic amidases, cyanide hydratases, and β-alanine synthase, although nitrilase catalyzes the cleavage reaction of a CN triple bond (from a nitrile to form an acid) different from aliphatic amidases cleaving a CN double bond (from an amide to form an acid) and cyanide hydratases cleaving a CN triple bond (from cyanide to form the amide). Further studies on nitrilases and the other members of the carbon–nitrogen hydrolase family at both protein and gene levels could provide information about their evolutionary implication.

We would like to dedicate this manuscript to Emeritus Prof. Teruhiko Beppu, who is the pioneer of genetic studies on a nitrile-degrading enzyme (nitrile hydratase), on his retirement from the University of Tokyo; for more than 40 years he has investigated and written about various microbial enzymes and genes in ways that have captured the interest of generations of students. We are indebted to Emeritus Prof. T. Beppu's group for providing the *Rhodococcus–E. coli* host–vector system. We thank Prof. K. F. Chater (John Innes Centre) for his helpful suggestions and critical reading of this manuscript. This work was supported in part by a grant from Kato Memorial Bioscience Foundation and by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. H.K. is a research fellow of the Japan Society for the Promotion of Science.

1. Kobayashi, M. & Shimizu, S. (1994) *FEMS Microbiol. Lett.* **120,** 217–224.
2. Asano, Y., Tani, Y. & Yamada, H. (1980) *Agric. Biol. Chem.* **44,** 2251–2252.
3. Kobayashi, M., Nagasawa, T. & Yamada, H. (1992) *Trends Biotechnol.* **10,** 402–408.
4. Bartling, D., Seedorf, M., Mithöfer, A. & Weiler, E. W. (1992) *Eur. J. Biochem.* **205,** 417–424.
5. Bartling, D., Seedorf, M., Schmidt, R. C. & Weiler, E. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6021–6025.
6. Bartel, B. & Fink, G. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6649–6653.
7. Kobayashi, M., Suzuki, T., Fujita, T., Masuda, M. & Shimizu, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 714–718.
8. Yamada, T., Tsukamoto, H., Shiraishi, T., Nomura, T. & Oku, H. (1990) *Ann. Phytopathol. Soc. Jpn.* **56,** 532–540.
9. Stalker, D. M., Malyj, L. D. & MacBride, K. E. (1988) *J. Biol. Chem.* **263,** 6310–6314.
10. Kobayashi, M., Nagasawa, T. & Yamada, H. (1989) *Eur. J. Biochem.* **182,** 349–356.
11. Kobayashi, M., Komeda, H., Yanaka, N., Nagasawa, T. & Yamada, H. (1992) *J. Biol. Chem.* **267,** 20746–20751.
12. Kobayashi, M., Yanaka, N., Nagasawa, T. & Yamada, H. (1990) *J. Bacteriol.* **172,** 4807–4815.
13. Kobayashi, M., Yanaka, N., Nagasawa, T. & Yamada, H. (1992) *Biochemistry* **31,** 9000–9007.
14. Nagasawa, T., Mauger, J. & Yamada, H. (1990) *Eur. J. Biochem.* **194,** 765–772.
15. Kobayashi, M., Izui, H., Nagasawa, T. & Yamada, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 247–251.
16. Nagasawa, T., Takeuchi, K. & Yamada, H. (1988) *Biochem. Biophys. Res. Commun.* **155,** 1008–1016.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
18. Hashimoto, Y., Nishiyama, M., Yu, F., Watanabe, I., Horinouchi, S. & Beppu, T. (1992) *J. Gen. Microbiol.* **138,** 1003–1010.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
20. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
21. Nagasawa, T., Kobayashi, M. & Yamada, H. (1988) *Arch. Microbiol.* **150,** 89–94.
22. Inouye, S., Nakazawa, A. & Nakazawa, T. (1986) *Gene* **44,** 235–242.
23. Wallace, R. G., Lee, N. & Fowler, A. V. (1980) *Gene* **12,** 179–190.
24. Gallegos, M.-T., Michan, C. & Ramos, J. L. (1993) *Nucleic Acids Res.* **21,** 807–810.
25. Garnier, J., Osguthorpe, D. J. & Bobson, B. (1978) *J. Mol. Biol.* **120,** 97–120.
26. Nakai, R., Horinouchi, S. & Beppu, T. (1988) *Gene* **65,** 229–238.
27. Finnerty, W. R. (1992) *Annu. Rev. Microbiol.* **46,** 193–218.
28. Goodfellow, M. (1986) in *Bergey's Manual of Systematic Bacteriology*, eds. Sneath, P. H. A., Mair, N. S., Sharpe, M. E. & Holt, J. G. (Williams & Wilkins, Baltimore), Vol. 2, pp. 1472–1481.
29. Ramos, J. L., Rojo, F., Zhou, L. & Timmis, K. N. (1990) *Nucleic Acids Res.* **18,** 2149–2152.
30. Strohl, W. R. (1992) *Nucleic Acids Res.* **20,** 961–974.
31. Bork, P. & Koonin, E. V. (1994) *Protein Sci.* **3,** 1344–1346.
32. Novo, C., Tata, R., Clemente, A. & Brown, P. R. (1995) *FEBS Lett.* **367,** 275–279.