Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4267–4272, April 1996
Applied Biological Sciences

Characterization of the gene cluster of high-molecular-mass nitrile hydratase (H-NHase) induced by its reaction product in *Rhodococcus rhodochrous* J1

(amide/regulation/AmiC/transposase/cobalt)

HIDENOBU KOMEDA, MICHIHIKO KOBAYASHI§, AND SAKAYU SHIMIZU

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Communicated by Takayoshi Higuchi, Momoyamacho, Kyoto, Japan, December 16, 1995 (received for review August 7, 1995)

ABSTRACT **The 4.6-kb region 5′-upstream from the gene encoding a cobalt-containing and amide-induced high molecular mass-nitrile hydratase (H-NHase) from *Rhodococcus rhodochrous* J1 was found to be required for the expression of the H-NHase gene with a host-vector system in a *Rhodococcus* strain. Sequence analysis has revealed that there are at least five open reading frames (H-ORF1~5) in addition to H-NHase α- and β-subunit genes. Deletion of H-ORF1 and H-ORF2 resulted in decrease of NHase activity, suggesting a positive regulatory role of both ORFs in the expression of the H-NHase gene. H-ORF1 showed significant similarity to a regulatory protein, AmiC, which is involved in regulation of amidase expression by binding an inducer amide in *Pseudomonas aeruginosa*. H-ORF4, which has been found to be uninvolved in regulation of H-NHase expression by enzyme assay for its deletion transformant and Northern blot analysis for *R. rhodochrous* J1, showed high similarity to transposases from insertion sequences of several bacteria. Determination of H-NHase activity and H-NHase mRNA levels in *R. rhodochrous* J1 has indicated that the expression of the H-NHase gene is regulated by an amide at the transcriptional level. These findings suggest the participation of H-ORF4 (IS*1164*) in the organization of the H-NHase gene cluster and the involvement of H-ORF1 in unusual induction mechanism, in which H-NHase is formed by amides (the products in the NHase reaction), but not by nitriles (the substrates).**

Nitrile compounds containing a cyano functional group such as cyanoglycosides, cyanolipids, indole-3-acetonitrile and β-cyano-L-alanine are formed by a wide range of plants (1). We have been studying microbial degradation of highly toxic nitriles (2) and found that their degradation can proceed through two enzymatic pathways; nitrile hydratase (NHase; EC 4.2.1.84) catalyzes the hydration of a nitrile to the corresponding amide which is then converted to the acid plus ammonia by amidase (3), while nitrilase catalyzes the direct hydrolysis of a nitrile to the corresponding acid plus ammonia (4). Interest in both nitrile-converting enzymes has increasingly focused on their versatile functions: biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile (5–9) and enzymatic production of useful compounds from nitriles (2).

In microorganisms that catabolyze nitriles by NHase, an interesting phenomenon is found; this enzyme, if inducible, is generally induced by amides (reaction products), not by nitriles (reaction substrates) (2). *Rhodococcus rhodochrous* J1 produces two kinds of NHases: high- and low-molecular-mass NHases (H-NHase and L-NHase, respectively), which exhibit different physicochemical properties and substrate specificities. When this strain is cultured in a medium containing urea and cyclohexanecarboxamide in the presence of cobalt ions, H-NHase and L-NHase are selectively induced, respectively (2). We have cloned and sequenced both H- and L-NHase genes from *R. rhodochrous* J1 (10). In each of the H- and L-NHase genes, an open reading frame (ORF) for the β subunit is located just upstream of that for the α subunit. This arrangement of the coding sequences is the reverse of the order found in the NHase genes of *Rhodococcus* sp. N-774 (11) and *Pseudomonas chlororaphis* B23 (12).

The genus *Rhodococcus*, a member of the class Actinomycetes (13), has recently received much attention in terms of its high ability on biodegradation and biotransformation (14). *Rhodococcus* has also been studied as a phytopathogen causing leafy galls (fasciation) on plants (15). However, genetic information of *Rhodococcus* has been extremely limited. We have examined the expression of both H- and L-NHase genes in *Escherichia coli* cells under the control of *lac* promoter, but the level of NHase activity in the cell-free extracts is much lower than those of H- and L-NHases in *R. rhodochrous* J1 (10), suggesting that an uncharacterized regulatory gene would be present in this strain. Research into the regulatory system of *Rhodococcus* species has so far been hampered by the lack of systems for genetic manipulation of *Rhodococcus*. In the present study, genes required for the expression of H-NHase have been identified by using a host-vector system in *Rhodococcus*.* The H-NHase gene cluster was also characterized to clarify its unusual induction mechanism in *R. rhodochrous* J1.

MATERIALS AND METHODS

Microbial Strains and Plasmids. *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 H-NHase gene. The plasmid pNHJ10H (10) carrying the H-NHase gene in a 6-kb *Sac* I fragment on pUC19 was used for subcloning and sequencing of genes.

Transformation of *R. rhodochrous* ATCC12674 by Electroporation. 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 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; external resistance, 400 Ω). Pulsed cells were diluted immediately

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NHase, nitrile hydratase; H-NHase, high molecular mass-NHase; L-NHase, low molecular mass-NHase; ORF, open reading frame.

§To whom reprint requests should be addressed.

*The sequence reported in this paper has been deposited in the GenBank/DDBJ data base (accession no. D67027).

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 kanamycin·ml^{-1}.

Preparation of Cell Extracts and Enzyme Assay. *R. rhodochrous* ATCC12674 transformants were grown at 28°C for 48 h in MYP medium containing 0.001% (wt/vol) $CoCl_2 \cdot 6H_2O$ supplemented with urea at several concentrations, harvested by centrifugation at 4000 × *g* at 4°C, and washed twice with 0.15 M NaCl. The washed cells were suspended in 0.1 M Hepes/KOH buffer (pH 7.2) containing 44 mM 1-butyric acid, 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 NHase as described (10). One unit of the enzyme catalyzes the formation of 1 μmol of benzamide per min from benzonitrile under the above conditions.

RNA Preparation. The subculture of *R. rhodochrous* J1 was done as described (19), and 5 ml of the subculture was then inoculated into a 500-ml shaking flask containing 60 ml of a culture medium (19) with or without the following additives—*i.e.*, urea (0.75%, wt/vol), $CoCl_2 \cdot 6\ H_2O$ (0.001%, 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 (40 μg) was electrophoresed on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane filter (Schleicher & Schuell) in 20× standard saline/citrate (SSC). Prehybridization and hybridization were done at 42°C in a solution consisting of 40% formamide, 5× SSC, 0.1% SDS, and 100 μg of sonicated salmon sperm DNA per ml. 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/0.1% SDS.

RESULTS

Expression of the H-NHase Gene in *R. rhodochrous* ATCC12674. *R. rhodochrous* ATCC12674 harboring a plasmid containing a 6-kb insert of pNHJ10H in the blunt-ended *Eco*RI site of pK4 showed no NHase activity (data not shown). To identify the sequence elements required for the expression of the H-NHase gene, the upstream region was cloned by the DNA-probing method with a *Sac*I-*Eco*RI 0.37-kb fragment as a probe, and a plasmid pNHU10 was obtained (Fig. 1). Plasmid pHJK15 contained a 4.3-kb *Eco*RI–*Xba*I fragment from pNHU10 and a 5.66-kb *Eco*RI fragment from pNHJ10H in the *Eco*RI–*Xba*I sites of the *Rhodococcus-E. coli* shuttle vector pK4. Other plasmids (pHJK13, pHJK18 and pHJK19) shown in Fig. 1 were constructed in the same manner, by inserting the various restriction fragments from pNHU10 and the 5.66-kb *Eco*RI fragment from pNHJ10H into the *Eco*RI-blunt-ended *Xba* I sites of pK4. Plasmid pHJK11 contained only the 5.66-kb *Eco*RI fragment in the *Eco*RI site of pK4. These plasmids were used to transform *R. rhodochrous* ATCC12674, and the resulting transformants were cultured in $CoCl_2$-containing MYP medium in the presence (0.75 g/liter or 3.75 g/liter) or absence of urea (the best inducer of the H-NHase formation). Enzyme assay using benzonitrile as a substrate for each cell-free extract has revealed that, in addition to H-NHase gene itself, at least a 4.6-kb upstream region (from the 5′ end terminus of the H-NHase gene to *Sca*I site) is required for the expression of the H-NHase gene (Table 1) as in pHJK19. Nagasawa *et al.* (19) have already found that urea (added to the culture medium supplemented with cobalt ions) acts as a powerful inducer of H-NHase formation in *R. rhodochrous* J1. However, in the *Rhodococcus–E. coli* host-vector system used in this experiment, H-NHase was much expressed even in the absence

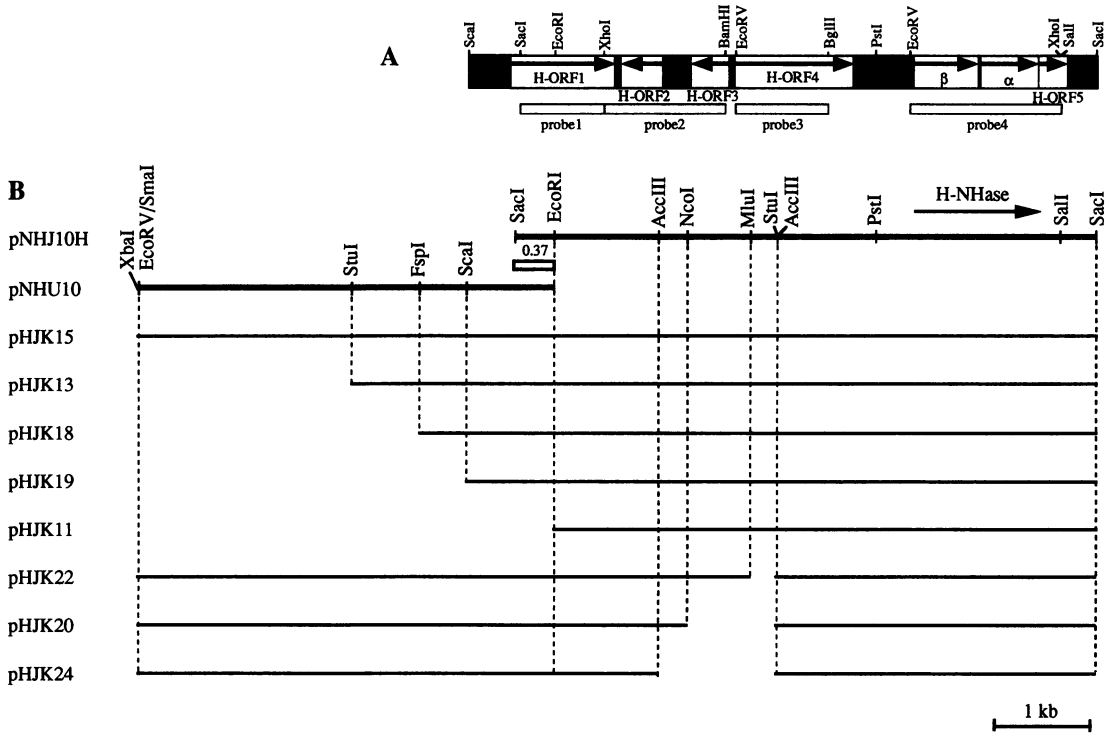

FIG. 1. Schematic view of the 6555-bp *Sca*I–*Sac*I fragment from pHJK19 (*A*) and construction of a set of plasmids (*B*). For clarity, only restriction sites discussed in the text are shown. The probes used in the experiment are shown (boxes). Various deletion plasmids are diagrammed below the restriction maps.

Table 1. NHase activity in *R. rhodochrous* ATCC12674 carrying various recombinant plasmids

Plasmid	NHase activity, units/mg·protein (0 g/l)	(0.75 g/l)	(3.75 g/l)
pK4	ND	ND	ND
pHJK15	7.41	11.3	1.86
pHJK13	8.83	10.2	3.28
pHJK18	7.49	12.9	4.44
pHJK19	9.39	10.2	4.88
pHJK11	1.20	0.21	0.22
pHJK22	9.09	17.0	NT
pHJK20	7.09	13.3	NT
pHJK24	1.65	1.24	NT

Urea at the concentrations in parentheses was added into the medium, and the cultivation was done as described. ND, not detected; NT, not tested;

of urea in the culture medium, and the presence of urea showed slight enhancement of H-NHase formation.

H-NHase formation in the transformants was examined by SDS/PAGE (Fig. 2). The transformant carrying pHJK19 expressed larger amounts of two proteins (26 kDa and 30 kDa), independently of urea concentration in the culture medium, than the transformant with pK4 or pHJK11. Both proteins formed were found to be the α and β subunits of H-NHase, respectively, by determination of their N-terminal amino acid sequences on a gas-phase amino acid sequencer (Applied Biosystems, model 470A). Expression of NHase activity shown above depended on the addition of cobalt ions into the medium because the transformant harboring pHJK19 cultured in the medium without cobalt ions had no NHase activity. Moreover, none of the *E. coli* JM109 harboring pK4-derivative plasmids used in this experiment gave NHase activity, even when these transformants were cultured in the medium supplemented with urea and $CoCl_2$.

Primary Structure of the Flanking Region of the H-NHase Gene. The *Pst*I–*Sal*I 1.97-kb fragment containing the H-NHase

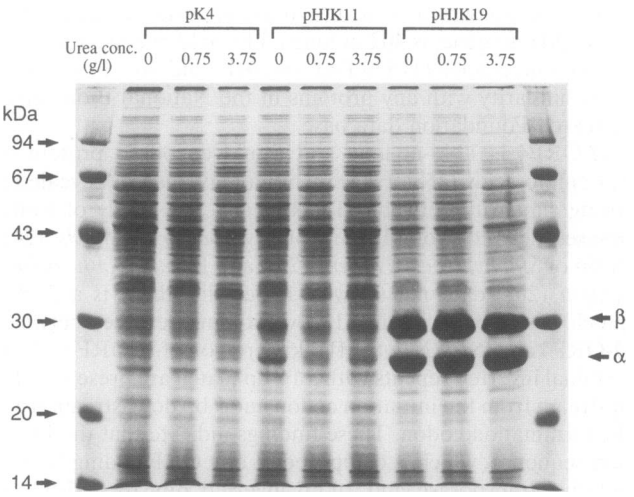

FIG. 2. Coomassie-stained SDS/PAGE, showing hyperformation of the H-NHase α- and β-subunit proteins in *R. rhodochrous* ATCC12674 transformants.

gene was sequenced (10). We further sequenced the *Sca*I–*Pst*I upstream region and *Sal*I–*Sac*I downstream region required for H-NHase production. Sequence analysis revealed that this fragment consisted of 6555-bp DNA and that five ORFs (ORF1~5) were newly found in the region in addition to the genes encoding α and β subunits of H-NHase (Fig. 1*A*).

H-ORF1 is 1083-nt long and would encode a protein of 361 amino acids (39155 Da). A computer-aided search for protein homology revealed that H-ORF1 was significantly similar to the negative regulator AmiC (21) of an aliphatic amidase gene in *Pseudomonas aeruginosa* (Fig. 3*A*). H-ORF2, which is located in the opposite orientation to the H-NHase gene, is 447 nt long and would encode a protein of 148 amino acids (16457 Da). H-ORF2 showed a similarity of amino acid sequence with the repressor genes *marR* (22) and *hpcR* (23) in *E. coli* (Fig.

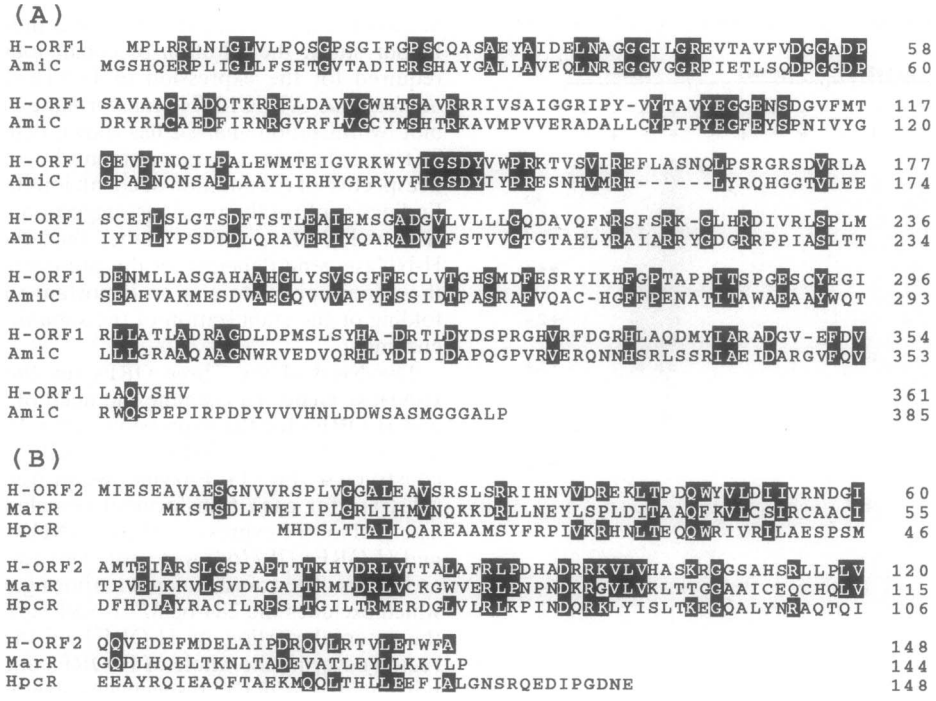

FIG. 3. Alignment of the deduced amino acid sequences of the *R. rhodochrous* J1 H-ORF1 and H-ORF2 with the respective homologous sequences. Identical residues are in black; dashes denote gaps introduced to maximize alignment. Abbreviations (refs.); AmiC, negative regulator of aliphatic amidase from *P. aeruginosa* (21); MarR, repressor of multiple antibiotic resistant operon from *E. coli* (22); HpcR, repressor of homoprotocatechuate-degradative operon from *E. coli* (23).

3*B*). H-ORF3, which is located in the opposite orientation to the H-NHase gene, is 402 nt long and would encode a protein of 133 amino acids (14578 Da). H-ORF3 did not show significant similarity with any proteins in the National Biomedical Research Foundation data base.

H-ORF4 is 1245-nt long and would encode a protein of 414-amino acids (45840 Da). A homology analysis revealed a strong similarity between H-ORF4 and the family of transposases derived from IS*1081* of *Mycobacterium bovis* (24), IS*256* of *Staphylococcus aureus* (25), and IS*Rm3* of *Rhizobium meliloti* (26) (data not shown). Especially, there is a 78.8% match of amino acids in 413 overlapping residues between H-ORF4 and IS*1081*. In the flanking region of H-ORF4, 19-bp terminal inverted repeats with a 16-bp match are present 38 bp upstream from the initiation codon and 9 bp downstream from the termination codon. These findings indicate that the insertion sequence, which is composed of one ORF coding for the putative transposase and 19-bp inverted repeats, exists upstream from the H-NHase gene. The designation number (IS*1164*) for this insertion sequence was assigned by the Plasmid Reference Center, Stanford University. Southern blot analysis at higher stringency using probe 3 specific for IS*1164* (see Fig. 1), against the *R. rhodochrous* J1 total DNA digested separately with several restriction enzymes, has suggested the existence of two or three insertion element-like sequences, which are homologous to IS*1164*, in the *R. rhodochrous* J1 DNA (data not shown). Although the distance between the TGA stop codon for H-ORF4 and the ATG start codon for the H-NHase β subunit is 637 bp, a search of the European Molecular Biology Laboratory and Genbank databases did not show any sequences closely related to the 637 spacer region.

H-ORF5 was found in the downstream region of the H-NHase gene (start and stop codons at nt 5942 ATG and 6256 TGA, respectively) in the same orientation as the H-NHase gene. H-ORF5 is 315 nt long and would encode a protein of 104 amino acids (11600 Da). H-ORF5 is homologous to the amino-terminal portion of each β subunit of H- and L-NHases (10) (data not shown). A strong hairpin structure was observed just downstream of the termination codon for H-ORF5 and

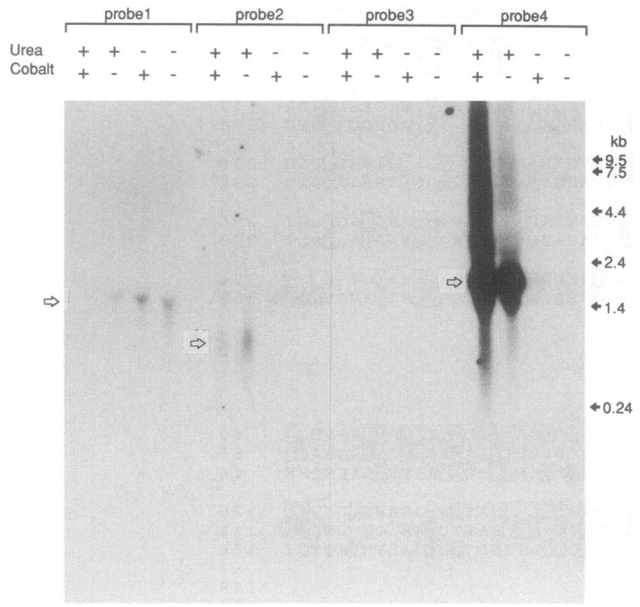

FIG. 4. Northern blots of RNA from *R. rhodochrous* J1 cultured in the medium in the presence (+) or in the absence (−) of urea and $CoCl_2$ and hybridized with probes 1–4 of Fig. 1. Open arrows point to prominent transcripts; those in probes 1, 2, and 3 regions correspond to 1.6, 0.9, and 1.8 kb, respectively.

may serve as a ρ-independent transcriptional termination signal.

Transcript Analysis of the H-NHase Gene Cluster. H-NHase activities in *R. rhodochrous* J1 cultured in the medium with urea (7.5%, w/v) and $CoCl_2$ (0.001%, w/v), with urea and without $CoCl_2$, without urea and with $CoCl_2$, and without urea and $CoCl_2$ were 1.21, 0.006, 0.053, 0.002 (units/mg·protein), respectively. These findings suggest that H-NHase formation is regulated by urea and cobalt ions at the transcriptional or translational level. As described above, the ORFs upstream the H-NHase gene are involved in the expression of the H-NHase gene. Therefore, four DNA fragments (Fig. 1) were used as probes (probe 1–4) against mRNA from *R. rhodochrous* J1 cultured in the medium in the presence or in the absence of urea and $CoCl_2$, to determine whether transcription of these ORFs and the H-NHase gene was altered by the culture conditions.

The region corresponding to probe 1 expressed one mRNA band, estimated at 1.6 kb, in the cells cultured in the medium in the absence of urea (Fig. 4). In the urea-induced cells, a mRNA band at the same size was faintly visible as well. Because the H-NHase gene was transcribed in large amounts (see below) in the urea-induced cells, the amount of RNA (corresponding to the probe 1 region) in the cells is relatively less than that in the urea-uninduced cells, suggesting that the probe 1 region, presumably H-ORF1 region, expresses constitutively. Probe 2 containing H-ORF2, H-ORF3, and part of H-ORF1 hybridized to one major RNA band, the length of which was estimated to be 0.9 kb, only in case of the cells cultured in the medium supplemented with urea; the RNA prepared from cells grown in the absence of urea did not give the 0.9-kb band of hybridization with probe 2. No hybridization signals with probe 3 were detected, suggesting that little or no H-ORF4 is expressed. Probe 4 containing the H-NHase gene and H-ORF5 hybridized to one dense RNA band, the length of which was estimated at 1.8 kb, when the RNA was prepared from the urea-induced cells.

Nagasawa *et al.* (16) have previously reported that the addition of cobalt ions to the culture medium is indispensable not only for catalytic activity but also for NHase formation in *R. rhodochrous* J1; and the enzyme is induced by cobalt ions. In our study, the addition of cobalt ions to the medium is required for the expression of H-NHase activity in the *R. rhodochrous* ATCC12674 transformants. However, Northern blot hybridization analysis has shown that the H-NHase gene is transcribed in a large amount by urea as an inducer, irrespective of the addition of cobalt ions to the medium. These findings confirm the previous suggestion (10) that the expression of H-NHase activity rather than the expression of the H-NHase gene depends on the presence of cobalt ions, and these ions appear to play an important role in enhancing the folding or the stabilization of the subunit polypeptides of the enzyme.

The Need of the Three ORFs for the Expression of the H-NHase Gene. To examine the need of H-ORF2, H-ORF3, and H-ORF4 for the expression of the H-NHase gene, three deletion plasmids (pHJK22, pHJK20, and pHJK24) were constructed (Fig. 1). Enzyme assays using benzonitrile as a substrate for each transformant revealed that H-ORF2 was essential for the expression of the H-NHase gene, but H-ORF3 and H-ORF4 (IS*1164*) were not. This result can be explained as follows. The transformants harboring pHJK22 or pHJK20, which excludes the 284-bp *Mlu*I–*Stu*I region corresponding to the internal portion of H-ORF4 or the 928-bp *Nco*I–*Stu*I region covering the whole of H-ORF3 and an amino-terminal portion of H-ORF4, respectively, exhibited NHase activity (Table 1). On the other hand, the transformant harboring pHJK24, which excludes the 1228-bp *Acc*III fragment covering the amino-terminal 10-amino acid residues of H-ORF2, in addition to the whole of H-ORF3 and amino-terminal portion

of H-ORF4, significantly decreased NHase activity (Table 1). These findings and the above experiments using pHJK11 suggest that both H-ORF1 and H-ORF2 are positive regulators involved in the expression of the H-NHase gene. The participation of H-ORF5 in the H-NHase gene expression remains to be determined.

DISCUSSION

Five ORFs (H-ORF1–5) that flank the H-NHase gene in *R. rhodochrous* J1 have been characterized. This gene organization is distinct from that of each NHase gene that has already been reported (2). Of these ORFs, H-ORF1 and H-ORF2 are indispensable for the intracellular formation of an active recombinant H-NHase in *R. rhodochrous* ATCC12674, whereas H-ORF3 and H-ORF4 (IS*1164*) have been found to have no influence on the expression of the H-NHase gene. H-ORF1, which was shown to express constitutively in *R. rhodochrous* J1 by Northern blot analysis, has significant similarity of the amino acid sequence to the negative regulator AmiC of the *P. aeruginosa* aliphatic amidase, which is induced by some low-molecular-mass amides such as acetamide and propionamide (27). Induction of the *P. aeruginosa* amidase is regulated by AmiC, which is considered to respond to the presence of amides as a sensor protein (28).

H-NHase of *R. rhodochrous* J1 is also induced by amide compounds—*i.e.*, acetamide, propionamide, acrylamide, methacrylamide, and urea, which are products of the nitrile hydration reaction catalyzed by NHase. The sequence analysis and the mapping experiment for the transcriptional initiation site of the H-NHase gene have demonstrated that no sequence homologous to amidase sequence exists in the upstream region of the H-NHase gene and that the transcription initiates at 71 and 48 bp upstream from the ATG initiation codon of the H-NHase β-subunit gene (data not shown); the H-NHase gene and the downstream region encoding H-ORF5 are transcribed in a single mRNA and are not part of a larger operon including the amidase gene. The finding that the AmiC-homologue (H-ORF1) region is responsible for the expression of the H-NHase gene that is not linked to any amidase gene, is noteworthy, while similarity of the amino acid sequence is not observed among amide-degrading enzymes; AmiE (the *Pseudomonas* amidase) does not show any similarity to amidases coupled with the *P. chlororaphis* B23 NHase, the *Brevibacterium* R312 NHase, and the *R. rhodochrous* J1 L-NHase (29). Assuming that the H-ORF1 gene product, as well as AmiC, functions as a sensor protein sensitive to amide compounds, it is suggested that the H-ORF1 gene product will be involved in the induction of H-NHase synthesis in some way, leading to occurrence of unusual induction mechanism in which NHase is formed by amides (the NHase reaction products).

Another ORF (H-ORF2) which shares a homology of amino acid sequence with putative repressor genes, *marR* and *hpcR*, from *E. coli*, is also required for the expression of the H-NHase gene in *R. rhodochrous* ATCC12674 (Table 1). Both of the *mar* (multiple antibiotic resistance) operon and the *hpc* (homoprotocatechuate)-degradative operon are shown to be negatively regulated by the *marR* (22) and *hpcR* (23) gene products, respectively. On the other hand, our findings that deletion of the amino-terminal portion of H-ORF2 significantly decreased NHase activity in the *R. rhodochrous* ATCC12674 transformant and that the transcription was stimulated by urea in the culture medium in *R. rhodochrous* J1 have indicated the necessity of H-ORF2 as a positive regulator in the process of H-NHase formation.

The occurrence of the insertion sequence IS*1164* in *Rhodococcus* species is, to our knowledge, the first documented example. While the sequences homologous to IS*1164* were observed in *R. rhodochrous* J1 DNA, whether IS*1164* functions as a mobile element in *R. rhodochrous* J1 remains to be determined. However, by the Southern hybridization method, we have already found the distribution of IS*1164* in some *Rhodococcus* species (unpublished results). The existence of IS*1164* upstream of the H-NHase β-subunit gene and the existence of the transcriptional terminator located just downstream of H-ORF5 support the finding in our Northern blot analysis that the H-NHase gene and its downstream region H-ORF5 constitute a single transcriptional unit; both genes are cotranscribed in a single polycistronic mRNA in the presence of urea irrespectively of the presence of cobalt ions. This is in contrast with the organization of gene clusters for NHases from *Rhodococcus* sp. N-774 (11, 30), *Rhodococcus* sp. (31), *Rhodococcus erythropolis* (32), *P. chlororaphis* B23 (12), and *Brevibacterium* sp. R312 (33), in which an amidase gene is located just upstream of each NHase gene with the same orientation. In the case of *R. rhodochrous* J1, the putative insertion sequence (IS*1164*) instead of an amidase gene is located in the upstream region from the H-NHase gene, suggesting the rearrangement of the H-NHase gene cluster by IS*1164* in the course of evolution.

The role of nitrile-converting enzymes in biosynthesis of the phytohormone, indole-3-acetic acid, is recently attracting increasing attention. cDNAs of nitrilase, which catalyzes the hydrolysis of indole-3-acetonitrile to indole-3-acetic acid plus ammonia, from a plant *Arabidopsis thaliana* have been cloned (6–8). We have also reported the occurrence of a biosynthetic pathway for indole-3-acetic acid from indole-3-acetonitrile *via* indole-3-acetamide by the combined action of NHase and amidase in phytopathogenic bacteria *Agrobacterium tumefaciens* and in leguminous bacteria *Rhizobium* (9). The existence of IS*1164* [homologous to the *Rhizobium* IS*Rm3*, which is a component of reiterated sequence IV of the *nod* megaplasmid (26)] in the upstream region of the H-NHase gene may be related with such biosynthesis of indole-3-acetic acid from indole-3-acetonitrile. Studies on nitrile metabolism in *Rhodococcus* at both protein and gene levels could provide information about biosynthesis of indole-3-acetic acid in plant-associated bacteria and plants and the evolutionary relationships of the former to the latter organisms.

Note Added in Proof. In the future, H-ORF1, H-ORF2, H-ORF3, H-ORF4, H-NHase β gene, H-NHase α gene, and H-ORF5 will be renamed as *nhhC*, *nhhD*, *nhhE*, *nhhF*, *nhhB*, *nhhA*, and *nhhG*, respectively.

This paper is dedicated to Emeritus Prof. Hideaki Yamada (Kyoto University) who is the discoverer of NHase. We are deeply indebted to Emeritus Prof. Beppu's group (University of Tokyo) and Mr. W. Mizunashi for providing the *Rhodococcus–E. coli* host-vector. We also thank Dr. M. Suyama for the computer search of amino acid sequences. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

1. Conn, E. E. (1981) in *Cyanide in Biology*, eds. Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J. & Wissing, F. (Academic, New York), pp. 183–196.
2. Kobayashi, M., Nagasawa, T. & Yamada, H. (1992) *Trends Biotechnol.* **10,** 402–408.
3. Asano, Y., Tani, Y. & Yamada, H. (1980) *Agric. Biol. Chem.* **44,** 2251–2252.
4. Kobayashi, M. & Shimizu, S. (1994) *FEMS Microbiol. Lett.* **120,** 217–224.
5. Kobayashi, M., Izui, H., Nagasawa, T. & Yamada, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 247–251.
6. Bartling, D., Seedorf, M., Mithöfer, A. & Weiler, E. W. (1992) *Eur. J. Biochem.* **205,** 417–424.
7. Bartling, D., Seedorf, M., Schmidt, R. C. & Weiler, E. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6021–6025.
8. Bartel, B. & Fink, G. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6649–6653.
9. Kobayashi, M., Suzuki, T., Fujita, T., Masuda, M. & Shimizu, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 714–718.
10. Kobayashi, M., Nishiyama, M., Nagasawa, T., Horinouchi, S., Beppu, T. & Yamada, H. (1991) *Biochim. Biophys. Acta* **1129,** 23–33.

11. Ikehata, O., Nishiyama, M., Horinouchi, S. & Beppu, T. (1989) *Eur. J. Biochem.* **181,** 563–570.
12. Nishiyama, M., Horinouchi, S., Kobayashi, M., Nagasawa, T., Yamada, H. & Beppu, T. (1991) *J. Bacteriol.* **173,** 2465–2472.
13. 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.
14. Finnerty, W. R. (1992) *Annu. Rev. Microbiol.* **46,** 193–218.
15. Crespi, M., Vereecke, D., Temmerman, W., Montagu, M. V. & Desomer, J. (1994) *J. Bacteriol.* **176,** 2492–2501.
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 Labolatory 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. Nagasawa, T., Takeuchi, K. & Yamada, H. (1991) *Eur. J. Biochem.* **196,** 581–589.
20. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
21. Wilson, S. & Drew, R. (1991) *J. Bacteriol.* **173,** 4914–4921.
22. Cohen, S. P., Hachler, H. & Levy, S. B. (1993) *J. Bacteriol.* **175,** 1484–1492.
23. Roper, D. I., Fawcett, T. & Cooper, R. A. (1993) *Mol. Gen. Genet.* **237,** 241–250.
24. Collins, D. M. & Stephens, D. M. (1991) *FEMS Microbiol. Lett.* **67,** 11–15.
25. Byrne, M. E., Rouch, D. A. & Skurray, R. A. (1989) *Gene* **81,** 361–367.
26. Wheatcroft, R. & Laberge, S. (1991) *J. Bacteriol.* **173,** 2530–2538.
27. Kelly, M. & Clarke, P. H. (1962) *J. Gen. Microbiol.* **27,** 305–316.
28. Wilson, S. A., Wachira, S. J., Drew, R. E., Jones, D. & Pearl, L. H. (1993) *EMBO J.* **12,** 3637–3642.
29. Kobayashi, M., Komeda, H., Nagasawa, T., Nishiyama, M., Horinouchi, S., Beppu, T., Yamada, H. & Shimizu, S. (1993) *Eur. J. Biochem.* **217,** 327–336.
30. Hashimoto, Y., Nishiyama, M., Horinouchi, S. & Beppu, T. (1994) *Biosci. Biotech. Biochem.* **58,** 1859–1865.
31. Mayaux, J.-F., Cerbelaud, E., Soubrier, F., Yeh, P., Blanche, F. & Petre, D. (1991) *J. Bacteriol.* **173,** 6694–6704.
32. Duran, R., Nishiyama, M., Horinouchi, S. & Beppu, T. (1993) *Biosci. Biotech. Biochem.* **57,** 1323–1328.
33. Mayaux, J.-F., Cerbelaud, E., Soubrier, F., Faucher, D. & Petre, D. (1990) *J. Bacteriol.* **172,** 6764–6773.