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Kyoto University
Organization and Regulation of Genes Involved in Nitrile Metabolism in *Rhodococcus rhodochrous* J1

Hidenobu Komeda

1996
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**ABBREVIATIONS**

<table>
<thead>
<tr>
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<tr>
<td>bp</td>
<td>Base pair(s)</td>
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<tr>
<td>CCCP</td>
<td>Carbonyl cyanide-m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl-</td>
</tr>
<tr>
<td>DR</td>
<td>Direct repeats</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
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<td>H-NHase</td>
<td>High molecular mass-nitrile hydratase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
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<td>IS</td>
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<tr>
<td>kb</td>
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<td>kDa</td>
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</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
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<td>Low molecular mass-nitrile hydratase</td>
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<tr>
<td>Mr</td>
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<td>NHase</td>
<td>Nitrile hydratase</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTH</td>
<td>Phenylthiohydantoin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SF6847</td>
<td>3,5-Di-tert-butyl-4-hydroxybenzilidenemalononitrile</td>
</tr>
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<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethan</td>
</tr>
<tr>
<td>TNB</td>
<td>5-Thio-2-nitrobenzoate</td>
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**INTRODUCTION**

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). Nitrile compounds are also widely manufactured and extensively used by the chemical industry; acetonitrile is used as a solvent, adiponitrile is a precursor of nylon-6,6 and acrylonitrile is produced as a precursor of acrylic fibers and plastics. Nitrile herbicides such as dichlobenil (= 2,6-dichlorobenzonitrile; commercial name Casoron), bromoxynil (= 3,5-dibromo-4-hydroxybenzonitrile; commercial name Brominil), ioxynil (= 3,5-diiodo-4-hydroxybenzonitrile; commercial name Bentrol) and Buctril (= 4-(octanoyloxy)-3,5-dibromobenzonitrile) are also widely used in agriculture for rice, wheat, barley, corn and berry. These nitriles have been widely distributed in our environment in forms of industrial waste water and residual agricultural chemicals, and if their release is not controlled, finally will threaten the environment. Therefore, nitrile is an urgent target from the standpoint of environmental purification and preservation. In the author's laboratory of Kyoto University, microbial degradation of highly toxic nitriles has been studied to find that their degradation can proceed through two enzymatic pathways (2); 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 ammonium by amidase (3), while nitrilase catalyzes the direct hydrolysis of a nitrile to the corresponding acid plus ammonium (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 (10).

*Rhodococcus rhodochrous* J1, which was found in the author's laboratory, has both nitrile-degrading pathways, depending on the inducer (10). When this strain is cultured in a medium containing isovaleronitrile as an inducer, only nitrilase is induced (11). The strain also produces higher (H-NHase) (12) and lower molecular mass (L-NHase) NHases (10). Both NHases require cobalt ions and amides for their induction. They are composed of two subunits, α and β (α-subunit differs in size from β-subunit in each, and α- and β-subunits of H-NHase differ from those of L-NHase). H-NHase and L-NHase are induced by urea and cyclohexanecarboxamide, respectively. Using immobilized cells containing H-NHase, the industrial production of acrylamide from acrylonitrile was started in 1991 (30,000 tons/year). This is the first case in which biotechnology was applied in the petrochemical industry and also
the first successful example of the introduction of an industrial bioconversion process for the manufacture of a commodity chemical (10).

Both H- and L-NHase genes (nhhBA and nhlBA, respectively) have been cloned from *R. rhodochrous* J1 and sequenced (13). In each of the H- and L-NHase genes, an open reading frame (ORF) for the β-subunit (nhhB and nhlB) is located just upstream of that for the α-subunit (nhhA and nhlA). This arrangement of the coding sequences is reverse of the order found in the NHase genes of *Rhodococcus* sp. N-774 (14) and *Pseudomonas chlororaphis* B23 (15).

*R. rhodochrous* J1 has great catalytic potential for the hydration of nitriles to the corresponding amides (16). In this process, contamination of acrylic acid formed by amidase in the strain causes the deterioration in the quality of the manufactured acrylamide; acrylamide is expected not to be changed into acrylic acid as much as possible by the amidase. Therefore, it is important from an applied standpoint to investigate amidases coupled with NHases in this strain.

Nitrilases that utilize benzonitrile and related aromatic nitriles as substrates have been purified from *Pseudomonas* (17,18) *Nocardia* sp. NCIB 11215 (19) and NCIB 11216 (20), *Fusarium solani* (21), *Arthrobacter* sp. (22), *R. rhodochrous* J1 (23) and *Escherichia coli* transformed with a *Klebsiella ozaenae* plasmid DNA (24). Nitrilases that act on aliphatic nitriles and arylacetonitriles are also purified from *Rhodococcus rhodochrous* K22 (25) and *Alcaligenes faecalis* JM3 (26), respectively, and characterized. The nitrilases from *R. rhodochrous* J1, *R. rhodochrous* K22 and *A. faecalis* JM3 are all strongly induced by the addition of isovaleronitrile to the medium (23,25,26), making large amounts of enzyme available for application in industrial production of a wide range of useful acids from nitriles. However, the mechanisms that regulate nitrilase expression have never been understood in these strains. All nitrilases so far reported are classified as sulphydryl enzymes since they are inactivated by thiol reagents. The *R. rhodochrous* J1 nitrilase is also inactivated by thiol reagents (23). An active cysteine residue has not yet been identified in any nitrilases.

The genus *Rhodococcus*, a member of the class Actinomycetes (27), has recently received much attention in terms of its high ability on biodegradation and biotransformation (28). However, genetic information of *Rhodococcus* has been extremely limited; research into the regulatory system of *Rhodococcus* species has so far been hampered by the lack of systems for genetic manipulation of *Rhodococcus*.

Chapter I describes the organization of the H-NHase gene cluster from *R. rhodochrous* J1. Two regulatory genes (nhhC and nhlD) required for the expression of H-NHase were 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. A possible insertion sequence, named IS1164, was fortuitously found in the intervening space between *nhhCD* and *nhhBA* and its primary structure was characterized. The distribution of IS1164-like element in the genomes of various *Rhodococcus* strains and other nitrile-metabolizing strains is also presented.

Chapter II describes the organization of the L-NHase gene cluster from *R. rhodochrous* J1. The author indicated the evidence of the occurrence of amidases in this strain. The author also cloned and sequenced an amidase gene (amdA), which is considered to be linked to the L-NHase gene. The recombinant *R. rhodochrous* J1 amidase protein in *E. coli* was also produced, purified and characterized. This chapter also describes the analyses on the mechanism of the L-NHase gene expression. In addition to nhlBA encoding L-NHase, two regulatory genes (nhIC and nhlD) were required for the amide-dependent induction of nhlBA by using the transformation system in *Rhodococcus*. nhIC has a sequence similarity to nhlC, which is involved in the H-NHase expression, and amIC, which is considered to be a sensor protein for inducer amides in the expression system of an aliphatic amidase in *Pseudomonas aeruginosa*, suggesting the products of both regulatory genes (NhIC and NhHC) are likely to sensor proteins for inducer amides. nhlF, which is situated between nhlBA and amdA, is similar to the bacterial genes encoding nickel transporters previously reported. The product of nhlF, NhIF transports cobalt ions into the *Rhodococcus* and *Escherichia coli* host cells. The transporter specific for cobalt ions was characterized using the transformation system in *Rhodococcus*.

Chapter III describes the analyses of the structure and induction mechanism of the nitrilase from *R. rhodochrous* J1, mainly at gene level. The nitrilase gene (nitA) from *R. rhodochrous* J1 was found to be similar to the bromoxynil nitrilase gene from *Klebsiella ozaenae*. An evidence that a cysteine residue (Cys-165) plays an important role in the function of the active site was also presented. A regulatory gene, nitR, which is situated downstream from nitA, was found to be responsible for the isovaleronitrile-dependent induction of nitA using the transformation system in *Rhodococcus*. Promoter region required for the regulation of nitA were also defined.
CHAPTER I  Analysis of High Molecular-Mass Nitrile Hydratase (H-NHase) Gene Cluster

Section 1  Regulatory genes for the expression of catalytically active H-NHase

In microorganisms that catabolize nitriles by NHase, an interesting phenomenon is found; this enzyme, if inducible, is generally induced by amides (reaction products), not by nitriles (reaction substrates) (10). As mentioned in the introduction to this thesis, *Rhodococcus rhodochrous* J1 produces two kinds of NHases; high- and low-molecular-mass-NHases (H-NHase and L-NHase), 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 (10). Both H- and L-NHase genes were cloned from *R. rhodochrous* J1 and sequenced (13). 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 reverse of the order found in the NHase genes of *Rhodococcus* sp. N-774 (14) and *P. chlororaphis* B23 (15). Expression of both H- and L-NHase genes in *E. coli* cells was examined 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 (13), suggesting that an uncharacterized regulatory gene would be present in this strain.

In this section, 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

Bacterial strains and plasmids

*R. rhodochrous* J1 was previously isolated from soil (29). *E. coli* JM109 (30) was the host for pUC plasmids. *R. rhodochrous* ATCC12674 was the host for a *Rhodococcus*-E. coli shuttle vector plasmid pK4 (31) and its derivatives, and was used for the expression of the H-NHase gene. The plasmid pNHJ10H (13) carrying the H-NHase gene in a 6-kb *SacI* 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 6,500 x 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 DNA in 1 μl of TE buffer (10 mM-Tris/1 mM EDTA, pH 8.0) in a 1-mm-gapped electroporvette (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, 400Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (31) 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 g/l CoCl2•6H2O supplemented with urea at several concentrations, harvested by centrifugation at 4,000 x 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 n-butyric acid, disrupted by sonication for 2 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 x g for 10 min at 4°C. The resulting supernatant was assayed for NHase as described previously (13). One unit of the enzyme catalyzes the formation of 1 μmol of benzamide/min from benzonitrile under the above conditions.

RNA preparation

The subculture of *R. rhodochrous* J1 was carried out as described previously (12), and 5 ml of the subculture was then inoculated into a 500-ml shaking flask containing 60 ml of a culture medium (12) with or without the following additives, i. e., urea (0.75%, w/v), CoCl2•6H2O (0.001%, w/v), 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 (32).

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 x SSC. Prehybridization and hybridization were carried out at 42°C in a solution
consisting of 40% formamide, 5 x 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 x SSC, 0.1% SDS, and then washed three times at room temperature in 2 x SSC solution with 0.1% SDS.

Fig. 1. Schematic view of the 6,555-bp Scal-SacI 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 diagramed below the restriction maps.

RESULTS

Expression of the H-NHase gene (nhhBA) in R. rhodochrous ATCC12674

R. rhodochrous ATCC12674 harboring a plasmid containing a 6-kb insert of pNHJ10H in the blunt-ended EcoRI site of pK4 showed no NHase activity (data not shown). To identify the sequence elements required for the expression of the H-NHase gene (nhhBA), the upstream region was cloned by the DNA-probing method with a Scal-EcoRI 0.37-kb fragment as a probe, and a plasmid pNHU10 was obtained (Fig. 1). Plasmid pHJK15 contained a 4.3-kb EcoRI-XbaI fragment from pNHU10 and a 5.66-kb EcoRI fragment from pNHJ10H in the EcoRI-XbaI 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 EcoRI fragment from pNHJ10H into the EcoRI-blunt-ended XbaI sites of pK4. Plasmid pHJK11 contained only the 5.66-kb EcoRI fragment in the EcoRI site of pK4. These plasmids were used to transform R. rhodochrous ATCC12674 and the resulting transformants were cultured in CoCl2-containing MYP medium in the presence of urea (0.75 g/l or 3.75 g/l) 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 nhhBA itself, at least a 4.6-kb upstream region (from the 5’ end terminus of the H-NHase gene to Scal site) is required for the expression of nhhBA (Table 1) as in pHJK19. Nagasawa et al. (12) 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 of urea in the culture medium, and the presence of urea showed slight enhancement of H-NHase formation.

<table>
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<th>Plasmid</th>
<th>Urea conc. (g/l)</th>
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<tr>
<td>pHJK15</td>
<td>7.41 11.3 1.86</td>
</tr>
<tr>
<td>pHJK13</td>
<td>8.83 10.2 3.28</td>
</tr>
<tr>
<td>pHJK18</td>
<td>7.49 12.9 4.44</td>
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<td>pHJK19</td>
<td>9.39 10.2 4.88</td>
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<tr>
<td>pHJK11</td>
<td>1.20 0.21 0.22</td>
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<tr>
<td>pHJK22</td>
<td>9.09 17.0 N.T.</td>
</tr>
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<td>pHJK20</td>
<td>7.09 13.3 N.T.</td>
</tr>
<tr>
<td>pHJK24</td>
<td>1.65 1.24 N.T.</td>
</tr>
</tbody>
</table>

N.D.: not detected, N.T.: not tested

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 was dependent 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 CoCl₂.

Primary structure of the flanking region of the H-NHase gene

The PstI-SalI 1.97-kb fragment containing the H-NHase gene was sequenced previously (13). The author further sequenced the ScaI-PstI upstream region and SalI-SacI downstream region required for H-NHase production. Sequence analysis revealed that this fragment consisted of 6,555 bp DNA and that five ORFs (nhhC, nhhD, nhhE, nhhF and nhhG) were newly found in the region in addition to nhhBA (Fig. 1-A).

nhhC is 1083-nucleotides long and would encode a protein of 361-amino acids (39,155 Da). A computer-aided search for protein homology revealed that the amino acid sequence deduced from nhhC was significantly similar to the negative regulator AmiC (33) of an aliphatic amidase gene in Pseudomonas aeruginosa (Fig. 3-A). nhhD, which is located in the opposite orientation to nhhBA, is 447-nucleotides long, and would encode a protein of 148-amino acids (16,457 Da). nhhD showed a similarity to the repressor genes marR (34) and hpcR (35) in E. coli (Fig. 3-B). nhhE, which is located in the opposite orientation to nhhBA, is 402-nucleotides long and would encode a protein of 133-amino acids (14578 Da). Amino acid sequence from nhhE did not show significant similarity with any proteins in the NBRF database.


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

Fig. 3. Alignment of the deduced amino acid sequences of the R. rhodochrous J1 NhhC and NhhD with the respective homologous sequences.

Residues in black boxes indicate identical sequences; dashes denote gaps introduced to maximize alignment. Abbreviations (references): AmiC, negative regulator of aliphatic amidase from P. aeruginosa (33); MarR, repressor of multiple antibiotic resistant operon from E. coli (34); HpcR, repressor of homoprotocatechuate-degradative operon from E. coli (35).

nhhF is 1245-nucleotides long and would encode a protein of 414-amino acids (45,840 Da). A homology analysis revealed a strong similarity between nhhF and the family of transposase genes derived from IS1081 of Mycobacterium bovis (36), IS256 of Staphylococcus aureus (37) and ISRms of Rhizobium meliloti (38). Especially, there is a 78.8% match of amino acids in 413 overlapping residues between nhhF and the transposase gene from IS1081. In the flanking region of nhhF, 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 nhhBA. The designation number (IS1164) for this insertion sequence was assigned by the Plasmid Reference Center, Stanford University. Southern blot analysis at higher stringency using probe 3 specific for IS1164 (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 IS1164, in the R. rhodochrous J1 DNA. Although the distance between the TGA stop codon for nhhf and the ATG start codon for the H-NHase β-subunit is 637 bp, a search of the EMBL and Genbank databases did not show any sequences closely related to the 637 spacer region.

nhhG was found in the downstream region of nhhA (start and stop codons at nucleotides 5942 ATG and 6256 TGA, respectively) in the same orientation as the H-NHase gene. nhhG is 315-nucleotides long and would encode a protein of 104 amino acids (11,600 Da). nhhG is homologous to the amino terminal portion of each nhhB and nhhA, which encode β-subunit of each H- and L-NHases, respectively (11). A strong hairpin structure was observed just downstream of the termination codon for nhhG and may serve as a p-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 CoCl₂ (0.015%, w/v), with urea and without CoCl₂, without urea and with CoCl₂, and without urea and CoCl₂ 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 nhh genes upstream nhhBA are involved in the expression of nhhBA. 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 CoCl₂, 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. Since 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 nhhC region, expresses constitutively. Probe 2 containing nhhD, nhhE and part of nhhC 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 nhhF is expressed. Probe 4 containing nhhBA and nhhG 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.

Fig. 4. Northern blots of RNA from R. rhodochrous J1 cultured in the medium in the presence (+) or in the absence (−) of urea and CoCl₂ and hybridized with probes 1–4 in Fig. 1. Open arrows point to prominent transcripts; those in the probe 1, probe 2 and probe 4 regions correspond to 1.6, 0.9 and 1.8 kb, respectively.

Nagawawa et al. (29) 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 the author’s 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 (13) 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 genes for the expression of nhhBA

To examine the need of nhhD, nhhE and nhhF for the expression of the nhhBA, three deletion plasmids (pHJK22, pHJK20 and pHJK24) were constructed (Fig. 1). Enzyme assays using benzoinitrile as a substrate for each transformant revealed that nhhD was essential for the expression of the H-NHase gene, but nhhE and nhhF (IS1164) were not. This can be explained as follows. The transformants harboring pHJK22 or pHJK20, which excludes the
284-bp MluI-Stul region corresponding to the internal portion of nhhF or the 928-bp NcoI-Stul region covering the whole of nhhE and an amino terminal portion of nhhF, respectively, exhibited NHase activity (Table 1). On the other hand, the transformant harboring pHJK24, which excludes the 1228-bp AccIII fragment covering the amino terminal 10 amino acid residues of nhhD in addition to the whole of nhhE and amino terminal portion of nhhF, significantly decreased NHase activity (Table 1). These findings and the above experiments using pHJK11 suggest that both nhhC and nhhD are positive regulators involved in the expression of the H-NHase gene. The participation of nhhG in the nhhBA expression remains to be determined.

**DISCUSSION**

Five ORFs (nhhC, nhhD, nhhE, nhhF and nhhG) that flank the nhhBA in *R. rhodochrous* J1 have been characterized. This gene organization is distinct from that of each NHase gene which has already been reported (10). Of these genes, nhhC and nhhD are indispensable for the intracellular formation of an active recombinant H-NHase in *R. rhodochrous* ATCC12674, whereas nhhE and nhhF (IS164) have been found to have no influence on the expression of the H-NHase gene. nhhC, 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 (39). Induction of the *P. aeruginosa* amidase is regulated by AmiC which is considered to respond to the presence of amides as a sensor protein (40).

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 nhhB (data not shown); nhhBA and the downstream region, nhhG, are transcribed in a single mRNA, and are not part of a larger operon including the amidase gene. The finding that the AmiC-homologue (nhhC) region is responsible for the expression of nhhBA which 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 *Rhodococcus* sp. N-774 NHase (14) and the *P. chlororaphis* B23 NHase (15). Assuming that NhhC as well as AmiC functions as a sensor protein sensitive to amide compounds, it is suggested that NhhC 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 (nhhD) which shares a homology of amino acid sequence with putative repressor genes, marR and hprR, from *E. coli*, is also required for the expression of nhhBA in *R. rhodochrous* ATCC12674 (Table 1). Both of the mar (multiple antibiotic resistance) operon and the hpr (homoprotocatechuate)-degradative operon are shown to be negatively regulated by the marR (34) and hprR (35) gene products, respectively. On the other hand, the author's findings that deletion of the amino terminal portion of nhhD 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 nhhD as a positive regulator in the process of H-NHase formation.

While the sequences homologous to IS1164 were observed in *R. rhodochrous* J1 DNA, whether IS1164 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 IS1164 in some *Rhodococcus* species. The existence of IS1164 upstream of nhhB and the existence of the transcriptional terminator located just downstream of nhhG support the finding in our Northern blot analysis that the H-NHase gene and its downstream region nhhG constitute a single transcriptional unit; both genes are cotranscribed in a single polycistrionic 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 (14, 41), *Rhodococcus* sp. (42), *Rhodococcus erythropolis* (43), *P. chlororaphis* B23 (15) and *Brevibacterium* sp. R312 (44), 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 (IS1164) instead of an amidase gene is located in the upstream region from nhhBA, suggesting the rearrangement of the H-NHase gene cluster by IS1164 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). 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 are also reported in phytopathogenic bacteria Agrobacterium tumefaciens and in leguminous bacteria Rhizobium (9). The existence of IS1164 [homologous to the Rhizobium ISRm3 which is a component of reiterated sequence IV of the nod megaplasmid (38)] in the upstream region of nhhBA 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.

**SUMMARY**

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 (nhhBA) with a host-vector system in a Rhodococcus strain. Sequence analysis has revealed that there are at least five open reading frames (nhhC, nhhD, nhhE, nhhF and nhhG) in addition to nhhBA. Deletion of nhhC, and nhhD resulted in decrease of NHase activity, suggesting a positive regulatory role of both genes in the expression of the nhhBA. nhhC showed significant similarity to a regulatory protein, AmiC, which is involved in regulation of amidase expression by binding an inducer amide in Pseudomonas aeruginosa. nhhF, 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 nhhF (IS1164) in the organization of the H-NHase gene cluster and the involvement of nhhC in unusual induction mechanism, in which H-NHase is formed by amides (the products in the NHase reaction), but not by nitriles (the substrates).

**Section 2**  
**Insertion sequence ISJ164 in the H-NHase gene cluster**

An insertion sequence (IS) is a mobile genetic element which is able to transpose to numerous sites on plasmids and chromosomes of microorganisms or plants, usually to give rise to their copies (45); however, only two studies have so far been reported on IS from Rhodococcus strains. IS1166 and IS1295 have recently been identified on a plasmid present in Rhodococcus sp. IGTS8 (46), whereas IS-Rf is isolated from Rhodococcus fascians using the Bacillus subtilis sacB gene as a selection marker (47).

As described in the previous chapter, studies on nhhBA regulation in R. rhodochrous J1 revealed the existence of five ORFs (nhhC, nhhD, nhhE, nhhF and nhhG) in addition to nhhBA in the H-NHase gene cluster. Two ORFs (nhhC and nhhD) were found to play positive regulatory roles in the process of the H-NHase formation; the intervening space between nhhBA and nhhCD was about 2.6 kb. The author has fortuitously found a possible IS in this region.

In this section, the author examined IS1164 (including nhhF) from R. rhodochrous J1. In addition, the author compared the structure of IS1164 and IS1081, which shows the highest similarity to IS1164, and found two pairs of terminal inverted repeats flanking their probable transposases. The distribution of IS1164-like elements in the genomes of various Rhodococcus strains and other nitrile-metabolizing strains is also presented.

**MATERIALS AND METHODS**

**Strains, culture conditions and plasmids**

Table 1 shows the microbial strains used in this chapter. For the preparation of total DNAs, Rhodococcus strains were cultured in a medium consisting of 5 g Tryptone (Difco), 5 g yeast extract (Oriental Yeast, Tokyo, Japan), 1 g glucose and 1 g K2HPO4/l distilled water (pH 7.0), and the other strains were cultured as described previously (9,26,48). The plasmid pNHJ10H (13) carrying nhhBA of R. rhodochrous J1 in the 6-kb SacI fragment on pUC19 was used for subcloning of gene.

**Enzymes and chemicals**

Restriction endonuclease was purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Toyobo Co. Ltd. (Osaka, Japan). [γ-32P]dCTP (110 TBq/mmol) was from Amersharm
(Tokyo, Japan). All other chemicals used were from commercial sources and were reagent-grade.

DNA manipulation

Total DNA of *R. rhodochrous* J1 and the other strains were isolated and purified as described by Saito and Miura (49). DNA manipulation was performed essentially as described by Sambrook *et al.* (30).

Southern hybridization

Southern blots against the restriction endonuclease-digested total DNA were prepared as described by Sambrook *et al.* (30). The 967-bp EcoRV-BgIII fragment in *IS1164* was purified from a low-melting agarose gel, labelled with α-[32P]dCTP using a Multiprime DNA labelling system (Amersham) and used to probe the Southern blots. Prehybridization and hybridization were performed at a higher stringency using a solution consisting of 50% (v/v) formamide, 2x SSC (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate), 1% (w/v) SDS and 10% (w/v) dextran sulfate at 42°C; the blots were then washed twice in 2x SSC for 5 min at room temperature, twice in 2x SSC containing 1% SDS for 20 min at 42°C and twice in 0.2x SSC containing 1% SDS for 20 min at 42°C.

RESULTS

Occurrence of *IS1164* in the H-NHase gene cluster

The author has identified the sequence elements (*nhhC* and *nhhD*) required for the expression of *nhhBA* encoding H-NHase (Fig. 1). Between their elements and *nhhBA*, there is *nhhf* encoding the putative transposase that had high similarities to the transposases from IS elements previously reported *i.e.*, *Mycobacterium bovis* IS1081 (78.8% identity) (36), *Staphylococcus aureus* IS256 (36.4% identity) (37) and *Pseudomonas cepacia* IS406 (36.0% identity) (50). In the flanking region of *nhhf*, two pairs of terminal inverted repeats (IR), 27-bp sequence with 18-bp matches for IR-1 and 19-bp sequence with 16-bp matches for IR-2 were observed; IR-1 are flanked by 9-bp direct repeats (DR). These findings suggest that *nhhf* encodes a presumed transposase and constitutes an insertion sequence, accompanied with the flanking direct and inverted repeats. The G + C content (66.9%) of *IS1164* resembled that of the *Rhodococcus rhodochrous* genome (67-73%, ref. 28).

Comparison of the ends of *IS1164* and *IS1081*

*IS1164* from *R. rhodochrous* J1 has the structural features shared by transposable elements as described above. Furthermore, it is noteworthy that *IS1164* has two pairs of IR that are accompanied with the intervening space of 47-bp between IR-1 (Left) and IR-2 (Left) and with 1-bp between IR-1 (Right) and IR-2 (Right) (Fig. 2). The alignment of extremities of *IS1164* and *IS1081* showed marked similarity between them (Fig. 2), indicating that *IS1081* also contains two pairs of IR flanked by 8-bp DR. To the author’s knowledge, existence of two pairs of IR in the flanking region of transposase gene has not been previously reported. The two pairs of IR are highly conserved between both ISs, suggesting the functional role for these IR sequences. On the contrary, DR corresponding to the possible target-site duplications show little sequence similarity.

Determination of copy number of *IS1164* in *R. rhodochrous* J1

To determine how many copies of the insertion sequence (*IS1164*) are present in *R. rhodochrous* J1 genome, Southern blot hybridization was carried out. Southern blots against the *R. rhodochrous* J1 total DNA that had been digested separately with restriction enzymes EcoRI, PstI, SacI, EcoRI plus SacI, and PstI plus BamHI, which do not have their sites within *IS1164*, were exposed to the *IS1164* internal probe of a 967-bp EcoRV-BgIII fragment.

Southern blot analysis at a higher stringency revealed that, in all cases, the probe hybridized
with a single bold band and two or three thin bands (Fig. 3). The bold band at all lanes was found to correspond to the DNA fragment containing IS1164 judging from the size of these fragments. This suggests that two or three insertion element-like sequences, which are homologous to IS1164 to some extent, are present and that IS1164 is a repeated sequence in the *R. rhodochrous* J1 genome.

![Image](image)

Fig. 3. Determination of the copy number of IS1164 in total DNA of *R. rhodochrous* J1.
Total DNA (4 μg) from *R. rhodochrous* J1 was digested with several endonucleases indicated (sites not present in IS1164), separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the labelled IS1164 probe (Materials and Methods). Sizes are shown in kilobase pairs.

Distribution of IS1164-like elements in other *Rhodococcus* and nitrile-metabolizing strains

To investigate the distribution of an IS1164-like element in other *Rhodococcus* and nitrile-metabolizing strains, we carried out Southern blot analysis against their EcoRI-digested total DNAs by using the 967-bp EcoRV-BglII fragment in IS1164 as a probe (Fig. 4). Difference in the intensity of the bands could correspond to multiple copies for one band or to less sequence similarity between the target DNA and the probe used. Eleven out of 16 *Rhodococcus* strains (lanes 2–17 in Fig. 4) were found to contain an IS1164-like element. In particular, *R. rhodochrous* NCIB9703 and *R. rhodochrous* NCIB11277 contained the element at a relatively higher copy number, at least 6 and 7 copies per genome, respectively. However, *P. chlororaphis* B23 (48), *Alcaligenes faecalis* JM3 (26) and *Agrobacterium tumefaciens* IAM B-261 (9), which are found to contain the enzymes involved in nitrile metabolism, just like *R. rhodochrous* J1, contained no DNA sequence showing similarity to IS1164 from *R. rhodochrous* J1.

![Image](image)

Fig. 4. Distribution of IS1164 among various *Rhodococcus* and other nitrile-degrading strains.
Total DNA (8 μg) from *R. rhodochrous* J1 (lane 1), *R. rhodochrous* K22 (lane 2), *R. rhodochrous* ATCC9356 (lane 3), *R. rhodochrous* ATCC19140 (lane 4), *R. rhodochrous* ATCC19149 (lane 5), *R. rhodochrous* JCM2157 (lane 6), *R. rhodochrous* JCM3202 (lane 7), *R. rhodochrous* NCIB9703 (lane 8), *R. rhodochrous* NCIB11277 (lane 9), *R. erythropolis* IFO12539 (lane 10), *R. erythropolis* IFO12682 (lane 11), *R. erythropolis* JCM2892 (lane 12), *R. erythropolis* JCM6823 (lane 13), *R. rubropertinctus* JCM3204 (lane 14), *Rhodococcus* sp. N774 (lane 15), *Rhodococcus* sp. NCIB11215 (lane 16), *Rhodococcus* sp. NCIB11216 (lane 17), *P. chlororaphis* B23 (lane 18), *A. faecalis* JM3 (lane 19), *A. tumefaciens* IAM B-261 (lane 20) was digested with EcoRI (site not present in IS1164), separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the labelled IS1164 probe (Materials and Methods). Sizes are shown in kilobase pairs.

**DISCUSSION**

Transposable elements are characterized by the presence of terminal inverted repeat sequences that are essential for transposition and the presence of direct repeat sequences that are probably duplicated upon insertion. *nhhF* encoding possible transposase has several features found in the known IS elements in its flanking region: terminal inverted repeats and target-site duplications. These structural features have been used to identify IS elements such as IS1081 from *Mycobacterium bovis* (36), IS1201 from *Lactobacillus helveticus* (51), IST2 from *Thiobacillus ferrooxidans* (52) without providing direct evidence for transposition. Sequence
comparisons of the putative transposase encoded by nhhF with those of other IS elements suggest that IS1164 is a new member of an IS family including IS1081 (36), IS256 (37), IS406 (50), Lactococcus lactis IS905 (53), Rhizobium meliloti ISRm3 (38), IS1201 (51), Mycobacterium smegmatis IS6120 (54), Rhodococcus sp. IGTS8 IS1166 (46) and IST2 (52). Of these members, IS406, IS905, ISRm3, IS6120 and IS1166 have been previously shown to have ability to transpose. IS1164 and IS1081 share a high degree of identity (78.8%), suggesting that these IS elements had a recent common ancestry. A search of the EMBL and Genbank databases did not show any homologous sequences in Streptomyces to IS1164, although Rhodococcus belongs to Actinomycete (27) including Streptomyces; Furthermore, Rhodococcus has significantly high G+C content as well as Streptomyces does.

IS1164 is located between nhhBA encoding H-NHase and nhhCD encoding positive regulators for nhhBA expression in R. rhodochrous J1 DNA. It seems reasonable to suppose that IS1164 has been inserted individually between nhhCD and nhhBA because IS1164 is bracketed by possible target site duplications. Southern hybridization revealed that IS1164-like elements are widely distributed among Rhodococcus: 11 out of 16 Rhodococcus strains tested showed positive hybridization signals. However, except for the case of R. erythropolis JCM2892 (43) (lane 12 in Fig. 4), no signal was detected in R. erythropolis JCM6823 (43), Rhodococcus sp. N-774 (14) or P. chlororaphis B23 (15), which possess NHase genes with significant similarities to nhhBA. On the other hand, IS-like elements have been detected even in R. rhodochrous K22 (25), R. erythropolis IFO12682, Rhodococcus sp. NCIB11215 (19) and NCIB11216 (20), which produce nitrilase. R. rhodochrous ATCC1949, R. rhodochrous NCIB9703 and R. rhodochrous NCIB11277, which gave hybridization signals, oxidize alkybenzenes, oxidize hydrocarbons and utilize hydrocarbons, respectively. Recently, a mechanism has been proposed by which tcbAB on Tns280 can be mobilized and joined with tcbCDE to form a novel catabolic pathway degrading chlorobenzenes (55). Moreover, five copies of IS6100 have also been identified on plasmid pOAD2, which carries genes encoding nylon oligomer-degrading enzymes (56). Although it is still unclear whether IS1164-like elements found in Rhodococcus by Southern hybridization method are involved in acquisition of ability that degrades recalcitrant highly toxic nitriles containing a cyan functional group and other aromatic compounds in Rhodococcus, the presence of these IS1164-like elements suggests such a possibility.

The genus Rhodococcus has been poorly characterized genetically. Only three kinds of ISs are so far known in Rhodococcus: IS1166 and IS1295 from Rhodococcus sp. strain IGTS8 (46) and IS-Rf from R. fascians DSM20131 (47). In terms of similarity, IS1164 and IS1166 appear to belong to the same family. On the other hand, IS1295 is dissimilar to IS1164. We could not compare IS-Rf with IS1164 because only IR of IS-Rf have been reported. Whether IS1164 functions as a mobile element in R. rhodochrous J1 remains to be determined. However, we have found IS1164-like elements in some Rhodococcus species including R. rhodochrous, R. erythropolis and R. rubroperticntus, indicating that these elements show a broad host range. Further investigations on IS1164 are needed to clarify whether IS1164 can be useful for genetic manipulation of the genomes of Rhodococcus, which are expected to be useful in various fields.

SUMMARY

An insertion sequence (IS1164) from Rhodococcus rhodochrous J1 has been identified by its nucleotide sequence. This 1430 bp-long IS has two pairs of imperfect terminal inverted repeats (IR-1 and IR-2) flanked by nine base pair direct repeats (DR), and contains an open reading frame encoding a putative transposase with similarities to those found in IS1081 from Mycobacterium bovis, IS256 from Staphylococcus aureus and IS406 from Pseudomonas cepacia. Structures of the terminal region containing inverted repeats were found to be highly conserved both in IS1164 and in IS1081. Hybridization analyses against total DNA from R. rhodochrous J1, 16 Rhodococcus strains and 3 other bacteria catabolizing nitriles as well as R. rhodochrous J1, using an internal DNA fragment within IS1164 as a probe, showed that R. rhodochrous J1 had three other IS1164-like elements and 11 out of 16 Rhodococcus strains contained IS1164-like elements varying in copy number from one to at least seven. Their elements are present not only in Rhodococcus producing recalcitrant nitriles-degrading enzymes (nitrile hydratase and nitrilase) but also in Rhodococcus utilizing alkybenzenes and hydrocarbons.
CHAPTER II Analysis of Low Molecular-Mass Nitrile Hydratase (L-NHase) Gene Cluster

Section 1 Occurrence of amidas in Rhodococcus rhodochrous J1c

Rhodococcus rhodochrous J1 has great catalytic potential for the hydration of nitriles to the corresponding amides (16). This strain produces two kinds of cobalt-containing nitrile hydratases (NHases); one is a high Mr-NHase (H-NHase) and the other is a low Mr-NHase (L-NHase) (10). When R. rhodochrous J1 was cultured in the medium containing urea or cyclohexanecarboxamide in the presence of cobalt ions, the H-NHase and L-NHase were inductively formed selectively. In fact, the industrial production of acrylamide from acrylonitrile using the R. rhodochrous J1 H-NHase was started in 1991 (30,000 tons per year). This is the first successful example of using a biotransformation process to produce a commodity chemical. In the nitrile-degrading pathway by NHase, amidas is essential for the growth of the strain, when the strain is cultured in a medium containing a nitrile as a sole source of carbon/nitrogen, because the amide formed by NHase has to be converted into the corresponding acid and ammonia (3). In this process, contamination of acrylic acid formed by amidas in the strain causes the deterioration in the quality of the manufactured acrylamide; acrylamide is required not to be changed into acrylic acid as much as possible by the amidas. Therefore, it is important from an applied standpoint to investigate amidases coupled with NHase. In this section, The author present the evidence that at least two amidases exist in R. rhodochrous J1.

MATERIALS AND METHODS

Strain and culture conditions

R. rhodochrous J1 (29) was subcultured at 26.5°C for 24 h in 8 ml of a basal medium consisting of 10 g of glycerol, 0.5 g of KH₂PO₄, 0.5 g of K₂HPO₄, 0.1 g of MgSO₄·7H₂O, 1 g of yeast extract (Oriental Yeast, Tokyo), and 5 g of Polypeptone (Daigo, Osaka)/liter of distilled water (pH 7.2). This subculture was inoculated into a 2-liter shaking flask containing 500 ml of the basal medium with one of the amidases listed on the Table in the presence or absence of CoCl₂·6H₂O (0.001%, mass/vol).

RESULTS AND DISCUSSION

As shown in the Table, whether cobalt ions are added to the basal medium or not, R. rhodochrous J1 cells cultured with each amide added to the culture medium showed amidas activity for propionamide as a substrate. Even when amide was not added to the basal medium, the activity degrading propionamide was found, but cells cultured in a synthetic medium consisting of glycerol, KH₂PO₄, K₂HPO₄, MgSO₄, NH₄Cl, NaCl and vitamin mixture (11) did not have any amidas activities; amidas activity may be induced by some amide-like compounds in the basal medium. When H-NHase was induced by urea, R. rhodochrous J1
cells were not able to hydrolyze benzamide as a substrate at all. On the other hand, when L-NHase was induced by cyclohexanecarboxamide, the cells could hydrolyze not only benzamide but also propionamide as substrates. The ratio of propionamide-degradation to benzamide-degradation (P/B) was not constant under each condition. The similar observation applied to the cell-free extract obtained from each cell suspension by sonication for 20 min with an insonator model 201M (Kubota, Tokyo). These findings demonstrate that there are at least two kinds of amidases in R. rhodochrous J1; they provided information leading to the manufacture of pure acrylamide to be established.

R. rhodochrous J1 cells cultured with L-NHase induced by cyclohexanecarboxamide did not attack acrylamide at all, but the cells containing H-NHase supplied in the industrial production just acted on acrylamide (0.967 U/mg dry cell mass) at 25°C, and had barely any activity at low temperatures in the reaction mixture. To prevent the formation of the by-product acrylic acid, the practical reaction has been done at a low temperature (2-4°C), because this reduces the amidase activity and the H-NHase is not easily inactivated even at this temperature. Acrylamide was also a good inducer for the formation of NHase in this strain. However, growth of the strain and NHase activity in this occasion were lower than those when H-NHase was induced. Thus, urea was selected instead of acrylamide has been used in the form of a polymer as an immobilizer of R. rhodochrous J1.

The addition of cobalt ions to the culture medium was indispensable for NHase activity both in R. rhodochrous J1 and in the Escherichia coli transformant containing H- or L-NHase gene. (13) On the contrary, in general, the cobalt addition lowered amidase activity for propionamide by 20-90% (Table). When amides such as N,N-dimethylpropionamide, crotonamide, methacrylamide, benzamide, and N-methyl-e-caprolactam were used as inducers, the addition of cobalt ions enhanced amidase activity for benzamide only 1.5-2.0 times, while NHase activity was strongly enhanced more than 60-times (data not shown) by the cobalt addition into the medium containing each amide. These findings indicate that amidases do not seem to require cobalt ions for their activity.

In Rhodococcus sp. N-774 (14), Pseudomonas chlororaphis B23 (15), and Rhodococcus sp. (42), each amidase gene is found in the same orientation and just upstream from each NHase gene, which is significantly similar to the nhhBA and nhlBA. Judging from the gene arrangement of both enzymes and the sequential nitrile degradation route through the combination of NHase and amidase, there might be amidases that are induced together with H- and L-NHase by urea and cyclohexanecarboxamide, respectively, and cobalt ions.

Amidase itself as well as NHase might be useful in the production of higher-value acids from the corresponding nitriles or amides in cooperation with an NHase. We have never isolated an amidase gene that is coupled with an H-NHase gene. Cloning of this amidase and overexpression of the amidase gene coupled with L-NHase gene from R. rhodochrous J1 in Escherichia coli are now being studied.

SUMMARY

R. rhodochrous J1, of which the high-Mr nitrile hydratase has been used for the industrial manufacture of acrylamide from acrylonitrile, produced at least two amidases differing in substrate specificity, judging from the effects of various amides on amidase activity in this strain. These amidases seemed to be inducible enzymes depending on amide compounds.
Section 2 Amidase coupled with L-NHase : Sequencing and expression of the gene and purification and characterization of the gene product

The microbial degradation of nitriles proceeds through two enzymatic pathways. One is the nitrilase pathway, in which nitrilase hydrolyzes nitriles directly to the corresponding carboxylic acids and ammonia. The other pathway is a combination of nitrile hydratase (NHase) and amidase. Firstly, nitriles are hydrated into the corresponding amides by NHase. Secondly, amides are hydrolyzed into the corresponding carboxylic acids and ammonia by amidase. *Rhodococcus rhodochrous* J1 has both nitrile-degrading pathways, depending on the inducer (10). When this strain is cultured in medium containing isovaleronitrile as an inducer, only nitrilase is induced (11). The strain also produces higher (H-NHase) and lower molecular mass (L-NHase) NHases (10). Both require cobalt ions and amides for their induction. They are composed of two subunits, \( \alpha \) and \( \beta \) (The \( \alpha \)-subunit differs in size from the \( \beta \)-subunit in each, and the \( \alpha \) - and \( \beta \)-subunits of H-NHase differ from those of L-NHase.). H-NHase and L-NHase are induced by urea and cyclohexane-carboxamide, respectively. In fact, the use of H-NHase in *R. rhodochrous* J1 as the third-generation strain for the industrial production (30,000 tons per year) of the important chemical commodity acrylamide from acrylonitrile was recently pioneered in Japan (10).

When the strain was cultured in the optimum medium, the amount of H-NHase in the cell-free extracts corresponded to more than 50% of the total soluble protein. However, the *R. rhodochrous* J1 amidase, along with amidases from *Rhodococcus* sp. N-774 (14,57) and *P. chlororaphis* B23 (15), which were used as 1st- and 2nd-generation strains respectively in the industrial manufacture of acrylamide, have never been investigated in form of protein, because of the low activity in these strains. Functional analysis of the amidase in *R. rhodochrous* J1 will certainly help to elucidate the hyperproduction of NHase and hypoproduction of amidase and subsequently allow the manufacture of pure acrylamide to be established. In the previous section, the author showed the evidence that at least two amidases, which are induced coordinately with H-NHase or L-NHase in *R. rhodochrous* 11. In this section, the author cloned and sequenced the amidase gene, which is considered to be linked to the L-NHase gene (*nhlBA*) (13). The recombinant *R. rhodochrous* J1 amidase protein in *Escherichia coli* was also produced, purified and characterized.

Bacterial strain and plasmids

*Escherichia coli* JM109 was the host for pUC18/19 plasmid transformation and phage M13 mp18/19 propagation (30). The plasmid pNH20L, carrying the L-NHase gene (*nhlBA*) of *R. rhodochrous* J1 in the 9.4 kb *SacI* fragment on pUC19 (13), was used for subcloning and sequencing the gene.

Materials, enzymes and chemicals

DEAE-Sephacel, Mono-Q™HR 5/5, Superose™12 and a low-molecular-mass standard kit were obtained from Pharmacia (Uppsala, Sweden). Marker proteins for molecular mass determination by HPLC were purchased from the Oriental Yeast Co. (Tokyo, Japan). All other chemicals used were from commercial sources and of reagent grade.

DNA sequencing

DNA fragments containing the amidase gene were cloned into M13 vectors and sequenced by dye-chain termination (58) using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, USA) and a *Tth* (*Thermus thermophilus*) Sequence Kit (Toyobo, Osaka, Japan).

Preparation of crude extracts from *Escherichia coli* transformants

Recombinant *E. coli* JM109 was cultured aerobically to full growth in 10 ml of 2 x YT medium containing 80 \( \mu \)g/ml ampicillin in 100 ml test tube at 37°C, and then transferred to 100 ml of the same medium in a 500-ml shaking flask with isopropyl-\( \beta \)-D-galactopyranoside (IPTG) added to a final concentration of 1 mM to induce the *lac* promoter. After a further 7- or 12-h cultivation, cells were harvested by centrifugation, suspended in 5 ml 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, disrupted by sonication for 10 min (19 kHz; Insonator Model 201M, Kubota, Tokyo, Japan) and centrifuged at 12,000 x g for 30 min. The resulting supernatants were dialyzed for 5 h against 21 0.02 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, and 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and 45% (mass/vol.) glycerol for 10 h. The resulting dialysates were assayed for the enzyme.
Enzyme assay

The standard reaction mixture (1 ml) for assaying the amidase activity contained 10 mmol potassium phosphate buffer (pH 7.5), 10 mmol benzamide and an appropriate amount of the enzyme. The reaction was performed at 30°C for 30 min and stopped by adding 0.1 ml 1 M HCl. The amount of benzoic acid formed in the reaction mixture was determined by HPLC under the same conditions as described previously (13) with the exception that the ratio of KH2PO4·H2PO4 to acetonitrile was 1:1 (by vol.). One unit of the enzyme was defined as the amount catalyzing the formation of 1 μmol benzoic acid/min from benzamide under the above conditions. Protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (59) using bovine serum albumin as a standard protein.

PCR amplification

A modified DNA fragment coding for the amidase was obtained by means of polymerase chain reaction (PCR). DNA was amplified using a thermal cycler (Perkin-Elmer/Cetus, USA). The reaction mixture contained 150 ng of template DNA, 100 pmol of each oligonucleotide pool, and Tth (Thermus thermophilus) DNA polymerase (Toyobo, Osaka, Japan) in a volume of 100 ml. One thermal cycle consisted of 95°C for 1 min, 42°C for 1 min, and 75°C for 3 min. A total of 30 cycles was performed. The PCR-synthesized DNA was purified from an agarose gel.

Purification of the amidase from Escherichia coli transformant

E. coli JM109 harboring pALJ30 was subcultured at 37°C for 12 h in a 100 ml test tube containing 10 ml of 2 x YT medium with 80 μg/ml ampicillin. One milliliter of the subculture was then inoculated into a 500 ml shaking flask containing 100 ml of the above medium. After a 4 h incubation at 37°C with reciprocal shaking, IPTG was added to the medium to a final concentration of 0.3 mM to induce the lac promoter, followed by a further incubation at 28°C for 4 h. Eight hours from the start, the cells were harvested by centrifugation for 10 min at 9,000 x g at 4°C, and washed with 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. All purification steps were performed at 0 to 4°C using potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol and 20% (mass/vol.) glycerol unless otherwise specified. Centrifugation was carried out for 30 min at 100,000 x g.

Step 1. Preparation of cell-free extracts. Washed cells from 2.4 liters of culture were suspended in 50 ml 0.1 M buffer and disrupted by sonication at 19 kHz or 20 min with an insonator model 201M (Kubota). The cell debris was removed by centrifugation. The resulting supernatant was used as the cell-free extract.

Step 2. DEAE-Sepharose column chromatography. The solution from step 1 was applied to a DEAE-Sepharose column (3.5 by 60 cm) equilibrated with 10 mM buffer. After the column was washed thoroughly with 10 mM buffer, followed by the same buffer containing 0.1 M KCl and 0.2 M KCl, the enzyme was eluted with 1.5 liters 10 mM buffer containing 0.3 M KCl. The active fractions were pooled.

Step 3. Ammonium sulfate fraction. Solid ammonium sulfate was added to the resulting enzyme solution to give 45% (mass/vol.) saturation. The pH was maintained at 7.5 with ammonia. After stirring for 4 h or more, the precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant to give 60% (mass/vol.) saturation. The suspension was then centrifuged, and the pellet was dissolved in 0.1 M buffer, followed by dialysis for 24 h against three changes of 2 liters 10 mM buffer.

Step 4. FPLC Mono-Q™ column chromatography. The enzyme solution from step 3 was applied to a Mono-Q™ HR 5/5 column equilibrated with 10 mM buffer, which was attached to a FPLC system (GP-250, Pharmacia, Sweden). After the column was washed with the same buffer, the enzyme was eluted by increasing the ionic strength of KCl in a linear fashion from 0 to 1 M in the same buffer, at a flow rate of 0.5 ml/min. The active fractions were pooled.

Step 5. FPLC Superose™12 column chromatography. The enzyme solution from step 4 was applied to a Superose™12 gel filtration column (1 cm x 30 cm) equilibrated with 0.1 M buffer. The rate of column elution was 0.5 ml/min using the FPLC system. The active fractions were combined.

Analytical measurements

SDS-PAGE was performed by the method of Laemmli (60). In order to estimate the molecular mass of the enzyme, the sample (20 μg) was applied to HPLC (Toyo Soda CO-8000 system; Tokyo, Japan) on a TSK G-3000SW column (0.75 x 60 cm; Toyo Soda, Japan), at a flow rate of 0.6 ml/min, with 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1 M Na2SO4 at room temperature. The absorbance of the eluate was recorded at 280 nm. The molecular mass of the enzyme was then calculated from the relative mobility compared to those of the standard proteins, glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome c (12.4 kDa) (products of Oriental
Yeast Co.). The purified amidase (1 mg in 1 mM potassium phosphate buffer, pH 7.5) was used directly for NH₂-terminal sequencing by automated Edman degradation with a Shimadzu protein sequencer PSQ-1 system equipped with a Wakosil phenylthiohydantoin column (4.6 x 250 mm; Wako Pure Chemical).

**Substrate specificity**

The standard reaction mixture (1 ml) was composed of 10 μmol potassium phosphate buffer (pH 7.5), 10 mmol amide and appropriate amount of the enzyme. The reaction proceeded at 30°C for 10-30 min, and was stopped by adding 0.1 ml 1 M HCl to the reaction mixture. The amount of NH₃ produced in the reaction mixture was colorimetrically estimated by the phenol/hypochlorite (61) method using a Conway micro-diffusion apparatus (62).

**Stereoisectivity**

The amounts of 2-phenylpropionic acid and 2-phenylpropionamide were assayed by HPLC under the same conditions as those used for the determination of benzoic acid with the exception that 205 nm instead of 230 nm was used as the detection wavelength. Diastereoisomeric amide derivatives of 2-phenylpropionic acid were determined at 254 nm. The configuration of 2-phenylpropionic acid was determined by a modification of the method of Hutt et al. (63). The solution (0.2 ml) containing no more than 2 μmol 2-phenylpropionic acid was mixed with 0.1 ml 1 M HCl and 0.75 ml CH₂Cl₂. After shaking and brief centrifugation, the water phase was evaporated and the remaining CH₂Cl₂ portion was reacted for 2 h at room temperature in a freshly prepared mixture (1 ml) with the following composition: S-(-)-1-(1-naphthyl)-ethylamine, 0.5 mg/ml; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 0.5 mg/ml; and 1-hydroxybenzotriazole anhydrous, 0.1 mg/ml. The sample was then dried in a rotary evaporator and resuspended in 0.2 ml of the above HPLC solvent. The retention times of the resultant diastereoisomeric amides of S-(+)- and R-(+)-2-phenylpropionic acids were 11.4 and 12.7 min, respectively.

**Acyl transferase activity**

The transferase activity was measured by estimating the amount of acylhydroxamate formed from hydroxylamine and an acyl donor. Amide, acid or ester was used as the acyl donor substrate. The standard reaction mixture (1 ml) was composed of 100 μmol potassium phosphate buffer (pH 7.5), 10 μmol of the acyl donor and 1 mmol hydroxylamine•HCl and an appropriate amount of the enzyme. The reaction proceeded at 30°C for 10-30 min and was stopped by adding 2 ml of FeCl₃ (8%, mass/vol.) in HCl (2%, mass/vol.). The extinction was read at 500 nm with a Shimadzu UV-240 spectrophotometer and related to the amount of hydroxamate formed in the reaction by comparison with a standard curve.

![Fig. 1. Construction of a set of plasmids for subcloning of the amidase gene. All of the restriction fragments were inserted at appropriate positions in pUC18/19. The location and direction of the lac promoter are indicated.](image-url)

**RESULTS**

**Locating the amidase gene by subcloning the downstream region of the L-NHase gene**

DNA fragments were prepared by digesting the cloned 9.4-kb insert of plasmid pNHJ20L (13) and inserted into pUC18 or pUC19 (Fig. 1). The ligated mixture was introduced by transformation into E. coli JM109 and ampicillin-resistant transformants were selected on 2 x YT agar medium containing 80 μg/ml ampicillin. These transformants were cultured and cell-free extracts were prepared as described in Materials and Methods. Enzyme assays using propionamide and benzamide as substrates for each cell-free extract showed that only the recombinant E. coli harboring pALJ10 had amidase activity and the transformants harboring the other plasmids had none. However, E. coli harboring pALJ10 and other subclones including pNHJ20L had no amidase activity in the presence of natural inducers (cobalt and cyclohexanecarboxamide) instead of IPTG. This finding suggested that a 1.96-kb EcoRI-SphI
region located 1.9 kb downstream of nhlA encoding the L-NHase α-subunit was essential for the expression of amidase activity in the presence of IPTG.

Nucleotide sequence of the amidase gene (amdA)

The sequencing strategy is shown in Fig. 2 and Fig. 3 shows the 1958-bp nucleotide sequence. The EcoRI-SphI fragment of pALJ10 contained an open reading frame encoding 515 amino acids (Mr = 54,626 Da) which started with ATG (methionine) and terminated with TAA. A typical Shine-Dalgarno sequence (64) was present 8 bp upstream from the initiation codon, but no consensus promoter sequences found in other prokaryotes (65) were observed in the upstream region. A strong hairpin structure (ΔG = -174 kJ/mol) just downstream of the termination codon of the open reading frame may serve as a ρ-independent transcriptional termination signal. The predicted amino acid sequence of the open reading frame was compared with those of amidases from Rhodococcus sp. N-774 (14), P. chlororaphis B23 (15), Rhodococcus sp. (42) and indole-3-acetamide hydrolase from Pseudomononas savastanoi (66) (Fig. 4). There was a 60.6% match of amino acids in 513 overlapping residues between R. rhodochrous J1 and Rhodococcus sp. N-774 whose nucleotide sequence was identical with that of Brevibacterium R312 (44).

Production of the amidase protein in E. coli

To produce the amidase in E. coli, a 1.7-kb NspV-SphI fragment was inserted between the AccI and SphI sites of pUC18, resulting in pALJ20 (Fig. 2). In this construction, the amidase gene (amdA) was under the control of the lac promoter. When E. coli harboring pALJ20 was cultivated in the presence of IPTG at 28°C or 37°C, amidase activity was detected in the supernatant of the sonicated cell-free extracts obtained at 12,000 x g. Culture condition of 12-h incubation at 28°C gave the highest activity [2.43 nmol·min⁻¹·(mg protein)⁻¹].
However, this amidase activity was even lower than that of *R. rhodochrous* J1 cultured in the presence of cyclohexancarboxamide and cobalt [13.5 nmol·min⁻¹·(mg protein)⁻¹].

To express the amidase gene at high level, we modified the sequence upstream of the ATG codon by means of PCR, using the recombinant plasmid as a template and the following two oligonucleotides as primers (Fig. 5). The sense primer contained a *SacI* recognition site, a ribosome binding site, a TAG stop codon in frame with the lac gene in pUC18 and 18 nucleotides of the amidase structural gene starting with the ATG start codon 9 nucleotides downstream of the ribosome binding site. The antisense primer contained 21 nucleotides of the gene (complementary to nucleotides 1824-1844 in Fig. 3) 61 nucleotides downstream from the end of the reading frame and a *HindIII* recognition site.

### Table 1. Amidase activities of *E. coli* transformants harboring pALJ30 under various conditions.

*E. coli* JM109 carrying pALJ30 was cultivated in 2× YT medium under various conditions. The inducer, IPTG, was added at a final concentration of 1 mM.

<table>
<thead>
<tr>
<th>Time for addition of inducer</th>
<th>Cultivation temperature</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>°C</td>
<td>nmol·min⁻¹·(mg protein)⁻¹</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>276</td>
</tr>
<tr>
<td>12</td>
<td>28</td>
<td>183</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>168</td>
</tr>
<tr>
<td>0</td>
<td>37</td>
<td>201</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>468</td>
</tr>
<tr>
<td>0</td>
<td>37</td>
<td>335</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>405</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>380</td>
</tr>
</tbody>
</table>

The PCR-product was inserted between the *SacI* and *HindIII* sites of pUC18, resulting in plasmid pALJ30, in which the amidase gene was under the control of the lac promoter. A protein corresponding to the predicted molecular mass of 54.6 kDa was synthesized only when the lac promoter was induced by IPTG. When *E. coli* JM109 harboring pALJ30 was cultivated for 7 h at 37°C, during which IPTG was added 4 h from the start, the level of amidase activity in the supernatant of the sonicated cell-free extracts of the transformant was 468 nmol·min⁻¹·(mg protein)⁻¹ (Table 1), which was much higher than that of *E. coli* containing pALJ20. At this time, as judged by quantitation of the SDS-PAGE track (Fig. 6) with a dual-wavelength

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**Fig. 4.** Comparison of the deduced amino acid sequences of amidases from various strains.

Amino acid sequences of amidases from *R. rhodochrous* J1 (J), *Rhodococcus* sp. N774 (N774), *P. chlororaphis* B23 (B23) and *Rhodococcus* sp. (Rho) and of indole-3-acetamide hydrolase from *P. savastanoi* (IndP) were aligned by introducing gaps (hyphens) to maximize identities. The dots indicate the presence of the same residue at each position.

**Fig. 5.** Sequences of oligonucleotide primers for the expression of the amidase gene.

Each sequence of the oligonucleotide primers is shown in the 5' to 3' end direction. The expression plasmid was constructed as described under Results. SD, ribosome-binding site.

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**Table 1.** Amidase activities of *E. coli* transformants harboring pALJ30 under various conditions.

*E. coli* JM109 carrying pALJ30 was cultivated in 2× YT medium under various conditions. The inducer, IPTG, was added at a final concentration of 1 mM.
TLC scanner (Shimadzu), the highest amount of amidase formed seemed to correspond to about 8% of the total soluble protein. When the transformant was cultivated at 37°C, a protein band corresponding to the amidase subunit was also observed in the precipitates of the sonicated cell-free extracts from the analysis of SDS-PAGE. The precipitates had no amidase activity, and so they may be inclusion bodies.

Purification of the amidase from E. coli transformant

Through the purification procedures described in Materials and Methods, the enzyme was purified with a yield of 30.4% from the cell-free extract of E. coli containing pALJ30, which was cultured under the optimum conditions for amidase activity, with benzamide as the substrate (Table 2). The purified enzyme showed only one band on SDS-PAGE. The purity of the enzyme preparation was also proven by HPLC on a TSK G3000SW column, which revealed a single symmetrical protein peak. The purified enzyme catalyzed the hydrolysis of benzamide to benzoic acid at 12.2 μmol·min⁻¹·(mg protein)⁻¹ under the standard reaction conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>14.10 mg</td>
<td>756 U</td>
<td>0.536 U/mg</td>
<td>100%</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>158 mg</td>
<td>490 U</td>
<td>3.10 U/mg</td>
<td>64.8%</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (45-60%)</td>
<td>75.4 mg</td>
<td>424 U</td>
<td>5.62 U/mg</td>
<td>56.1%</td>
</tr>
<tr>
<td>FPLC MonoQ HR5/5</td>
<td>29.6 mg</td>
<td>307 U</td>
<td>10.4 U/mg</td>
<td>40.6%</td>
</tr>
<tr>
<td>FPLC Superose 12</td>
<td>18.9 mg</td>
<td>230 U</td>
<td>12.2 U/mg</td>
<td>30.4%</td>
</tr>
</tbody>
</table>

Molecular mass and subunit structure

The molecular mass of the enzyme was determined to be 110 kDa by analytical HPLC. When the enzyme was treated with 1% (mass/vol.) SDS and 50 mM 2-mercaptoethanol, a single band was visualized by protein staining; the molecular mass corresponding to the band was estimated to be 55 kDa, consistent with that estimated from the nucleotide sequence. Thus, the enzyme probably consists of 2 subunits identical in molecular mass. The purified amidase from the recombinant E. coli had an NH₂-terminal sequence of Ser-Ser-Leu-Thr-Pro-Pro-Asn-Ser-Pro-Pro-Met-Ser-His-Phe-Arg-Phe. This is the same as that deduced from the DNA sequence with the exception that it lacks a N-terminal methionine.

Effect of temperature and pH

The activity was measured at various temperatures from 10 to 60°C and the optimum was found to be 55°C. Above 60°C, the enzyme activity was rapidly lost (Fig. 7-A). The effect of pH on the activity of the enzyme was examined using benzamide as the substrate (Fig. 7-B). The enzyme showed maximal activity at pH 7.9.

Stability

The enzyme was incubated for 30 min in 10 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol under various temperature conditions. Aliquots were removed and the amidase activity was assayed under the standard conditions. It exhibited the following
activity: 60°C, 0%; 55°C, 1.0%; 50°C, 2.7%; 45°C, 27%; 40°C, 48%; 35°C, 95%; 30°C, 100%; 25°C, 100%; 20°C, 100%. This enzyme was unstable at its optimal temperature (55°C). After the enzyme was incubated at 30°C for 20 min in buffers at various pH, an aliquot was assayed for amidase activity under the standard conditions. The enzyme was most stable in the broad pH range of 6.7-10.

Fig. 7. Effects of temperature and pH on amidase activity. 
(A) Reactions proceeded for 20 min at various temperatures. (B) Reactions proceeded for 20 min at 30°C in the following buffers (final concentration, 0.1 M): acetate ( ), potassium phosphate ( ), Tris/HCl ( ), borate ( ) and glycine ( ). The relative activity is expressed as a percentage of the maximum activity attained under the experimental conditions.

Inhibitors

The inhibitory effects of various compounds on the enzyme activity were investigated. Incubation proceeded at 30°C for 20 min in standard reaction mixtures containing test compounds at 1 mM. The enzyme was highly sensitive towards HgCl₂ and AgNO₃ (7 and 22%, respectively, of the original activity). Other metals such as FeSO₄, CuSO₄ and CdCl₂ were also inhibitory to some extent (58, 58 and 87%, respectively). The enzyme was inhibited by 5,5'-dithiobis(2-nitrobenzoic acid) and p-chloromercuribenzoic acid to some extent (71 and 64%, respectively), but other thiol reagents such as N-ethylmaleimide and iodoacetic acid had no significant effect. Phenylhydrazine caused appreciable inhibition (64%), but other carbonyl reagents such as cysteamine, DL-penicillamine, d-cycloserine and semicarbazide did not influence the activity. Chelating reagents including EDTA, 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt, o-phenanthroline, 8-hydroxyquinoline and α,α’-dipyridyl also had no significant effect on the enzyme.

Substrate specificity

The ability of the enzyme to catalyze the hydrolysis of various amide compounds was examined (Table 3). The synthesis of benzoic acid from benzamide, corresponding to 12.2 µmol.min⁻¹·(mg protein)⁻¹, was taken as 100%. Aliphatic amides such as propionamide, butyramide, isobutyramide, valeramide and capronamide were remarkably active as substrates. Aromatic amides such as benzamide, nicotinamide, 2-thiophenecarboxamide, m- or p-toluamide and 2-aminobenzamide were also hydrolyzed by the enzyme. When the Michaelis constants for benzamide and propionamide were estimated from Lineweaver-Burk plots, the former (KM = 0.15 mM) was found to exhibit somewhat higher affinity for the enzyme than did the latter (KM = 0.48 mM). Amino acids containing an amide group such as glutamine and asparagine were inactive as substrates, whereas L-methioninamide was a good substrate.

Table 3. Substrate specificity of the amidase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzamide</td>
<td>100</td>
<td>o-Hydroxybenzamide</td>
<td>2.6</td>
</tr>
<tr>
<td>Formamide</td>
<td>0</td>
<td>p-Hydroxybenzamide</td>
<td>14.4</td>
</tr>
<tr>
<td>Acetamide</td>
<td>23.8</td>
<td>Nicotinamide</td>
<td>55.9</td>
</tr>
<tr>
<td>Propionamide</td>
<td>236</td>
<td>Isocitroninamide</td>
<td>8.0</td>
</tr>
<tr>
<td>Butyramide</td>
<td>288</td>
<td>Pyrazinamide</td>
<td>11.8</td>
</tr>
<tr>
<td>Isobutyramide</td>
<td>656</td>
<td>2-Thiophenecarboxamide</td>
<td>54.2</td>
</tr>
<tr>
<td>Valeramide</td>
<td>519</td>
<td>2-Phenylacetamide</td>
<td>82.8</td>
</tr>
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<td>Pelvalamide</td>
<td>90.8</td>
<td>3-Indololacetamide</td>
<td>23.3</td>
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<td>Hexanamide</td>
<td>241</td>
<td>Urea</td>
<td>0</td>
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<tr>
<td>Acrylamide</td>
<td>40.6</td>
<td>Methylurea</td>
<td>25.9</td>
</tr>
<tr>
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<td>112</td>
<td>Ethyleneurea</td>
<td>8.1</td>
</tr>
<tr>
<td>Crotonamide</td>
<td>19.6</td>
<td>Phenyleurea</td>
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</tr>
<tr>
<td>Cyclohexanecarboxamide</td>
<td>61.3</td>
<td>L-Glutamine</td>
<td>0</td>
</tr>
<tr>
<td>Fluoroacetamide</td>
<td>0</td>
<td>D-Glutamine</td>
<td>0</td>
</tr>
<tr>
<td>Chloroacetamide</td>
<td>96.2</td>
<td>L-Asparagine</td>
<td>0</td>
</tr>
<tr>
<td>Lactamide</td>
<td>83.7</td>
<td>D-Asparagine</td>
<td>0</td>
</tr>
<tr>
<td>Malonamide</td>
<td>14.4</td>
<td>Glicinamide</td>
<td>8.5</td>
</tr>
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<tr>
<td>p-Aminobenzamide</td>
<td>15.3</td>
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</table>

Stereoselectivity

The ability of the enzyme to hydrolyze racemic amides to optically active acids was investigated. The optical purity of 2-phenylpropionic acid converted from (+/-) 2-phenylpropionamide by the amidase was determined as described in Materials and Methods.
Figure 8 shows the enantioselective hydrolysis of 2-phenylpropionamide to 2-phenylpropionic acid. Until 40 min from the start of the reaction, the S-enantiomer was selectively formed (enantiomer excess >95). This amidase appeared to be highly specific for the S-enantiomer of 2-phenylpropionamide. Enantioselectivity of the enzyme for 2-chloropropionamide was examined in the same way as used for 2-phenylpropionamide. R-(+)- and S-(−)-2-Chloropropionamides were converted to (+)- and (−)-2-chloropropionic acids at the same rate in the reaction time, suggesting that the enzyme cannot recognize the configuration of 2-chloropropionamide. Steric hindrance of the enzyme by the chlorine atom of the substrate was less effective upon the stereoselectivity than that by the phenyl group.

Acyl transferase activity

The relative activities of the transferase reactions (amide transferase, acid transferase and ester transferase) catalyzed by the amidase were determined. Only amide transferase activity was observed, whereas acid transferase and ester transferase activities were below the detection limits. Compared with that towards benzamide (100%), the activity towards propionamide was 145%. Other amides, namely acetamide (64%), butyramide (734%), isobutyramide (544%), valeramide (943%), acrylamide (542%), crotonamide (139%), nicotinamide (20%), isonicotinamide (26%) and pyrazinamide (39%) functioned as acyl-donors. The substrate spectrum of the amides as acyl donors was in close agreement with the finding of substrate specificity of the amidase, except that transferase activity using unsaturated aliphatic amides such as acrylamide and crotonamide as a substrate was relatively high.

DISCUSSION

In this section, the author describe the purification and characterization of the protein derived from the cloned amidase gene (amdA) linked to the L-NHase gene (dH/BA) in a "practical microorganism" R. rhodochrous J1, which produces two NHases (H-NHase and L-NHase) depending on the inducer. These NHases differ in substrate specificity: H-NHase preferentially acts on aliphatic nitriles, while L-NHase acts on aromatic nitriles as well as aliphatic nitriles as substrates. Under the conditions in which H-NHase is induced by urea, R. rhodochrous J1 cells cannot hydrolyze benzamide as a substrate. On the other hand, under the conditions in which L-NHase is induced by cyclohexanecarboxamide, the cells can hydrolyze benzamide as well as propionamide as a substrate (67). Furthermore, this purified amidase can hydrolyze aromatic amides as well as aliphatic amides as shown in Table 3. The L-NHase and this amidase showed similar trend in substrate specificity for aliphatic and aromatic compounds: for example, L-NHase acts on propionamide (100%), benzonitrile (75%) and 3-cyanopyridine (50%), while this amidase acts on propionamide (100%), benzamide (42.4%) and nicotinamide (23.7%). These findings suggest that amidase activity derived from the present amidase gene was detected along with L-NHase activity; both enzymes seemed to be induced by the same inducer and cooperate in degrading several nitriles efficiently.

The author found amdA 1.9 kb downstream of dH/BA. This is the first demonstration that an amidase gene involved in nitrile metabolism locates downstream from the NHase gene. In Rhodococcus sp. N-774 (14) and another (42) and P. chlororaphis B23 (15), each amidase gene is found in the same orientation and just upstream from the genes coding for α- and β-subunits of each NHase. There are two patterns in gene order of nitrile hydratase subunits. In Rhodococcus sp. N-774 and P. chlororaphis B23, the order is amidase-NHase(α-β), whereas the order is amidase-NHase(β-α) in Rhodococcus sp. In this way, gene construction of the nitrile metabolism operon is variously organized. In order to examine the possibility that another amidase gene may be located upstream from dH/BA, cloning of the upstream region of dH/BA by a gene walking method is now in progress. In P. chlororaphis B23, two open reading frames (P47K and OrfE) are present just downstream of the NHase β-subunit gene. The additional sequence of a 38 kDa protein, which is expressed in E. coli transformant...
harboring NHase-amidase expression plasmid (pPCN4), is also found just upstream of the amidase gene (15). Although nitrile metabolism appears to be simple in the two step reaction of NHase and amidase, other open reading frames such as P38K, P47K and OrfE also might have some functions in the nitrile degradation pathway in *P. chlororaphis* B23. The region between *nhBA* and *amidA* is of about 1.9 kb long enough to contain one or more open reading frames, which may be involved in nitrile metabolism or control of the expression of L-NHase or amidase. Further analysis of this region is needed for the interpretation of the L-NHase-amidase operon.

*Pseudomonas aeruginosa* amidase can hydrolyze formamide, acetamide propionamide, hydroxycetamide and acrylamide but cannot hydrolyze butyramide, isobutryamide, valeramide and lactamide (39). The *Arthrobacter* amidase can act on acetamide, acrylamide and propionamide but cannot hydrolyze formamide, butyramide, valeramide, isobutryamide, methacrylamide, malonamide, succinamide, lactamide, benzamide and nicotinamide (68). The *Brevibacterium* sp. R312 wide spectrum amidase can degrade all the amides mentioned above, but the activities for butyramide, isobutryamide, valeramide and benzamide are very low (69).

On the other hand, the *R. rhodochrous* J1 amidase had a wider substrate specificity. Thus, the *R. rhodochrous* J1 amidase is distinct from the above-mentioned enzymes. Furthermore, the *R. rhodochrous* J1 amidase showed enantiomer-selectivity toward 2-phenylpropionamide as the substrate. Purification and characterization of enantiomer-selective amidases from *Brevibacterium* sp. R312 (44) and *Rhodococcus* sp. (42) and cloning of their genes has been reported. Each amidase from these strains is also genetically coupled with each nitrile hydratase, indicating that the enantioselectivity is probably a common feature among the amidas linked with the NHases. However, H- and L-NHases cannot act upon various nitriles enantioselectively (unpublished results). Yamamoto et al. (70) reported the production of S-(+)-2-(4′-isobutylphenyl) propionic acid [S-(+)-ibuprofen], which is useful as a non-steroidal anti-inflammatory drug, from racemic 2-(4′-isobutylphenyl)propionitrile by *Acinetobacter* sp. AK226 nitrilase. Similar reactions can proceed by using a NHase followed by an enantio-selective amidase. The recombinant amidase from *R. rhodochrous* J1 might be useful in the production of higher-value acids from the corresponding nitriles or amides in cooperation with a NHase.

Amidases coupled with NHase have been studied at the gene level in *Rhodococcus* species including N-774, *P. chlororaphis* B23, *Brevibacterium* sp. R312 but their activities regarding transfer of the acylgroup from amides to hydroxylamine have not been mentioned.

The present amidase demonstrated acyl transferase activity as efficient as that of amidases, which are considered not to be involved in nitrile metabolism, such as the wide spectrum amidase from *Brevibacterium* sp. R312 (69) and the aliphatic amidase from *P. aeruginosa* (39).

Under the optimum culture conditions for acrylamide production, *R. rhodochrous* J1 produces a large amount of H-NHase (50% of the total soluble protein) in cell-free extracts whereas amidase activity is very low. Judging from the sequential nitrile degradation by NHase and amidase, and the gene construction of both enzymes from *Rhodococcus* species including N-774, *P. chlororaphis* B23 and here, we consider that *R. rhodochrous* J1 has an amidase, which is induced together with H-NHase by cobalt ions and urea. The amidase gene linked to the H-NHase gene is now being studied.

**SUMMARY**

The cloned 9.4-kb insert of plasmid pNHJ20L containing low-molecular-mass nitrile hydratase (L-NHase) gene from *Rhodococcus rhodochrous* J1 was digested with various restriction enzymes, and the trimmed fragments were inserted into pUC18 or pUC19. A 1.96-kb EcoRI-SphI region located 1.9 kb downstream of the L-NHase gene was found to be essential for the expression of amidase activity in *Escherichia coli*; the gene arrangement of the amidase and the NHase in *R. rhodochrous* J1 differed from those in *Rhodococcus* species including N-774, *Pseudomonas chlororaphis* B23. The nucleotide-determined sequence indicated that the amidase consists of 515 amino acids (54,626 Da) and the deduced amino acid sequence of the amidase had high similarity to those of amidases from *Rhodococcus* species including N-774 and *P. chlororaphis* B23 and to indole-3-acetamide hydrolase from *Pseudomonas savastanoi*.

The amidase gene (*amidA*) modified in the nucleotide sequence upstream from its start codon expressed 8% of the total soluble protein in *E. coli* under the control of lac promoter. The level of amidase activity in cell-free extracts of *E. coli* was 0.468 units/mg using benzamide as a substrate. This amidase was purified to homogeneity from extracts of the *E. coli* transformant with 30.4% overall recovery. The molecular mass of the enzyme estimated by HPLC was about 110 kDa, and the enzyme consists of two subunits identical in molecular mass (55 kDa). The enzyme acted upon aliphatic amides such as propionamide and also upon aromatic amides such as benzamide. The apparent Km values for propionamide and benzamide were 0.48 and 0.15 mM, respectively. This amidase was highly specific for the S-enantiomer.
of 2-phenylpropionamide, but could not recognize the configuration of 2-chloropropionamide. It also catalyzed the transfer of an acyl group from an amide to hydroxylamine to produce the corresponding hydroxamate.

Section 3 Regulatory genes required for the amide-dependent induction of L-NHase

In microorganisms that catabolize nitriles by NHase, this enzyme, if inducible, is generally induced by amides (reaction products), not by nitriles (reaction substrates): an interesting unique phenomenon (10). *Rhodococcus rhodochrous* J1 produces two kinds of NHases: high and low molecular-mass NHases (H-NHase and L-NHase), which exhibit different physicochemical properties and substrate specificities; both H- and L-NHases are composed of α- and β-subunits (α differs in size from β in each case, and the α- and β-subunits of H-NHase differ from those of L-NHase). 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 (10). Using immobilized cells containing H-NHase, the industrial production of acrylamide from acrylonitrile was started in 1991 (30,000 tons/year); this is the first case in which biotechnology was applied in the petrochemical industry and also the first successful example of the introduction of an industrial bioconversion process for the manufacture of a commodity chemical. Furthermore, the industrial production of a vitamin nicotinamide from 3-cyanopyridine using cells containing L-NHase, which is induced by crotonamide, is due to start in Europe in 1997. Both H- and L-NHases contain cobalt ions as a cofactor in contrast with ferric-NHases from *Rhodococcus* sp. N-774 (10) and *Pseudomonas chlororaphis* B23 (71), which had been used for the acrylamide manufacture as 1st- and 2nd-generation strains, respectively.

Both H- and L-NHase genes (*nhhBA* and *nhlBA*) were cloned from *R. rhodochrous* J1 and sequenced (13). In each of *nhhBA* and *nhlBA*, an open reading frame (ORF) for the β-subunit (*nhhB* and *nhlB*) is located just upstream of that for the α-subunit (*nhhA* and *nhlA*). These arrangements of the coding sequences are reverse of the order found in the NHase genes of *Rhodococcus* sp. N-774 (13), *P. chlororaphis* B23 (15) and *Rhodococcus erythropolis* JCM6823 (43). In *R. rhodochrous* J1, The author has found an amidase gene (*amdA*) 1.9-kb downstream of *nhlA* (72); however, in *Rhodococcus* sp. N-774 (14), *P. chlororaphis* B23 (15), *Brevibacterium* sp. R312 (44) and *Rhodococcus* sp. (42), each amidase gene is found just upstream from the genes coding for α-subunit of each NHase. In this manner, construction of the genes responsible for nitrile metabolism is variously organized, suggesting that gene rearrangement had occurred in the genomes of these microorganisms.
Research into the regulatory system of *Rhodococcus* species has recently started by use of a transformation system with a *Rhodococcus*-E. coli shuttle vector (31). In the *nhhBA* gene cluster, the author has found two ORFs (*nhhC* and *nhhD*), which play a positive regulatory role in the process of the H-NHase formation (73). *NhHC* shares a homology with AmIC, a regulator protein for an aliphatic amidasase gene (*amIC*) from *Pseudomonas aeruginosa* (33) and *NhHD* has similarity to possible repressors MarR (34) and HpcR (35) from *E. coli*. However, the H-NHase gene cluster does not contain an amidasase gene. There are no reports on the relationship between NHase and amidasase from a standpoint of the gene-regulation mechanism.

In this section, genes required for the amide-dependent induction of *nhhBA* have been identified by using the transformation system in *Rhodococcus*. The author has also shown the coregulation of *nhhBA* and *amLA* in the experiment using various deletion-mutants.

**MATERIALS AND METHODS**

**Strains and plasmids**

*R. rhodochrous* J1 was previously isolated from soil (29). *E. coli* JM109 was the host for pUC plasmid transformation and phage M13 mp18/19 propagation (30). *R. rhodochrous* ATCC12674 was the host for a *Rhodococcus*-E. coli shuttle vector plasmid pK4 (31) and its derivatives, and used for *nhhBA* expression. The plasmid pNH20L (13) carrying *nhhBA* of *R. rhodochrous* J1 in the 9.4-kb *SacI* fragment on pUC19 was used for subcloning and sequencing of genes.

**Enzymes and chemicals**

Restriction endonuclease, T4 DNA ligase and *E. coli* alkaline phosphatase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Toyobo Co. Ltd. (Osaka, Japan). [γ-32P]ATP (180 TBq/mmol) and [α-32P]dCTP (110 TBq/mmol) were from Amersham (Tokyo, Japan). All other chemicals used were from commercial sources and were of reagent-grade.

**DNA manipulation**

Total DNA of *R. rhodochrous* J1 was prepared as described previously (13). Plasmid DNA was routinely prepared by the alkaline lysis method (30) and, if necessary, was purified by ethidium bromide-cesium chloride centrifugation. DNA manipulation was performed essentially as described by Sambrook et al. (30). The DNA sequence was determined by the dideoxynucleotide chain termination method (58). [α-32P]dCTP and Sequenase (United States Biochemicals, Cleveland, USA) or [γ-32P]ATP and a 7th Sequence kit (Toyobo) were used for sequencing.

**Transformation of *R. rhodochrous* ATCC12674 by electroporation**

A mid-exponential culture of *R. rhodochrous* ATCC12674 was centrifuged at 6,500 x g for 10 min at 4°C and washed three times with demineralized cold water. Cells were then concentrated 20-fold in the demineralized cold water and kept on ice. Ice-cold cells (100 μl) was mixed with 1 μg DNA in 1 μl of TE buffer (10 mM-Tris/1 mM EDTA, pH 8.0) in a 1-mm-gapped electroporation cuvette (Bio-Rad, Richmond, USA), 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, 400Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (31) 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 24 h in MYP medium containing 0.01% (w/v) CoCl₂·6H₂O with (2 g/l) or without crotonamide at several concentrations, harvested by centrifugation at 4,000 x 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 n-butric acid, disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo, Japan), and centrifuged at 12,000 x g for 10 min at 4°C. The resulting supernatants were used for the enzyme assay. NHase activity was assayed in a reaction mixture (2 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 6 mM benzonitrile and an appropriate amount of the enzyme. The reaction was carried out at 20°C for 10 min and stopped by the addition of 0.2 ml 1 M HCl. The amount of benzamidase formed in the reaction mixture was determined as described previously (13). Amidase activity was assayed in a reaction mixture (1 ml) consisting of 10 mM potassium phosphate buffer (pH 7.5), 10 mM benzamide and an appropriate amount of the enzyme. The reaction was carried out at 30°C for 30 min and stopped by the addition of 0.1 ml 1 M HCl. The amount of benzonic acid formed in the reaction mixture was determined as described previously (72). One unit of these enzymes was defined as the amount catalyzing the formation of 1 μmol of benzamide and benzoic
acidity/min from benzonitrile and benzamide, respectively, under the above conditions. Protein
was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (59)
using a dye reagent supplied by Bio-Rad.

Western blot analysis

The anti-(L-NHase) antiserum and the anti-(amidase) antiserum were raised in young
white female rabbits immunized with the L-NHase purified from R. rhodochrous J1
(unpublished results) and the amidase purified from E. coli JM109/pALJ30 (72), respectively.
Cell extracts prepared by sonication were applied onto SDS-polyacrylamide gel, and transferred
to a nitrocellulose membrane by a standard procedure (30). Western blots were probed with
anti-(L-NHase) antiserum or anti-(amidase) antiserum and then with anti-rabbit IgG conjugated
to horseradish peroxidase. Probing with antibodies and color development were carried out as
described by the supplier, Bio-rad.

Computer analysis of amino acid sequences

The DNA sequence was analyzed using the GENETYX sequence analysis program
(Software Development Co., Tokyo, Japan). A search of the National Biomedical Research
Foundation (NBRF) protein sequence data bank for sequence similarities was carried out with
the BLAST algorithm.

RESULTS

Cloning of the 5' upstream region of nhlBA

The cloning and characterization of the L-NHase gene (nhlBA) of Actinomycete R.
rhodochrous J1 has previously been described (13). The author cloned the upstream region of
nhlBA by the DNA-probing method with a 1.35-kb fragment, which was isolated from
pNHJ20L digested by SacI plus EcoRI, as a probe (Fig. 1). Southern hybridization using this
probe against total DNA from R. rhodochrous J1 digested with EcoRI revealed that this probe
hybridized with a single 7.5-kb fragment (data not shown). This DNA fragment was separated
by agarose gel electrophoresis, ligated with pUC18 digested with EcoRI, and introduced into
E. coli JM109 by transformation. Colony hybridization with the probe for screening
ampicillin-resistant transformants containing the restriction fragment yielded pNLU10 (Fig. 1).
This plasmid pNLU10 contained the 7.5-kb fragment derived from R. rhodochrous J1 DNA.

Analyses by restriction endonuclease and by sequencing of the fragment showed that two
inserts from pNLU10 and pNHJ20L shared a common 1.35-kb SacI-EcoRI region. Plasmid
pNLUD30 was constructed by inserting a 6.15-kb EcoRI-SacI fragment from pNLU10 and a
9.4-kb SacI fragment from pNHJ20L into the EcoRI-SacI sites of pBluescriptSK+ (Toyobo).

Expression of nhlBA in R. rhodochrous ATCC12674

To identify the sequence elements required for the amide-dependent expression of
nhlBA, we constructed a set of plasmids (pLJK10-pLJK60) (Fig. 1). Plasmids pLJK10,
pLJK30 and pLJK40 contained blunt-ended 15.55-kb, 12.8-kb and 11.3-kb fragments,
respectively, from pNLUD30, in the blunt-ended EcoRI site of the Rhodococcus-E. coli shuttle
vector pK4. Plasmids pLJK20 and pLJK60 were constructed by inserting 8.3-kb and 3.1-kb
KpnI fragments, respectively, from pNLUD30 into the KpnI site of pK4. Plasmid pLJK50
contained a 5.5-kb PstI fragment from pNLUD30 in the PstI site of pK4. These plasmids were
used to transform R. rhodochrous ATCC12674 and the resulting transformants were cultured
in the CoCl2-containing medium in the presence or absence of crotonamide. In this experiment,
nhlBA expression was induced by crotonamide, which induces a greater amount of L-NHase.

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Fig. 1. Genetic organization of the L-NHase gene cluster and construction of a set of plasmids (left panel) and
NHase activity of each R. rhodochrous ATCC12674 transformant (right panel).
(Left panel) For clarity, only restriction sites discussed in the text are shown. nhlB and nhlA encode L-NHase β-
and α- subunits, respectively, and ωnA encodes an amidase (72). nhid and ωdC are the newly
identifed genes described in this section. The probe used in the experiment are shown by box. Various deletion-
plasmids are diagrammed below the restriction maps. (Right panel) NHase activity of whole cells, which were
cultivated in the medium with (2 g/l) or without crotonamide, was detected as described in Materials and
Methods using benzonitrile as a substrate. +, much; -, trace.
and

The L-NHase formation in *R. rhodochrous* ATCC12674 transformants was examined by SDS-PAGE and Western blot analysis (Fig. 2). Immunostaining of the Western blots in this experiment showed two bands of 29 and 26 kDa corresponding to the accumulated protein detected by Coomassie brilliant blue staining. No immunoreacting bands were detected in the transformant was cultured in the medium without crotonamide. Expression of NHase activity shown above was dependent on the addition of cobalt ions into the medium, because *R. rhodochrous* ATCC12674 transformants cultured in the medium without cobalt ions had no NHase activity (data not shown). 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 CoCl₂ and crotonamide.

![Western blot of a similar gel after immunostaining with antibodies for the L-NHase.](image)

**Table 1.** NHase activity in cell-free extracts of *R. rhodochrous* ATCC12674 transformants containing various plasmids.

<table>
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<th>Plasmid</th>
<th>NHase activity (U/mg)</th>
<th>Crotonamide</th>
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</thead>
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<td>pK4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
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<td>pLJK10</td>
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N.D.: Not detected

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</table>

N.D.: Not detected

The L-NHase formation in *R. rhodochrous* ATCC12674 transformants was examined by SDS-PAGE and Western blot analysis (Fig. 2). Immunostaining of the Western blots in this experiment showed two bands of 29 and 26 kDa corresponding to the accumulated protein detected by Coomassie brilliant blue staining. No immunoreacting bands were detected in the transformant carrying pK4 without any inserts, even though the transformant was cultured in the medium supplemented with crotonamide. Expression of NHase activity shown above was dependent on the addition of cobalt ions into the medium, because *R. rhodochrous* ATCC12674 transformants cultured in the medium without cobalt ions had no NHase activity (data not shown). Moreover, none of the *E. coli* JM109 harboring pK4-

![Western blot of a similar gel after immunostaining with antibodies for the L-NHase.](image)
family, for example, BraC from 

P. aeruginosa (74). NhIC also showed a relatively weak match with BraC (17.2% identity, 43.3% similarity) (Fig. 4-A).

and these homologs is relatively low, they are of similar size (112-125 amino acid residues) and 11 consensus amino acid residues are distributed all over the sequence (Fig. 4-B).

The second ORF named nhID, is 336 nucleotides long, and would encode a protein of 112 amino acids. A computer-aided FASTA search of the SwissProt protein data base indicated that nhID showed similarity to regulatory genes, merR from Streptomyces lividans (75) (24.8% identity, 44.8% similarity), cadC from Staphylococcus aureus (76) (24.7% identity, 43.0% similarity) and arsR from E. coli (77) (24.3% identity, 45.9% similarity). These genes homologous to nhID are all located upstream of the heavy metal resistance genes (merAB, cadA and arsBC, respectively) and are supposed to have transcriptional regulatory functions for the resistance genes. Although the identity between NhID (the product of nhID) and these homologs is relatively low, they are of similar size (112-125 amino acid residues) and 11 consensus amino acid residues are distributed all over the sequence (Fig. 4-B).

There are extensive noncoding sequences between nhID and nhIC (357 bp) and nhIC and nhIB (884 bp), possibly indicating regulatory independence of these three genes thus separated.
The requirement of nhIC and nhID for the amide-dependent expression of nhIBA

The transformant carrying pLJK30 exhibited the amide-inducible L-NHase expression, whereas the transformant carrying pLJK40, which excludes nhID, expressed L-NHase constitutively (Fig. 1, Table 1). Considering that nhIC is present in both pLJK30 and pLJK40, in the presence of amide as an inducer, nhID functions negatively, whereas nhIC functions positively for the L-NHase expression. To examine the necessity of nhIC for nhIBA expression, a plasmid pLJK70 containing the 1477-bp EcoRV.1-EcoRV.2 fragment in the blunt-ended XbaI site of pLJK60 was constructed (Fig. 1). The transformant harboring pLJK70 greatly decreased NHase activity (0.291 and 0.225 U/mg-protein for the uninduced and the induced cells, respectively) compared to the transformant harboring pLJK60, irrespective of the presence of crotonamide in the culture medium. This finding and the above obtained using pLJK30 and pLJK40 suggest that when amide is added to the culture medium, NhIC inhibited the action of repressor NhID, leading to the L-NHase expression, while NhIC could not function in the absence of amide to the medium, leading to repression of the L-NHase expression by NhID.

Table 2. Amidase activity in cell-free extracts of R. rhodochrous ATCC12674 transformants containing various plasmids.

<table>
<thead>
<tr>
<th>Amidase activity (U/mg)</th>
<th>Crotonamide</th>
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<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>pLJK10</td>
<td>N.D.</td>
</tr>
<tr>
<td>pLJK30</td>
<td>N.D.</td>
</tr>
<tr>
<td>pLJK40</td>
<td>0.111</td>
</tr>
<tr>
<td>N.D.: Not detected</td>
<td></td>
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</tbody>
</table>

Expression of the L-NHase and amidase genes

Of seven pLJK plasmids constructed above, pLJK10, pLJK30 and pLJK40 contain intact amdA. Assay of amidase activity using benzamide as a substrate (Table 2) and Western blot analysis with anti-(amidase) antiserum (Fig. 5) for the transformants harboring each of these three plasmids showed that the transformants carrying pLJK10 or pLJK30 inducibly produced amidase in the presence of crotonamide in the culture medium, but the transformant carrying pLJK40 produced amidase constitutively. This expression pattern of the amidase is the same as that of L-NHase (Table 1), suggesting that both nhIBA and amdA are coordinately controlled or cotranscribed in a single mRNA in spite of the relatively long intervening space (1.9 kb). Sequence analysis and characterization of the 1.9-kb region are now in progress.

Similarity among NhID and the bacterial transcriptional regulators, MerR, CadC and ArsR for the metal resistance suggested that the induction of L-NHase and amidase could be affected by the presence of cobalt ions, which is indispensable for the formation of catalytically active L-NHase. To test this possibility, we further investigated the formation of L-NHase and amidase of the transformant carrying pLJK10 cultured in the presence or absence of cobalt ions and in the presence or absence of crotonamide. Western blot analyses with anti-(L-NHase) antiserum (Fig. 6-A) and anti-(amide) antiserum (Fig. 6-B) for the transformant demonstrated that the formation of each L-NHase and amidase was affected only by the presence of the inducer amide and not by the presence of cobalt ions. Assay for amidase activity of the four kinds of cells cultured as described above agreed with the results of the Western blot analyses; the extracts of cells cultured in the crotonamide-containing medium in the presence and in the absence of cobalt ions showed amidase activities of 0.043 and 0.037 U/mg, respectively, although those in the medium without crotonamide showed no detectable amidase activity irrespective of the presence of cobalt ions. Since cobalt ions are indispensable for the formation of catalytically active L-NHase, we could not investigate the effect of cobalt ions on the expression of nhIBA by measuring NHase activity.
are products of the nitrile hydration reaction catalyzed by NHase. The finding that the nhlC (amiC-homolog) is responsible for the amide-inducible expression of nhlBA is very interesting, while similarity in amino acid sequence is not observed among amide-degrading enzymes; AmiE (the *P. aeruginosa* amidase) (80) does not show any similarity to AmdA from *R. rhodochrous* J1. Assuming that NhlC as well as AmiC functions as a sensor protein sensitive to amide compounds in the culture medium, we suggest that nhlC is involved in induction of the L-NHase synthesis in some way. nhlC has higher similarity with *nhlC* than with *amiC*.

The author showed the coordinate expression of *nhlBA* and *amdA* in the experiments including enzyme assays and Western blot using various transformants. It has been already reported (72) that L-NHase and the amidase showed similar trends in substrate specificity for aliphatic and aromatic compounds: L-NHase acts on benzonitrile (the synthesis of benzamide from which was taken as 100%), 3-cyanopyridine (66.7%), *n*-capronitrile (200%), methacrylonitrile (97.3%) and crotononitrile (28.0%), while the amidase acts on benzamide (the synthesis of benzoic acid from which was taken as 100%), nicotinamide (55.9%), *n*-capronamide (241%), methacrylamide (112%) and crotonamide (19.6%). These findings suggest that both enzymes are induced by the same inducer (amide) and then cooperate in degrading nitriles efficiently.

**DISCUSSION**

In this section, the author reports two ORFs (*nhlC* and *nhlD*), which are located upstream *nhIB* in *R. rhodochrous* J1. This gene organization is distinct from that of each NHase gene which has so far been reported. *nhlC* seems to play a positive role for formation of active L-NHase in the presence of amide-inducer. Amino acid sequence deduced from *nhlC* has marked similarity 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 (39,78). Formation of the *Pseudomonas* amidase derived from the *amiE* gene is positively regulated by AmiR via a transcription anti-termination mechanism (79), and negatively regulated by AmiC which is considered to function by inhibiting the action of AmiR through protein-protein interaction. The AmiC protein has been shown to bind acetamide in equilibrium dialysis studies, and therefore appears to respond to the presence of amides as a sensor protein (40). L-NHase is also induced by amide compounds (not by nitriles), *i.e.*, acetamide, propionamide, acrylamide, methacrylamide, crotonamide and cyclohexane-carboxamide, which

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Fig. 6. Effects of cobalt ions and crotonamide on the formation of L-NHase and amidase proteins.

*R. rhodochrous* ATCC12674 harboring pJK10 was cultured in the medium with (+) or without (-) CoCl₂·6H₂O (0.01 g/l) and with (+) or without (-) crotonamide (2 g/l).

Western blot analysis was carried out using anti-(L-NHase) antiserum (A) and anti-amidase antiserum (B). Lane 1, cell-free extracts prepared from the transformant cultured in the medium without cobalt ions and crotonamide; lane 2, with cobalt ions and without crotonamide; lane 3, without cobalt ions and with crotonamide; lane 4, with cobalt ions and crotonamide.

**Fig. 6.** Effects of cobalt ions and crotonamide on the formation of L-NHase and amidase proteins.
active form. These findings raise the possibility that the repressor NhlD might have lost a function as a heavy metal sensor during the course of evolution.

![Diagram of gene clusters nhlDCBA and nhhCDEFBA in R. rhodochrous J1.](image)

**Fig. 7.** Organization of the gene clusters nhlDCBA and nhhCDEFBA involved in nitrile metabolism in R. rhodochrous J1. Amino acid sequence similarity (percentages of identity) between equivalent genes of both gene clusters are indicated. nhlBA and nhhBA encode each of β- and α-subunits of L-NHase and H-NHase, respectively. Possible transcriptional terminators are indicated by solid triangles.

Fig. 7 compares the organization of nhlBA and nhhBA and their flanking genes. In nhh genes, both nhhC and nhhD are required for nhhBA expression, whereas nhhE and nhhF seem to have no effect on nhhBA expression. nhhF encodes a possible transposase and constitutes a possible insertion sequence (IS1164) together with its flanking inverted repeats (84), and nhhE is similar to the gene (ORF5) found in a potential transposable element R46 (85). Compared to the arrangement of the nhl gene cluster, two extra nhh genes (nhhE and nhhF) exist in the region between the regulatory and structural genes; the insertion event of the nhhEF unit into the upstream region of nhhBA occurred in the course of evolution. Considering the gene order and relative high homology of both nhICBA and nhhCBA, together with the finding that both nhIC and nhhC may play roles in the L- and H-NHases expression, respectively, although nhID is different from nhhD in their structure and regulatory function, it is likely that the gene duplication took place in the R. rhodochrous J1 genome, after which extra genes were added.

**SUMMARY**

The 3.5-kb of 5'-upstream region from nhlBA encoding a cobalt-containing low molecular-mass nitrile hydratase (L-NHase) from Rhodococcus rhodochrous J1 was found to be required for the amide-dependent expression of nhlBA in experiments using a Rhodococcus transformation system. Sequence analysis of the 3.5-kb fragment revealed the presence of two open reading frames (nhlD and nhlC) in this fragment. NhlD has similarity to regulators MerR, CadC and ArsR. NhlC has similarity to the regulators AmiC for the expression of an aliphatic amidase from Pseudomonas aeruginosa and NhBC for the expression of a high molecular-mass nitrile hydratase from R. rhodochrous J1. Assay for NHa activity of transformants carrying nhlD deletion or nhlC deletion suggests a negative regulatory role for nhlD and a positive regulatory role for nhlC in the process of the L-NHase formation. Assay for NHase and amidase activities and Western blot analysis of each Rhodococcus transformant carrying various deletion-plasmid, have shown that nhlBA and amdA encoding an amidase, which is located 1.9-kb downstream of nhlBA, were regulated in the same manner. These findings present the genetic evidence for a novel gene cluster of L-NHase induced by the reaction product (amide) in the 'practical microorganism' R. rhodochrous J1.
Section 4  Cobalt transporter linked to L-NHasef

Cobalt is necessary as a trace element for all cells but is toxic at higher concentrations, a fact of considerable environmental importance. It is the central metal cofactor in the corrin ring of vitamin B12 (86) and also plays an important role in biological functions. Methionyl aminopeptidase, which catalyzes the removal of the initiator methionine from nascent polypeptide chains, contain cobalt ions in both prokaryotes and eukaryotes; the N-terminal modification caused by this enzyme appears to be involved in functional regulation, intracellular targeting and protein turnover, although its physiological importance is incompletely understood (87).

Methylmalonyl-CoA carboxytransferase from Propionibacterium shermanii (88) and glucose isomerase from Streptomyces albus (89) are also cobalt-containing enzymes.

Several transition metals, which play an essential role as cofactors in many biochemical processes, must be transported into cells against concentration gradients, i.e., trace concentrations outside and substantial amounts within the cells. Divalent cations of Zn2+, Co2+, Ni2+ and Cd2+ are transported into the cells by a broad-substrate-range Mg2+ transport system in Alcaligenes eutrophus (90). The transport of a broad range of metal ions by the relatively unspecific uptake system is an economical solution for most cells and allows the accumulation of trace elements inside the cells for future needs. On the contrary, there seem to be other transport systems for Zn2+, Co2+ and Ni2+ with higher ion selectivity (91,92). The Ni-specific transporter was identified as a part of the plasmid-encoded hydrogenase (a Ni-containing enzyme) gene cluster in A. eutrophus (93). However, there are no reports on the structure and function involved in the uptake of cobalt ions other than by the broad specificity Mg2+ transport system in both prokaryotes and eukaryotes.

Nitrile hydratases (NHase; EC 4.2.1.84), which catalyze the hydration of nitriles to the corresponding amides followed by their conversion to the acids plus ammonia by amidase, contain cobalt atoms in an actinomycete Rhodococcus rhodochrous J1 (10). Culture of this strain in a medium containing urea and cyclohexanecarboxamide in the presence of cobalt ions, results in the respective production of a high molecular-mass NHase (H-NHase) and a low molecular-mass NHase (L-NHase) with different physicochemical properties and substrate specificities are selectively induced, respectively. Both H- and L-NHases are composed of α- and β-subunits (α differs in size from β in each case, and the α- and β-subunits of H-NHase differ from those of L-NHase).

In the nhlBA gene cluster, the author has found two ORFs (nhIC as a positive regulator and nhlD as a negative regulator required for the amide-dependent induction of nhlBA)(94). An amidase gene (amdA) is located 1.9-kb downstream of nhlA (72), and the expression of nhlBA and amdA is coordinately regulated (94).

In this section, the author describes the identification of a gene, nhlF, which is situated between nhlBA and amdA and is similar to the bacterial genes encoding nickel transporters previously reported. Furthermore, the author presented evidence that the product of nhlF, NhIF transports cobalt ions into the Rhodococcus and Escherichia coli host cells. By use of the transformation system in Rhodococcus, the transporter specific for cobalt ions has been also characterized.

MATERIALS AND METHODS

Strains, plasmids and media

E. coli JM109 (30) was used as the host strain for recombinant plasmids. R. rhodochrous ATCC12674 was the host for a Rhodococcus-E. coli shuttle vector pK4 (31) and its derivatives, and was used for the expression of the L-NHase gene (nhlBA) and the presumed cobalt transporter gene (nhlF). E. coli transformants were grown in LB medium (30). R. rhodochrous ATCC12674 transformants were grown in MYP medium (31).

Enzymes and chemicals

Restriction endonucleases, calf intestine alkaline phosphatase and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd. Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Wako Pure Chemicals (Tokyo, Japan). [α-32P]dCTP (110 TBq/mmol), [γ-32P]ATP (180 TBq/mmol) and 57CoCl2 (17.3 TBq/mmol) were from Amersham Japan. Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) was from Nakalai (Kyoto, Japan). 3,5-Di-tert-butyl-4-hydroxybenzilidenemalononitrile (SF6847) was kindly provided from Dr. H. Miyoshi. All other chemicals were of the highest purity commercially available.

DNA manipulation

DNA manipulation was performed essentially as described by Sambrook et al. (30). The DNA sequence was determined by the dideoxynucleotide chain termination method (58).
[α-32P]dCTP and Sequenase (United States Biochemicals, Cleveland, USA) or [γ-32P]ATP and a T7h Sequence kit (Toyobo, Osaka, Japan) were used for sequencing.

Transformation of R. rhodochrous ATCC12674 by electroporation

A mid-exponential culture of R. rhodochrous ATCC12674 was centrifuged at 6,500 x 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 DNA in 1 μl of TE buffer (10 mM-Tris/1 mM EDTA, pH 8.0) in a 1-mm-gapped electroporator (Bio-Rad, Richmond), 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, 400Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (31) and incubated for 2 h at 26°C. They were then spread on MYP medium containing 75 μg kanamycin/ml.

Preparation of cell suspension and enzyme assay

R. rhodochrous ATCC12674 transformants were grown at 28°C for 24 h in MYP medium containing CoCl2·6H2O at several concentrations, harvested by centrifugation at 6,500 x 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 n-butyril acid. NHase activity was assayed in a reaction mixture (2 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 6 mM benzonitrile and an appropriate amount of the cell. The reaction was carried out at 20°C for 10 min and stopped by the addition of 0.2 ml 1 M HCl. The amount of benzamide formed in the reaction mixture was determined as described previously (13).

Cobalt uptake experiment

Assay for cobalt uptake was performed using cells grown in MYP medium without cobalt ions for 24 h at 28°C as described above. Cells were centrifuged at 6,500 x g for 10 min at 4°C and washed twice with 150 mM NaCl. The washed cells were suspended in buffer A [50 mM Tris·HCl (pH 7.5) containing 10 mM MgCl2] to a concentration of about 10 mg dry cell mass/ml. Cobalt uptake assays were performed in 30 ml Erlenmeyer flasks containing 0.5 mg of dry cell mass in 10 ml of buffer A. Cells were preincubated for 5 min at 30°C before addition of 57CoCl2. Assays were shaken at 30°C. To determine the cobalt content of the cells, 0.1 ml of the assay volume was taken at appropriate times and immediately passed through a membrane filter (pore size 0.45 μm; diameter 2.5 cm; Millipore, USA). Cells were immediately washed on the filter thrice with 3 ml of buffer A. The filters were dried and counted in a γ counter (Packard, USA).

Construction of nhIF expression plasmid

To express nhIF in E. coli, the author improved the sequence upstream from the putative start codon (TTG, nucleotides 5644-5646) by PCR with pLJK50 as a template and two oligonucleotides (Primer 1 and 2) as primers. Primer 1 (5′CTGCAAGCTTAAAGGAGAATTAGCGTATGACCAGCACCACCACCACAC3′) contained a HindIII recognition site, a ribosome-binding site, a TAG stop codon in frame with the lacZ gene in pUC19 and 22 nucleotides (nucleotides 5644-5665 in Fig. 2) of nhIF with the ATG start codon instead of the TTG codon. Primer 2 (5′GTATCTCGGTGCGTACGTACGTG3′) contained 26 nucleotides of the gene (complementary to nucleotides 6710-6735 in Fig. 2) 8 nucleotides downstream from the end of the reading frame and a PstI recognition site. DNA was amplified by the polymerase chain reaction (PCR) using a thermal cycler (Perkin-Elmer, USA). Reaction mixtures contained 1 μg of template DNA, 100 pmol each of oligonucleotide pool, and Thermus thermophilus DNA polymerase (Toyobo) in a volume of 100 μl. Thirty thermal cycles consisted of 94°C for 1 min, 55°C for 1 min and 75°C for 3 min each. The plasmids designated as pLCO10 and pLCO20 were constructed by ligation of the gel-purified and HindIII-PstI digested PCR product with pUC19/HindIII-PstI and pSTV29/HindIII-PstI, respectively, and were transformed into E. coli JM109.

Fig. 1. Genetic organization of the L-NHase gene cluster and the constructed fragments. nhIF and nhA are genes encoding L-NHase β- and α- subunit proteins, respectively (13). nhBC and nhD have recently been found to be responsible for the amide-dependent L-NHase induction (94). amA encodes the amidasine (72). nhE and nhIF are the newly identified genes described in this section. Solid triangle represents putative transcription terminator (72). The bold line demonstrates the region corresponding to the sequence shown in Fig. 2.
RESULTS

Primary structure of the intervening region among nhlBA and amdA

A 1.73-kb SacI-EcoRI region containing nhlBA (13) and a 1.96-kb EcoRI-SphiI region containing amdA (72) from R. rhodochrous 1J was previously cloned and sequenced; nhlA and amdA are separated by the 1.9-kb intervening region (Fig. 1). Furthermore, the author showed that both enzyme genes are coordinately regulated by a positive regulator (nhlC) and a negative regulator (nhlD), which are located upstream of nhlB (94). Here, the author determined the nucleotide sequence of the 1.5-kb EcoRI region. Fig. 2 shows the nucleotide sequence of the 1.5-kb EcoRI region and its flanking sequence previously determined, as well as amino acid sequences of two ORFs (nhlE and nhlf) newly found in the region. The presumptive ATG start codon was found for nhlE, but the initiation codon TTG was less frequent for nhlf. nhlE and nhlf were preceded by Shine-Dalgarno sequences located within reasonable distances from the respective presumptive start sites. The first 1.5-kb ORF (nhlF) located just downstream from nhlA is 447 nucleotides long, and may encode a protein of 148 amino acids (16,887 Da). nhlE showed a low similarity of amino acid sequence with nhlG from R. rhodochrous 1J (73) (35.6% identity) (data not shown). nhlG is also located just downstream from nhlA encoding the H-NHase α-subunit protein, and its function has not yet been determined. The second ORF named nhlf, is 1059 nucleotides long, and may encode a highly hydrophobic polypeptide of 352 amino acids (37,187 Da); the deduced amino acid sequence of Nhlf includes a substantial number of hydrophobic residues (63%). A computer-aided FASTA search of the SwissProt protein database indicated that Nhlf showed similarity to nickel transporters such as HoxN (93) from A. eutrophus (36.1% identity), HupN (95) from Bradyrhizobium japonicum (37.8% identity), NixA (96) from Helicobacter pylori (37.8% identity) and UreH (97) from Bacillus sp. (16.9% identity) (Fig. 3). hoxN and hupN are located in each nickel-containing hydrogenase gene cluster and ureH is located in the nickel-containing urease gene cluster. nixA is isolated as the gene complementing urease activity in E. coli harboring urease structural genes under nickel limitation; this gene is not closely linked to the urease gene cluster. A hydrophathy plot by the method of Kyte and Doolittle (98) revealed that Nhlf was a markedly hydrophobic protein and contained eight possible transmembrane helices, numbered as 1–8 (Fig. 4).
NHase activity under cobalt limitation

L-NHase enzyme encoded by nhlBA, contains 1.7 atoms cobalt/mol enzyme, and L-NHase is formed as an active form in *R. rhodochrous* J1 only in the presence of cobalt ions in the culture medium (10). The position of nhIF close to nhlBA and the similarity in the amino acid sequence between NhIF and the bacterial nickel transporters suggested that NhIF is a protein that transports cobalt ions into the cell. First, we examined the effect of nhIF on the activity of cobalt-dependent L-NHase using a *Rhodococcus*-*E. coli* host-vector system. As shown in Fig. 1, plasmid pLJK50 contained the *Pst*I fragment covering intact nhlBAEF and a part of *amdA* on a *Rhodococcus*-*E. coli* shuttle vector pK4, and pLJK60 contained the *KpnI* fragment covering a part of nhIC and intact nhlBAE on pK4. We transformed each plasmid into *R. rhodochrous* ATCC12674 as a host strain and cultured the resulting transformants in the medium changing final concentrations of CoCl₂. NHase assays using benzonitrile as a substrate for each cell suspension prepared as described in Materials and Methods demonstrated that the presence of nhIF allows the formation of catalytically active NHase even at very low cobalt concentrations (1, 2, 3, 4, 5 μM) (Fig. 5); in particular, nhIF increased NHase activity 3.7-fold in the case of 1 μM of CoCl₂. This suggested that nhIF encodes a transporter with high affinity for cobalt ions. With 5 μM of CoCl₂, however, NHase activity with the pLJK50-containing transformant was to 2-fold. Furthermore, both transformants showed almost the same NHase activities when they were cultured in the medium supplemented with 0.001% CoCl₂ (w/v) (data not shown), corresponding to 42 μM of CoCl₂, which is the optimum concentration for the NHase formation in *R. rhodochrous* J1 (99). This indicates the presence of nonspecific transport system with low affinity for cobalt ions in the *R. rhodochrous* ATCC12674 host strain.

![Graph](image)

**Fig. 4.** Hydropathy plot of NhIF. Hydropathy was calculated for NhIF by using the algorithm of Kyte and Doolittle (98) with a window of 15 amino acid residues.

**Fig. 5.** Effect of nhIF on NHase activity of the recombinant *R. rhodochrous* ATCC12674. Solid boxes, R *rhodochrous* ATCC12674/pLJK50; shaded boxes, R *rhodochrous* ATCC12674/pLJK60. Strains were grown for 24 h at 28°C in MYP medium containing CoCl₂ as indicated. The NHase activity was measured as described in Materials and Methods.

**Fig. 6.** Cobalt uptake of the recombinant *R. rhodochrous* ATCC12674 transformants

The synthesis of catalytically active NHase by the transformant harboring pLJK50 led the author to examine whether NhIF could function as a cobalt transporter. We measured ^57^Co²⁺ uptake of the *Rhodococcus* transformants. Cell suspensions of *R. rhodochrous* ATCC-12674 containing either pLJK50, pLJK60 or pK4 were prepared. Uptake of Co²⁺ was determined by the addition of ^57^CoCl₂ (final concentration, 10 nM) to the cell suspension (0.5 mg dry cell mass in buffer A) followed by vacuum filtration after 5, 10, 15, 20 and 25 min. Identical assay without the cells showed that non-specific binding of ^57^Co²⁺ to the membrane filter was negligible. As illustrated in Fig. 6, the presence of nhIF significantly increased cobalt uptake.

**Effects of uncouplers and divalent cations on cobalt uptake**

The effects of uncouplers on the nhIF-conferring cobalt uptake were examined. CCCP was added to the cell suspension of the *Rhodococcus* transformant harboring pLJK50 in buffer A, 10 min prior to the addition of ^57^CoCl₂. CCCP at the final concentration of 1 μM and 10 μM in the reaction mixture inhibited the uptake by 25% and 85%, respectively, after 25 min of the reaction time. However, CCCP has also been shown to exhibit side effects besides the fun-
cation of a protonophore; it blocks sulfhydryl groups in membrane proteins of *E. coli* and *Staphylococcus aureus* (100,101). Therefore the effect of an uncoupler SF6847 on the cobalt uptake was investigated. SF6847 at the final concentration of 0.1 μM, 1 μM and 10 μM inhibited the uptake by 55%, 85% and 85%, respectively, after the reaction time of 25 min. These findings demonstrated that proton gradients are involved in the cobalt uptake conferred by nhlF.

Other divalent cations such as Mn2+, Fe2+, Ni2+ and Cu2+ were added at the final concentration of 5 μM, 10 min before the addition of 57CoCl2 into the cell suspension. The measurement of the cobalt uptake in each condition showed that none of each Mn2+, Fe2+ or Cu2+ affected the cobalt uptake, while the addition of Ni2+ led a marked decrease of the cobalt uptake (Fig. 7).

**Expression of the cobalt transporter in *E. coli***

Plasmid pLJK50 containing nhlBAEF and a part of amdA conferred the energy-dependent cobalt uptake upon the *Rhodococcus* host, whereas pLJK60 containing a part of nhlC and nhlBAE did not. These observations suggest that NhIF is a single component responsible for the cobalt uptake. To test this possibility, nhlF was introduced into an *E. coli* strain, and its cobalt uptake activity was investigated. To enhance nhlF expression in *E. coli*, the author altered the sequence upstream from TTG start codon by PCR as described in Materials and Methods. The resulting nhlF was introduced on a high-copy-number-plasmid pLCO10 derived from pUC19 or a low-copy-number-plasmid pLCO20 de-rived from pSTV29 into an *E. coli* JM109. The *E. coli* transformants harboring each pLCO10, pLCO20 and pUC19 were cultured in LB medium supplemented with 1 mM of IPTG for 12 h at 28°C. Cells were harvested and the cell suspensions were prepared by the method as in the case of the *Rhodococcus* transformants. The cobalt uptake experiments indicated that pLCO10 and pLCO20 confers significantly cobalt uptake activity upon the *E. coli* strain (Fig. 8), suggesting that only nhlF is required for the functional cobalt uptake and that the NhIF poly-peptide folded in a functionally active form probably in the *E. coli* inner membrane. The uptake activity seemed to be independent of the copy number of the plasmids within the *E. coli* cells.

**DISCUSSION**

*H*- and L-NHases are selectively produced in *R. rhodochrous* J1 cultured only in the presence of cobalt ions with each inducer (10). Both purified enzymes contain cobalt atoms as a prosthetic metal and require this divalent cation for the catalytically active enzyme; these cobalt atoms bind tightly to the enzyme and are not released from the protein even after dialysis for five days (12). No other metals such as Ni, and Fe which is a cofactor of NHases from *Pseudomonas chlororaphis* B23 (71) and *Brevibacterium* sp. R312 (102), can replace cobalt ions in both NHases. To provide the NHase enzyme with sufficient cobalt, the metal ions should be actively transported into the *R. rhodochrous* J1 cell.

The present study in this section on the nucleotide sequence of the intervening region between nhlBA and amdA suggests that nhlF located in this region possesses significant similarities to the previously known genes encoding potential nickel transporters from Gram-negative and Gram-positive bacteria. The author also found that nhlF significantly enhanced nhlBA-derived L-NHase activity in *Rhodococcus* transformants in the cobalt-limiting conditions and that nhlF on the vector plasmid conferred the cobalt uptake activity upon *Rhodococcus* and *E. coli* hosts. The studies using uncouplers suggested that proton gradients are involved in the cobalt transport. These findings suggest that NhIF located in cell membrane energy-dependently mediates the transport of cobalt ions into the cell and therefore facilitates its incorporation into the L-NHase enzyme.

It is interesting to note that nhlF is part of a L-NHase gene cluster spanning a DNA region of 8.6-kb. This region contains the structural genes involved in nitrile metabolism by the combination of L-NHase and the amidase, together with the regulatory genes responsible for the amide-dependent induction of both enzymes (94). The cobalt uptake experiments using
the Rhodococcus transformant harboring pLJK10 (see Fig. 1) cultured in the medium supplemented with or without an inducer crotonamide for the formation of L-NH\textsubscript{2}ase and the amidase showed that NhfF activity appeared only in the presence of crotonamide (data not shown). These findings suggested the coordinate regulation of nhlBA, nhlF and amdA, which is probably due to a cotranscription of these genes in a single mRNA, consistent with the presence of p-independent potent transcriptional terminator found in the downstream region of amdA (72) and with the absence of such a sequence between nhlA and nhlF or nhlF and amdA.

NhfF-related proteins previously reported have been directly or indirectly shown to be involved in the uptake of nickel ions. Among these nickel transporters, HoxN from A. eutrophus has been most intensively studied. In A. eutrophus, two transport systems for nickel ions exist: a nonspecific high-capacity magnesium transport system and a high-affinity low-capacity nickel transporter, HoxN. Nickel uptake by the magnesium transport system was competitively inhibited by Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Zn\textsuperscript{2+} and Co\textsuperscript{2+}, whereas the activity of the HoxN-mediated transport system was inhibited by only Co\textsuperscript{2+} (103). In this study, the author demonstrated that the cobalt uptake activity derived from nhlF was markedly inhibited by adding excess Ni\textsuperscript{2+} to the cell suspension; the other metals tested, i.e., Mn\textsuperscript{2+}, Fe\textsuperscript{2+} and Cu\textsuperscript{2+} had no effect on the cobalt uptake. These observations suggested that NhfF is responsible for nickel uptake as well as cobalt uptake and also that HoxN is involved in cobalt uptake as well as nickel uptake; unfortunately, HoxN has not been reported to be involved in the cobalt uptake from A. eutrophus. However, the author could not detect nickel uptake by pLJK50-containing Rhodococcus using radioactive \( ^{65}\text{NiCl}_2 \) (data not shown), which indicates that nickel ion is not a substrate for NhfF. Consequently, NhfF is a cobalt-specific transporter.

Hydropathy plots of amino acid sequence of NhfF, the topological model for HoxN proposed by Eitinger \textit{et al.} (104) and the positive inside rule developed by von Heijne (105) suggested that NhfF is a membrane protein including eight transmembrane helices with the orientation of locating N-terminus in the cytoplasm (Fig. 3). Alignment of amino acid sequences of NhfF and its related nickel transporter proteins (Fig. 3) provides some information about the structure of NhfF. Wolfram \textit{et al.} (106) presented two segments conserved among the nickel transporters as potent domains involved in the high-affinity nickel binding or in the translocation process; their positions are from aa. 44 to 72 and from aa. 89 to 99 of HoxN. Both segments include histidine residues (aa. 62, 68 and 97 of HoxN), which are generally considered to be potential metal ligands. The regions corresponding to both segments are highly conserved in NhfF, and the above-mentioned histidine residues also exist in NhfF (aa. 68, 74 and 103 of NhfF). On the other hand, compared with the nickel transporters, quite different amino acid residues appeared in the corresponding sequence of NhfF; histidine (aa. 10), tryptophan (aa. 15), tyrosine (aa. 44), alanine (aa. 50), leucine (aa. 87), threonine (aa. 143, 254 and 284), arginine (aa. 179), serine (aa. 200), where different amino acids are conserved among the nickel transporters. Therefore, these amino acid residues may be involved in the cobalt-specificity.

Conklin \textit{et al.} reported that \textit{COT1}, isolated as a suppressor of cobalt toxicity, is responsible for the reduction of the cytoplasmic Co\textsuperscript{2+} ion concentration within cells of \textit{Saccharomyces cerevisiae} (107,108). The increased tolerance to cobalt ions in the \textit{COT1}-overexpressing yeast is due to the \textit{COT1}-dependent increased sequestration or compartmentalization within the mitochondria of cobalt ions that cross the plasma membrane. \textit{COT1} was presumed to be a 48-kDa membrane protein with six membrane-spanning domains and found in the mitochondrial membrane fraction of cells. The similar function of cobalt uptake suggests some similarity in the primary or secondary structures of NhfF and \textit{COT1}. Computer analysis of the amino acids sequences of NhfF and \textit{COT1} did not show overall similarity between them. However, as shown in Fig. 3, helix 1 of NhfF contains a segment highly similar to a segment in helix 5 of \textit{COT1} (8 of 10 residues). In this region, both NhfF and \textit{COT1} contain a histidine residue which is a potential metal-binding amino acid, but neither HoxN nor HupN contains histidine at the corresponding site, suggesting the functional role for the cobalt-specific recognition.

On the other hand, the \textit{COT2} gene (presently \textit{GRRI}) was also isolated from \textit{Saccharomyces cerevisiae} (108,109); \textit{COT2} mutant tolerates increased level of Co\textsuperscript{2+} through the reduction in the rate of glucose-dependent transport of cobalt into cells. However, \textit{COT2} does not appear to be responsible for the cobalt transport; this protein may play a more general role in yeast physiology that indirectly controls the permeability of the membrane to cobalt ions or the driving force for the uptake, and \textit{COT2} might be involved in the glucose activation of the plasma membrane ATPase. As expected, there is no sequence similarity between NhfF and \textit{COT2}.

NhfF also exhibited no sequence similarity to the genes involved in the active efflux system of broad specificity for metals of Ca\textsuperscript{2+}, Zn\textsuperscript{2+} and Co\textsuperscript{2+}, which have been characterized in detail by genetic analyses on resistance of these metals in \textit{A. eutrophus} (95,110,111).
SUMMARY

Cobalt is an essential component of a low molecular-mass nitrile hydratase (L-NHase) from *Rhodococcus rhodochrous* J1. A new gene, *nhlF*, was found in the DNA region between *nhlBA* and *amdA*, which are involved in the degradation of nitriles. The product of *nhlF* shows a significant sequence similarity with those of *hoxN* from *Alcaligenes eutrophus*, *hupN* from *Bradyrhizobium japonicum*, *nixA* from *Helicobacter pylori* and *ureH* from *Bacillus* sp., which are considered to be involved in nickel uptake into these cells. Sequence and hydropathy plot analyses have shown that NhlF would be a 352-amino acid protein with eight hydrophobic putative membrane-spanning domains. The *nhlF* expression in *R. rhodochrous* ATCC12674 and *E. coli* JM109 confers uptake of $^{57}$Co in their cells, but not of $^{63}$Ni. These findings together with the finding that the cobalt uptake was inhibited by the addition of uncouplers such as CCCP and SF6847 have suggested that NhlF specially mediates the cobalt transport into the cell energy-dependently.

CHAPTER III Genetic Analysis of Nitrilase

Section 1 Sequencing and overexpression of the nitrilase gene (*nitA*) and identification of an essential cysteine residue

Nitrilase catalyzes the direct cleavage of nitriles to yield the corresponding acids and ammonia. When *Rhodococcus rhodochrous* J1 is cultivated in medium containing isovaleronitrile as an inducer, only nitrilase is inducibly formed (11). Nitrilases have also attracted increasing attention as catalysts for organic chemical processing, because of the mild conditions, quantitative yields, absence of by-products and in some cases, enantio- or regioselectivity that result from their reactions (112,113).

Nitrilases that utilize benzonitrile and related aromatic nitriles as substrates have been purified from *Pseudomonas* (17,18), *Nocardia* sp. NCIB 11215 (19) and NCIB 11216 (20), *Fusarium solani* (21), *Arthrobacter* sp. (22), *Rhodococcus rhodochrous* J1 (23) and *Escherichia coli* transformed with a *Klebsiella ozaenae* plasmid DNA (24). In the author's laboratory, nitrilases that act on aliphatic nitriles and arylacetonitriles were also purified from *Rhodococcus rhodochrous* K22 (25) and *Alcaligenes faecalis* JM3 (26), respectively and characterized. However, there is only one report of cloning the nitrilase gene for *K. ozaenae* bromoxynil nitrilase (24).

All nitrilases so far reported are classified as sulfhydryl enzymes since they are inactivated by thiol reagents. The *R. rhodochrous* J1 nitrilase is also inactivated by thiol reagents (23). An active cysteine residue has not yet been identified in any nitrilase.

In this section, the author describes the cloning and sequencing of the nitrilase gene (*nitA*) from *R. rhodochrous* J1, and presents evidence that a cysteine residue (Cys-165) plays an important role in the function of the active site.

MATERIALS AND METHODS

Bacterial strains and plasmids

*Rhodococcus rhodochrous* J1 was previously isolated from soil samples (29). *Escherichia coli* JM105 (30) was used for pUC plasmid 18/19 transformation.
Media

*R. rhodochrous* J1 was cultivated as described previously (23). M13 phage was propagated and the nitrilase gene was expressed in 2 x YT medium (30).

Enzymes and chemicals

Lysyl-endopeptidase and 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) were obtained from Wako Pure Chemicals (Tokyo, Japan). Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd. [γ-32P]ATP (180 TBq/mmol), [α-32P]dATP (15 TBq/mmol) were from Amerham Japan. A low molecular mass standard kit was obtained from Pharmacia LKB Biotechnology Inc. Cellulofine GCL-2000 superfine was purchased from Seikagaku Kogyo Co. (Tokyo). All other chemicals used were from commercial sources and of reagent grade.

NH₂-Terminal sequence analysis

Nitrilase was purified from *R. rhodochrous* J1 as described previously (23), and the enzyme (1 mg in 1 mM potassium phosphate buffer, pH 7.5) was used directly for the NH₂-terminal sequence analysis by automated Edman degradation with an Applied Biosystems 470A gas-phase amino acid sequencer. The phenylthiohydantoin-derivatives were separated and identified by an on-line phenylthiohydantoin analyzer (Model 120A, Applied Biosystems Japan, Tokyo) with a phenylthiohydantoin C18 column.

Isolation of peptide fragments and peptide sequencing

Nitrilase (1.56 mg) was incubated in 20% (v/v) trichloroacetic acid at 37°C for 6 h and digested with 0.41 μg lysylendopeptidase in 0.6 ml 0.01 M Tris/HCl (pH 7.6) at 37°C for 24 h. The mixture (70 μl) was applied to high-pressure liquid chromatography equipped with a Ultron N C18 (4.6 x 150 mm Shinwa Kako, Kyoto, Japan) and eluted with a linear gradient of acetonitrile (0-60%, v/v) in the presence of 0.1% (v/v) trifluoroacetic acid at a flow rate 1.0 ml/min. The peptide fragments isolated were sequenced by automated Edman degradation.

Cloning of a nitrilase gene from *R. rhodochrous* J1

Oligonucleotide primers were synthesized based upon the amino acid sequences of the NH₂ termini and the internal fragment generated with lysyl-endopeptidase. The amino acid sequence Val-Ala-Ala-Val-Gln-Ala-Gln-Pro-Val-Trp-Phe-Asp-Ala was used to model the oligodeoxynucleotide pool 5'-GTCGCTGCGAT(C,G,T)CAAGCC(A,C)CA(A,G,T)GTY
(A,C,G)TGTT(C,G)GA(C,T)GC-3' (sense strand), and Phe-Ala-Arg-Ile-Ile-Gly-Pro-Asp-Gly to model 5'-CC(A,G)TC(A,G,G)GG(A,C,G)CC(A,G,T)ATGAT(C,G)CG(C,G)GC(A,G)AA-3' (antisense strand). These oligonucleotides were synthesized by the phosphoramidite method (114) using an Applied Biosystems Model 381A automatic synthesizer. Total DNA of *R. rhodochrous* J1 was prepared after cell lysis (wet mass, 15 g) with lysozyme and *Achromobacter* peptidase (Wako Pure Chemicals, Tokyo, Japan) following the method of Saito and Miura (49). DNA was amplified by the polymerase chain reaction (PCR) using a thermal cycler (Perkin-Elmer/Cetus, U.S.A.). Reaction mixtures contained 10 μg of DNA, 100 pmol of each oligonucleotide pool, and *Thermus thermophilus* DNA polymerase (Toyobo) in a volume of 100 μl. Thirty thermal cycle consisted of 93°C for 1 min, 55°C for 2 min, and 73°C for 3 min each. The gel-purified PCR-synthesized product (750 base pairs (bp)) was cloned into the Pst-SmaI sites of M13mp18 replicative-form DNA and designated as pNJ1. The gel-purified PCR-product was further used as a radiolabeled probe by random priming (115) to clone the full-length nitrilase gene. Nucleotides were sequenced by chain-termination (58) using Sequenase version 2 (United States Biochemical Corp., Cleveland, U.S.A.). Deoxy-ITP or 2'-deoxy-7-deaza GTP was used as a substitute for dGTP during M13 sequencing, to minimize compression.

Preparation of crude extracts from *Escherichia coli* transformants

Recombinant *E. coli* JM105 was cultured to full growth in 10 ml of 2 x YT medium containing 50 μg/ml ampicillin in a 50-ml test tube at 37°C, then transferred to 100 ml of the same medium in a 500-ml shaking flask with IPTG added to a final concentration of 1 mM, to induce the *lac* promoter. After various culture periods, the cells were harvested by centrifugation, suspended in 3 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, disrupted by sonication for 5 min (19 kHz, Insonator model 201M; Kubota, Tokyo, Japan) and centrifuged at 12,000 x g for 30 min. The resulting supernatants were used in the enzyme assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (60).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the oligonucleotide directed in vitro mutagenesis system of Amersham Japan according to the procedure recommended by the
suppliers, and which was basically developed by Eckstein and co-workers (116). To prepare both sense and antisense single-stranded DNAs, EcoRI and BamHI sites of replicative-form M13mp18 and M13mp19 were ligated with the 593-bp EcoRI-BamHI fragment (nucleotide positions 318-910) isolated from plasmid pNJ10. After transformation into the competent strain JM105, the recombinant phages were screened initially for white plaques on plates containing IPTG and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Two oligonucleotides were synthesized for mutagenesis: 5′-CGCTCAACA*GCTGGGAGC-3′ for replacement of Cys-165 by Ser and 5′-GGCGCGCTCAACG*C*TGGGAGCATTTC-CAG-3′ for replacement of Cys-165 by Ala (the positions of the introduced PvuII and MluI restriction sites are underlined, respectively), where asterisks indicate mismatched bases. The mutants were initially screened by restriction enzyme mapping on replicative-form DNA prepared from several plaques and then by DNA sequencing. Typically, 65-100% of the transformants contained the desired mutation. A 198-bp EcoRV-Xhol fragment (nucleotide positions 537-734) was excised from each of the mutated phage DNAs and inserted between the EcoRV and Xhol sites of plasmid pNJ20 instead of the parental fragment. The two mutant plasmids thus obtained were designated pNJ20-165S and pNJ20-165A, using the one-letter code for each substituted amino acid residue. The constructs were confirmed by restriction mapping.

**Reaction with DTNB**

Various amounts of DTNB (1.56 μmol) were incubated with the enzyme (2.8 μmol of subunit) in 10 mM potassium phosphate buffer (pH 7.2) at 0°C for 2 h (final volume, 1 ml). DTNB was replaced by water in a blank. After the reaction, the 412 nm was monitored, and an aliquot of the reaction mixture was removed to determine the remaining activity. The amount of 5-thio-2-nitrobenzoate (TNB) released was estimated with the published molar absorption coefficient in 8 M urea, 14,290 M⁻¹ cm⁻¹ (117).

**Enzyme assays**

Nitrite activity for *R. rhodochrous* J1 was assayed by the same method as previously described (23). The protein concentration was determined according to Bradford (59). One unit of nitrite activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol benzoic acid/min under the above conditions. The specific activity was expressed as units/milligram of protein.

**RESULTS AND DISCUSSION**

**Cloning and nucleotide sequence of the nitrite gene from *R. rhodochrous* J1**

The nitrite from *R. rhodochrous* J1 was purified and the amino acid sequences of the peptides were determined by digesting the nitrite with lysyl-endopeptidase. The purified enzyme had an NH₂-terminal sequence of Val-Glu-Tyr-Thr-Asn-Arg-Phe-Lys-Val-Ala-Ala-Val-Gln-Val-Pro-Val-Arg-Asp-Ala-Lys-Thr-Val-Lys-Thr-Val-Ser-Arg-Ala-Ala-Glu-Ala. Sequences of two peptides were Phe-Ala-Arg-Tyr-His-Glu-Asn and Leu-Ile-Gly-Arg-Gly-Gly-Gly-Phe-Ala-Arg-Ile-Gly-Gly-Pro-Asp-Gly-Arg-Asp-Leu-Ala-Thr-Pro-Leu-Ala-Glu-Asp, respectively (see Fig. 2). The oligonucleotide sense primer containing the PstI site synthesized was a 38 mer with 288 variants (corresponding amino acid positions 10-22 in Fig. 2), and the antisense primer was a 26 mer with 432 variants (corresponding amino acid positions 253-261 in Fig. 2). The nucleotide sequence corresponding to both primers was detected in the cloned pNJ1 containing a 750-bp-length fragment generated by PCR amplification.

![Fig. 1. Restriction map, sequencing strategy of plasmid pNJ10, and construction of plasmid pNJ11 for expression of nitrite gene.](image-url)

Plasmid pNJ10 contains a 5.4-kb fragment at the PstI site of pUC19. A sequencing strategy for the nitrite gene is shown under the restriction map of pNJ10. Arrows indicate the direction and extent of the sequence determination. Plasmid pNJ11 contains a 1.3-kb fragment at the Smal and AccI sites of pUC19. The location and direction of the lac promoter are also indicated.
endonucleases. A single restriction fragment was found by hybridization of the total DNA with several restriction endonucleases. Southern hybridization was performed using the radiolabeled PCR-synthesized probe with the following modifications. Hybridization proceeded at higher stringency, using a buffer containing 35% (v/v) formamide, 5 X SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate) and 0.1% (m/v) SDS at 42°C for 12 h. A single 5.4-kb band was detected from a Psrl digest of the total DNA. This fragment was recovered and ligated with T4 DNA ligase to linear pUC19 DNA successively treated with Psrl and bacterial alkaline phosphatase. The ligated mixture was introduced by transformation into E. coli JM105 and ampicillin-resistant transformants were selected on 2 X YT agar medium containing 50 µg/ml ampicillin. Colony hybridization with the PCR-generated probe for screening the clones containing the restriction fragment resulted in pNJ10 containing a 5.4-kb Psrl fragment (Fig. 1). Restriction endonuclease digestion together with Southern hybridization with the above probe indicated the location of the nitrate gene (nitA). The nucleotide sequence was determined in both orientations and all the restriction sites used for cloning were verified by determination as part of an overlapping sequence. An open reading frame encoding 366 amino acids (Fig. 2), which started with methionine and terminated with a TGA codon, also encoded sequences corresponding precisely to those determined using purified nitratease. The amino acid composition of the enzyme calculated from the nucleotide sequence is similar to that obtained by chemical analysis of the purified enzyme (23). This sequence encodes a putative polypeptide of a molecular mass of 40189 Da, which is in close agreement with that of 41.5 kDa separated by SDS-PAGE (23). A typical Shine-Dalgarno sequence (64) was present 8 bp upstream from the initiation codon, but none of the consensus promoter sequences found in other prokaryotes (65) were observed in the upstream region. A strong hairpin structure (AG = -44.6 kcal/mol) located just downstream of the termination codon of the nitA. The overall G + C composition of positions 1, 2 and 3 of the codons for the nitrate is 64.3, 43.6 and 80.1%, respectively. This gene was characterized by the high frequency of G/C at the third letter of the codon within the coding region, as observed in some organisms such as Streptomyces (118) and Thermus (119) having DNA with a high G + C content.

To obtain the entire gene, after digestion of the total DNA with several restriction endonucleases, Southern hybridization was performed using the radiolabeled PCR-synthesized probe with the following modifications. Hybridization proceeded at higher stringency, using a buffer containing 35% (v/v) formamide, 5 X SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate) and 0.1% (m/v) SDS at 42°C for 12 h. A single 5.4-kb band was detected from a Psrl digest of the total DNA. This fragment was recovered and ligated with T4 DNA ligase to linear pUC19 DNA successively treated with Psrl and bacterial alkaline phosphatase. The ligated mixture was introduced by transformation into E. coli JM105 and ampicillin-resistant transformants were selected on 2 X YT agar medium containing 50 µg/ml ampicillin. Colony hybridization with the PCR-generated probe for screening the clones containing the restriction fragment resulted in pNJ10 containing a 5.4-kb Psrl fragment (Fig. 1). Restriction endonuclease digestion together with Southern hybridization with the above probe indicated the location of the nitrate gene (nitA). The nucleotide sequence was determined in both orientations and all the restriction sites used for cloning were verified by determination as part of an overlapping sequence. An open reading frame encoding 366 amino acids (Fig. 2), which started with methionine and terminated with a TGA codon, also encoded sequences corresponding precisely to those determined using purified nitratease. The amino acid composition of the enzyme calculated from the nucleotide sequence is similar to that obtained by chemical analysis of the purified enzyme (23). This sequence encodes a putative polypeptide of a molecular mass of 40189 Da, which is in close agreement with that of 41.5 kDa separated by SDS-PAGE (23). A typical Shine-Dalgarno sequence (64) was present 8 bp upstream from the initiation codon, but none of the consensus promoter sequences found in other prokaryotes (65) were observed in the upstream region. A strong hairpin structure (AG = -44.6 kcal/mol) located just downstream of the termination codon of the nitA. The overall G + C composition of positions 1, 2 and 3 of the codons for the nitrate is 64.3, 43.6 and 80.1%, respectively. This gene was characterized by the high frequency of G/C at the third letter of the codon within the coding region, as observed in some organisms such as Streptomyces (118) and Thermus (119) having DNA with a high G + C content.
halogen atoms, but it does not act upon benzonitrile, which is a good substrate for the *R. rhodochrous* J1 nitrilase (23). A small difference in amino acid sequence between both nitrilases seems to bring about a large difference in substrate specificity. The protein sequence data base of the NBRF (National Biochemical Research Foundation) was searched for sequences similar to the nitrilases from *R. rhodochrous* J1 and *K. ozaenae*. However, no homology was found. Comparison with the amino acid sequences of the rhodochrous gene revealed no similarity to those of two nitrile hydratases from *R. rhodochrous* J1 (13), although both nitrilase and nitrile hydratases act upon a nitrile compound, suggesting that they have different enzymatic reaction mechanisms.

**Production and purification of the nitrilase protein in E. coli**

To overproduce the nitrilase in *E. coli*, a 1.3-kb *PvuI-NarI* fragment was inserted between the *SalI* and *AccI* sites of pUC18, resulting in pNJ11 (Fig. 1), in which the nitrilase gene was under the control of the lac promoter. When *E. coli* harboring pNJ11 was cultivated in the presence of IPTG at 37°C, nitrilase activity was detected in the supernatant of the sonicated cell-free extracts obtained at 12,000 x g. The author analyzed the production of the nitrilase in the supernatant of the sonicated extracts by SDS-PAGE and detected a 41.5-kDa synthesized protein corresponding to the subunit of the purified *R. rhodochrous* J1 nitrilase. However, the level of nitrilase activity in the supernatant was 0.080 unit/mg, which was low compared with that in cell-free extracts of *R. rhodochrous* J1 cells cultivated with isovaleronitrile (with benzonitrile as a substrate) (23).

**primer 1**

\[
5'\text{CCCCAAGCTTTAAGGAGGAATAGGACATGGTCGAATAC ACAAA C} 3'
\]

HindIII

Stop Start

**primer 2**

\[
5'\text{ATCTCTAGATGCCCTGCTCA} 3'
\]

XbaI

Fig. 4. Sequences of the oligonucleotide primers for expression of nitrilase gene. Primers 1 and 2 are the sense and antisense primers, respectively. The sequences are shown in the 5’ to 3’ direction.

To enhance the nitrilase activity, the author improved the sequence upstream of the ATG codon using PCR with pNJ10 as a template and two oligonucleotides as primers (Fig. 4). Primer 1 contained a *HindIII* recognition site, a ribosome binding site, and a TAG stop codon in-frame with the *lacZ* gene in pUC19 and 18 nucleotides (nucleotides 90-107 in Fig. 2) of *nitA* starting with the ATG start codon 9 nucleotides downstream of the ribosome-binding site. Primer 2 contained 22 nucleotides of the gene (complementary to nucleotides 1239-1260 in Fig. 2) 46 nucleotides downstream from the end of the reading frame, and an *XbaI* recognition site. The plasmid designated as pNJ20 was constructed by ligation of the PCR-product with pUC19/HindIII-XbaI, and transformed into *E. coli* JM105.

| Table 1. Nitrilase activities of *E. coli* transformants under various culture conditions |
|----------------------------------|-------------------|-----------------|------------------|
| **IPTG feeding time** | **Cultivation time** | **Cultivation temperature** | **Specific activity** |
| h | h | °C | unit/mg |
| 0° | 6 | 28 | 0.150 |
| 0° | 12 | 28 | 0.166 |
| 4° | 7 | 28 | 0.140 |
| 4° | 12 | 28 | 0.190 |
| 0° | 6 | 37 | 3.68 |
| 0° | 12 | 37 | 7.87 |
| 4° | 7 | 37 | 3.72 |
| 4° | 12 | 37 | 8.04 |

* IPTG was added to 2 x YT medium at the same time the culture was started.
* IPTG was added to 2 x YT medium 4 h after the start of culture.

In this construction, the *nitA* was under the control of the *lac* promoter. A protein corresponding to the purified *R. rhodochrous* J1 nitrilase subunit was synthesized only when the *lac* promoter was induced by IPTG. When *E. coli* carrying pNJ20 was cultured in the presence of IPTG, nitrilase activity was observed in the supernatant of the sonicated cell-free extracts, but not in the pellet obtained at 12,000 x g. The maximum level of nitrilase activity in the supernatant was 8.04 units/mg (Table 1). This value corresponded to 50.6% compared with the specific activity in the purified nitrilase from *R. rhodochrous* J1. The expression of the *nitA* was examined in the supernatant of the sonicated cell-free extracts by SDS-PAGE (Fig. 5). As judged by quantitative evaluation of the gel track with a dual-wavelength TLC Scanner CS-930 system (Shimadzu, Kyoto, Japan), the highest amount of the nitrilase formed corresponded to about 50% of the total soluble protein. Therefore, hyperproduction of the nitrilase was attained in the active form, whereas nitrile hydratases from *R. rhodochrous* J1 were produced as the insoluble form in *E. coli* (13).
The nitrilase produced in the recombinant cells was purified through the following one-step column procedure at 0-4°C. Cell-free extract was fractionated with ammonium sulfate (33-50%, m/v), followed by dialysis against 0.01 M potassium phosphate buffer pH 7.5, containing 1 mM dithiothreitol. The dialyzed enzyme solution was applied to a Cellulofine GCL-2000 superfine column and eluted with 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. Active fractions were precipitated with 60% saturated ammonium sulfate and dialyzed against the above buffer. The purified enzyme, which showed only one band on SDS-PAGE (Fig. 5), gave almost the same physicochemical properties such as, specific activity and molecular mass, as the purified parental nitrilase from R. rhodochrous J1.

Reactivity with DTNB

Previous studies on R. rhodochrous J1 nitrilase (23) indicated that the enzyme is susceptible to thiol reagents and is therefore classified as a sulfhydryl enzyme. In previous studies (23), incubation of the enzyme with DTNB for 10 min at 0°C resulted in loss of 92.2% of the activity. After incubation of the enzyme with DTNB for 2 h at 0°C in the presence of 8 M urea, 3.82 mol of TNB was detected per mol of enzyme subunit. By analyzing the amino acid composition (23) and the nucleotide sequence, the enzyme subunit was found to contain 4 mol of half-cystine. These findings indicate that each subunit has 4 free cysteine residues, but no disulfide bonds. Thereafter, DTNB was incubated with the enzyme in the absence of urea. The enzyme was fully inactivated when ~1 mol of DTNB reacted with 1 mol of enzyme subunit (Fig. 6). The relationship between the inactivation and modification (i.e., TNB release) was proportional, that is, among four free sulfhydryl groups present in a subunit, only one reacted with DTNB. This relationship between the quantity of DTNB that reacted and the degree of inhibition, showed that modification of one sulfhydryl group per subunit by DTNB resulted in the complete loss of the catalytic activity of the parental nitrilase.

![Fig. 5. SDS/PAGE of supernatant prepared from E. coli containing pNJ20.](image)

Lanes 1 and 11 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 (43 kDa), carbonic anhydrase (14 kDa). Lanes 2-9 show supernatants of sonicates (60 μg protein). Lane 2, E. coli containing pNJ20, sample taken after a 6-h incubation at 28°C with IPTG; lane 3, same as lane 2, but after a 12-h incubation; lane 4, same as lane 2, but after a 7-h incubation, during which IPTG was added 4 h from the start; lane 5, same as lane 4, but after a 12-h incubation; lane 6, same as lane 2, but after a 6-h incubation at 37°C; lane 7, same as lane 6, but after a 12-h incubation; lane 8, same as lane 6, but after a 7-h incubation, during which IPTG was added 4 h from the start; lane 9, same as lane 8, but after a 12-h incubation; lane 10, purified nitrilase (15 μg of protein) from the sample derived from lane 9 as described in the text, as a control.

![Fig. 6. Titration of sulfhydryl group of nitrilase with DTNB.](image)

TNB released was determined by measuring the absorbance at 412 nm. Residual activity (○) and TNB released (●) were determined as described under 'Materials and Methods'.

The R. rhodochrous J1 nitrilase contains 4 cysteiny1 residues (at positions 41, 165, 224, 238) (Fig. 3). Of these cysteine residues, only Cys-165 is conserved at the corresponding
position in the *K. ozaenae* nitrilase, and there is high sequence similarity around this cysteine. Judging from the comparison of overall amino acid sequences between these nitrilases, it seems that there is a considerable structural similarity. To determine whether Cys-165 is essential for nitrilase activity, this residue was replaced by site-directed mutagenesis.

**Expression of mutant nitrilase genes**

The author constructed two mutant enzymes in which Cys-165 was replaced with Ala or Ser. SDS-PAGE of cell-free extracts from the transformant cells harboring plasmids pNJ20-165S or pNJ20-165A, which were cultured under the same conditions used to overproduce the active nitrilase in the transformant carrying pNJ20, revealed a predominant protein band with a mobility identical to that of the non-mutationed nitrilase. Both mutant proteins were purified to homogeneity by SDS-PAGE according to the procedure used to purify nitrilase from extracts of the transformant cells harboring pNJ20. The purified C165S and C165A enzymes were immunochemically indistinguishable from the parental enzyme when examined by Ouchterlony double-diffusion analysis (121) using the anti-(nitrilase) anti-serum prepared as previously described (23). Their circular dichroism spectra in the far-UV region and molecular mass were also identical with those of the parental enzyme (data not shown). These results demonstrate that essentially no major change in the overall conformation of the enzyme protein was elicited by mutation of Cys-165 to Ser or Ala. Whereas the parental enzyme produced benzoic acid from benzonitrile within 5 min at 25°C, no products were detected from the mutant enzymes even after a 24 h reaction. That is, the specific activity of the mutant enzymes is below the detection threshold due to changing Cys-165 to Ala or Ser. These findings demonstrate that Cys-165 is essential for catalytic activity.

*R. rhodochrous* J1 produces one nitrilase and two nitrile hydratases depending on the inducer. This strain exhibited versatile nitrile metabolism. That in one strain the metabolic pathway completely changes according to the inducer, is an interesting phenomenon. Studies on nitrile-metabolizing enzymes are also significant from not only an academic standpoint such as the analysis of their gene-regulation but also from a biotechnological perspective including the production of useful acids or amides.

**SUMMARY**

The amino acid sequences of the NH$_2$ terminus and internal peptide fragments of a *Rhodococcus rhodochrous* J1 nitrilase were determined in order to prepare synthetic oligonucleotides as primers or the polymerase chain reaction. A 750 base DNA fragment thus amplified was used as the probe to clone a 5.4-kb *PstI* fragment coding for the whole nitrilase. The nitrilase gene (*nitA*) modified in the sequence upstream of the presumed ATG start codon was expressed to about 50% of the total soluble protein in *Escherichia coli*. The predicted amino acid sequence from the *nitA* showed similarity with that of the bromoxynil nitrilase from *Klebsiella ozaenae*. The 5,5'-dithiobis(2-nitrobenzoic acid) modification of the nitrilase from *R. rhodochrous* J1 resulted in inactivation with the loss of one sulfhydryl group per enzyme subunit. Of four cysteine residues in the *Rhodococcus* nitrilase, only Cys-165 is conserved in the *Klebsiella* nitrilase. Mutant enzymes containing Ala or Ser instead of Cys-165 did not exhibit nitrilase activity. These findings suggest that Cys-165 plays an essential role in the function of the active site.
Section 2 Transcriptional regulation of nitA

Four cDNAs encoding nitrilases that convert indole-3-acetonitrile to IAA have been cloned and characterized from Arabidopsis thaliana (6-8). Pathogenic fungal species such as Taphrina virescens, Taphrina deformans and Taphrina pruni, which cause hyperplastic diseases in plants such as cherry, peach and plum, respectively, also form nitri-lase involved in IAA biosynthesis, resulting in growth and division of infected plant cells (122).

In the author's laboratory, nitrilases from Rhodococcus rhodochrous J1 (23,123), Rhodococcus rhodochrous K22 (25,124) and Alcaligenes faecalis JM3 (5,26), which act on aromatic nitriles, aliphatic nitriles and arylacetonitriles, respectively, were characterized in the protein and genetic studies. Stalker et al. have also cloned the bacterial Klebsiella oxytoca gene encoding nitri-lase that degrades bromoxynil (24). However, the mechanisms that regulate nitri-lase expression have not been reported. The nitrilases from R. rhodochrous J1, R. rhodochrous K22 and A. faecalis JM3 are all strongly induced by the addition of isovaleronitrile to the medium (23,25,26), making large amounts of enzyme available for application in industrial production of a wide range of useful acids from nitriles. In this section, the author describes characterization of the promoter and regulation of the nitri-lase gene (nitA) in R. rhodochrous J1.

MATERIALS AND METHODS

Strains and plasmids

R. rhodochrous J1 was previously isolated from soil (29). E. coli JM109 (30) was the host for pUC plasmids. R. rhodochrous ATCC12674 was the host for a Rhodococcus-E. coli shuttle vector plasmid pK4 (31) and its derivatives, and was used for the expression of the nitrilase gene (nitA). The plasmid pNJ10 (123) carrying nitA in a 5.4-kb PstI fragment on pUC19 was used for subcloning and sequencing of genes.

Transformation of R. rhodochrous ATCC12674 by electroporation

DNA manipulation was performed essentially as described by Sambrook et al. (30). A mid-exponential culture of R. rhodochrous ATCC12674 was centrifuged at 6,500 x 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 DNA in 1 µl of TE buffer (10 mM-Tris/1 mM EDTA, pH 8.0) in a 1-mm-gapped electrorcuvette (Bio-Rad, Richmond), 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, 400Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (31) and incubated for 2 h at 26°C. They were then spread on MYP medium containing 75 µg 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% v/v), harvested by centrifugation at 4,000 x 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% (v/v) glycerol, disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo, Japan), and centrifuged at 12,000 x g for 10 min at 4°C. The resulting supernatant was assayed for nitri-lase as previously described (123). One unit of nitri-lase catalyzes the formation of 1 µmol of benzoic acid/min under the above conditions. The protein was determined according to Bradford (59). 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, Japan), 5 g meat extract (Mikuni, Tokyo, Japan), 0.5 g yeast extract (Oriental Yeast, Tokyo, Japan) and 2 g NaCl/l 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 and 3 g yeast extract/l tap water (pH 7.2)) with or without isovaleronitrile (0.1%, w/v), 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 (32).

Northern (RNA) blot hybridization

For Northern blot hybridization, each RNA sample (30 µg) was subjected to electrophoresis on a 1% agarose-formaldehyde gel, and transferred to a nitrocellulose membrane filter (Schleicher & Schuell, Germany) in 20 x SSC (1 x SSC = 0.15 M NaCl, 15
mM sodium citrate). Prehybridization and hybridization were carried out at 42°C in a solution consisting of 40% (v/v) formamide, 5 x SSC, 0.1% (w/v) SDS and 100 mg/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 x SSC, 0.1% SDS, and then washed three times at room temperature in 2 x SSC solution with 0.1% SDS.

Primer extension analysis

The primer 5'-GAATGTGTTTGTGATTCCGACCATG-3', complement-ary to positions 620 to 644 (Fig. 3), was synthesized, and then labeled with [32P] at the 5' end by polynucleotide kinase. Reverse transcriptase-mediated primer extension was performed by the method of Sambrook et al. (30).

![Fig. 1. Construction of a set of plasmids (left column) and nitrilase activity of each R. rhodochrous ATCC12674 transformant (right column).](image)

(Left column) 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 PstI site or PstI site that had been filled in with T4 DNA polymerase, of pK4. In the case of pYHJ40 and pYHJ50, the Nhel 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 boxes. (Right column) Nitrilase activity of whole cells was detected as described in Materials and Methods using benzonitrile as a substrate. ++++, much; +, trace; -, not detected.

**RESULTS**

Expression of nitA in R. rhodochrous ATCC12674

To identify the sequence element required for the expression of nitA, I constructed a set of plasmids containing sub-fragments of a 5.4-kb PstI fragment from pNJ10 (123) inserted at the PstI 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 (31) with or without isovaleronitrile (0.1% v/v). 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 Nhel site to the 5' end terminus of nitA) and a 1.4-kb downstream region (from the 3' end terminus of nitA to the EcoT22I site) are required for the appearance of nitrilase activity, as in pYHJ40. As previously found in R. rhodochrous J1 (11, 23), 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].

**Table 1. Nitrilase activity in cell-free extracts of R. rhodochrous ATCC12674 transformants containing various plasmids.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Isovaleronitrile</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>pYHJ10</td>
<td>+</td>
<td>0.003</td>
</tr>
<tr>
<td>pYHJ20</td>
<td>-</td>
<td>0.193</td>
</tr>
<tr>
<td>pYHJ30</td>
<td>+</td>
<td>0.537</td>
</tr>
<tr>
<td>pYHJ40</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>pYHJ50</td>
<td>+</td>
<td>0.002</td>
</tr>
</tbody>
</table>

N.D.: not detected.

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 Japan, 470A, Tokyo) (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 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 *BsmI*-PvuI 1.29-kb fragment containing *nitA* was sequenced previously (123). The author further sequenced the *Nhel*-BsmI upstream region and the *PvuI*-EcoT221 downstream region required for nitrase production (Fig. 3).

An 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* (125) and to AraC, the positive regulator of arabinose-metabolism in *E. coli* (126); 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 carboxy termini of the proteins. The similarity of NiTR to XylS and AraC, and the importance of nitR for nitrase production, provided strong evidence that NiTR is a positive regulator of *nitA* expression.

Fig. 2. Coomassie-stained SDS-PAGE showing nitrase formation in *R. rhodochrous* ATCC12674 transformants. Lanes indicated by 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 indicated by −, 100 μg of cell-free extracts from the *R. rhodochrous* ATCC12674 transformants cultured in the medium supplemented with (0.1%, v/v) isovaleronitrile; Lanes indicated by −, 100 μg of cell-free extracts from the *R. rhodochrous* ATCC12674 transformants cultured in the absence of isovaleronitrile. The open arrows indicate the band corresponding to the nitrase.

Fig. 3. Nucleotide sequence of flanking region of *nitR* 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 nitrase determined by Edman degradation. Shine-Dalgarno sequences are underlined, and the codons are indicated by asterisks. The cytidine residue in a box is the transcriptional start site of *nitR* determined by primer extension analysis. The *nitR* sequence (denoted with gaps) was published earlier (13, GenBank accession no. D11425). Endpoints of deletions introduced from upstream are marked by vertical arrows. Inverted repeats are shown by converging arrows.

Fig. 4. Comparison of amino acid sequences of NiTR from *R. rhodochrous* J1 and homologous proteins. Amino acid identity of NiTR from *R. rhodochrous* J1, XylS from *P. putida* (125) and AraC from *E. coli* (126) 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.
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). A single mRNA species of 1.4 kb corresponding to nitA was found only in induced cultures (Fig. 5), indicating that nitrilase formation is regulated at the transcriptional level. On the other hand, almost no mRNA corresponding to the nitR-coding region could be detected irrespective of the culture conditions, demonstrating that nitA and nitR are separately transcribed. This is consistent with the presence of a putative p-independent transcriptional terminator (ΔG = -44.6 kcal/mol) in the non-coding region between nitA and nitR (Fig. 3). The divergent transcription of the regulatory and cognate structural genes observed for most members of the xylS/aroC family (127) was not found for nitR.

Mapping of nitA transcript

Since nitrilase is produced at high levels in R. rhodochrous J1 (23) 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, the author 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 (TTCA TG) 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] (128).

![Fig. 5. Northern blot analysis of R. rhodochrous J1](image)

An RNA ladder (Bethesda Research Laboratories) was used as a size marker. The probes used in the experiment are shown in Fig. 1. Lanes indicated by +, 30 µg of RNA extracted from the R. rhodochrous J1 cultured in the medium supplemented with (0.1%, v/v) isovaleronitrile. Lanes indicated by -, 30 µg of RNA extracted from the R. rhodochrous J1 cultured in the absence of isovaleronitrile.

![Fig. 6. Mapping of the 5' end of the nitA transcript](image)

Primer extension analysis using total RNA isolated from R. rhodochrous J1 cultured in the presence (lane indicated by +) or absence (lane indicated by -) 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.
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 Nhel-EcoT221 fragment (= the inserted fragment of pYHJ40) from pNJJ10 was inserted into the EcoRV-PstI sites of pBluescript SK(+) after the Nhel end was filled in with T4 DNA polymerase. The resulting plasmid pNITUP was used to generate deletions from the Nhel/EcoRV 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 ATCC 12674. 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, 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.

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Isovaleronitrile</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYHJ40</td>
<td>-</td>
<td>0.002</td>
</tr>
<tr>
<td>pYHJ101</td>
<td>+</td>
<td>0.297</td>
</tr>
<tr>
<td>pYHJ102</td>
<td>-</td>
<td>0.008</td>
</tr>
<tr>
<td>pYHJ103</td>
<td>+</td>
<td>0.425</td>
</tr>
<tr>
<td>pYHJ104</td>
<td>+</td>
<td>0.009</td>
</tr>
<tr>
<td>pYHJ105</td>
<td>+</td>
<td>0.433</td>
</tr>
<tr>
<td>pYHJ106</td>
<td>+</td>
<td>0.004</td>
</tr>
<tr>
<td>pYHJ107</td>
<td>+</td>
<td>0.514</td>
</tr>
</tbody>
</table>

N.D.: not detected.

In this section, a Rhodococcus host-vector system was used to examine the mechanism involved in the control of nitA expression, using as host R. rhodochrous ATCC12674, which exhibited no detectable nitrilase activity (Fig. 1, Table 1). The author has 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 ATCC 12674. 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. 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 (127, 129). Among this family, for regulators recognizing chemical signals (inducers), the non-conserved 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. In the previous section, the author showed 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 (123). This finding as well as the result from the Northern blot analysis indicates that nitR functions as a transcriptional activator for the formation of NitA.

The author described the mapping of a transcript in Rhodococcus species for the first time. Since 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 Streptomyces consensus promoter sequence, TTAGC(A/G)-17bp-TAg(A/G)(A/G)T (130). 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 promoter 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 (131,132) by several conserved motifs, one of which contains an invariant cysteine as demonstrated in the nitrilases (5,123,124). Nitrilases are significantly similar to aliphatic amidases, cyanide hydratases and β-alanine synthase. The expression of an aliphatic amidase, which is induced by amides such as acetamide and propionamide, is regulated by both a negative regulator, AmiC and a positive regulator, AmiR in *Pseudomonas aeruginosa* (40). The regulation mechanism of *nitA* in *R. rhodochrous* J1 is different from that of the aliphatic amidase. Further studies on nitrilases at both protein and gene levels could provide information about their evolutionary implication.

**SUMMARY**

The 1.4-kb downstream region from a nitrilase gene (*nitA*) of *Rhodococcus rhodochrous* J1 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 *Mr* of 35,100. Its deduced amino acid sequence showed similarity to a positive regulator family including XylS from *Pseudomonas putida* and AraC from *Escherichia coli*. Deletion of the central and 3′ terminal portion of *nitR* resulted in the complete loss of nitrilase activity. The *nitR* protein product was required for nitrilase synthesis. These findings strongly demonstrate that *nitR* codes for a transcriptional positive regulator in *nitA* expression. 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. Almost no transcripts for *nitR* were detected even in the presence of isovaleronitrile in the medium, suggesting that its transcription was relatively poor. 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 bp -52, in induction of *nitA* transcription.

**CONCLUSION**

In this thesis, the author investigated the organization and regulation of genes involved in nitrile metabolism in *Rhodococcus rhodochrous* J1, which is used in the industrial production of useful amides. The results described in each chapter are summarized as follows:

**CHAPTER 1**

This chapter described the gene organization of high molecular-mass nitrile hydratase (H-NHase) from *R. rhodochrous* J1.

Genes required for the expression of (H-NHase) were identified by using a host-vector system in *Rhodococcus*. Sequence analysis of the DNA region responsible for *nhhBA* expression revealed the presence of at least five open reading frames (*nhhC, nhhD, nhhE, nhhF* and *nhhG*) in addition to *nhhBA*. Deletion of *nhhC* or *nhhD* resulted in decrease of NHase activity, suggesting a positive regulatory role for both genes in the expression of the *nhhBA*. *NhhC* showed significant similarity to a regulatory protein, AmiC, which is involved in regulation of amidase expression by binding an inducer amide in *Pseudomonas aeruginosa*. *nhhF*, which has been found to be uninvolved in regulation of *nhhBA* expression by enzyme assay for its deletion transformant and by Northern blot analysis for *R. rhodochrous* J1, showed high similarity of the amino acid sequence to transposases from insertion sequences of several bacteria. Determination of H-NHase activity and *nhhBA* mRNA levels in *R. rhodochrous* J1 indicated that the expression of *nhhBA* is regulated by an amide at the transcriptional level (Section 1).

The author investigated the insertion sequence (IS1164) from *Rhodococcus rhodochrous* J1. In addition, the author compared the structure of IS1164 and IS1081, which shows the highest similarity to IS1164, and found two pairs of terminal inverted repeats flanking their probable transposases. This 1430 bp-long IS has two pairs of imperfect terminal inverted repeats (IR-1 and IR-2) flanked by nine base pair direct repeats (DR), and contains an open reading frame encoding a putative transposase. Structures of the terminal region containing inverted repeats were found to be highly conserved both in IS1164 and in IS1081 from *Mycobacterium bovis*. Hybridization analyses against total DNA from *R. rhodochrous* J1, 16 *Rhodococcus* strains and 3 other bacteria catabolizing nitriles as well as *R. rhodochrous* J1 showed that *R. rhodochrous* J1 had three other IS1164-like elements, and 11 out of 16
Rhodococcus strains contained IS1164-like elements varying in copy number from one to at least seven (Section 2).

CHAPTER II

This chapter described the structure of flanking region of low molecular-mass nitrile hydratase (L-NHase) gene from R. rhodochrous J1 and its regulation.

The author presented the evidence that at least two amidases exist in R. rhodochrous J1. R. rhodochrous J1 produced at least two amidases differing in substrate specificity, judging from the effects of various amides on amidase activity in this strain. These amidases seemed to be inducible enzymes depending on amide compounds (Section 1).

The author cloned and sequenced the amidase gene, which was considered to be linked to the L-NHase gene (nhlBA). This amidase gene (amdA) was located 1.9-kb downstream of nhlA. Therefore, gene arrangement of the amidase and the NHase in R. rhodochrous J1 differed from those in Rhodococcus sp. N-774, Pseudomonas chlororaphis B23 and Rhodococcus sp. The nucleotide sequence indicated that the amidase consisted of 515 amino acids (Mr = 54,626 Da), and the deduced amino acid sequence of the amidase had high similarity to those of amidases from Rhodococcus species including N-774 and P. chlororaphis B23 and indole-3-acetamide hydrolase from Pseudomonas savastanoi. The amidase gene (amdA) modified in the nucleotide sequence upstream from its start codon expressed 8% of the total soluble protein in E. coli under the control of lac promoter, and the amidase was purified from extracts of the E. coli transformant and characterized. The relative molecular mass of the enzyme estimated by HPLC was about 110 kDa, and the enzyme consists of two subunits identical in molecular mass (55 kDa). The enzyme acted upon aliphatic amides such as propionamide and also upon aromatic amidases such as benzamide. It was highly specific for the S-enantiomer of 2-phenylpropionamide, but could not recognize the configuration of 2-chloropropionamide. It also catalyzed the transfer of an acyl group from an amide to hydroxylamine to produce the corresponding hydroxamate (Section 2).

The author identified the genes required for the amide-dependent induction of low molecular-mass nitrile hydratase (L-NHase). The 3.5-kb of 5'-upstream region from nhlBA was required for the amide-dependent expression of nhlBA in a Rhodococcus host strain. Sequence analysis of the region revealed the presence of two open reading frames, nhlD and nhlC. nhlD had similarity to regulatory genes merR, cadC and arsR, which are all located upstream of the heavy metal resistance genes (merAB, cadA and arsBC, respectively) and are supposed to have transcriptional regulatory functions for the resistance genes. NhlC had similarity to the regulators AmiC for the expression of an aliphatic amidase from P. aeruginosa and NhlB for the expression of nhhBA from R. rhodochrous J1. Assay for NHase activity of transformants carrying nhlD deletion or nhlC deletion suggested a negative regulatory role for nhlD and a positive regulatory role for nhlC in the process of the L-NHase formation. Assay for NHase and amidase activities and Western blot analysis of various Rhodococcus transformants carrying each deletion-plasmid, showed that nhlBA and amdA were regulated in the same manner. These findings presented the genetic evidence for a novel gene cluster of L-NHase and amidase induced by amide compounds in R. rhodochrous J1 (Section 3).

Cobalt is an essential component of L-NHase from R. rhodochrous J1. The author identified a new gene, nhlF, in the intervening region between nhlBA and amdA. The product of nhlF, NhlF, showed a significant sequence similarity with those of hoxN from Alcaligenes eutrophus, hupN from Bradyrhizobium japonicum, nixA from Helicobacter pylori and ureH from Bacillus sp., which are considered to be involved in nickel uptake into these cells. Sequence and hydrophathy plot analyses showed that NhlF was a 352-amino acid protein with eight hydrophobic putative membrane-spanning domains. nhlF expression conferred uptake of $^{57}$Cu upon R. rhodochrous ATCC12674 and E. coli JM1109 host cells. The studies using uncouplers suggested that proton gradients are involved in the cobalt transport. These findings suggested that NhlF located in cell membrane energy-dependently mediates the transport of cobalt ions into the cell and therefore facilitates its incorporation into the L-NHase enzyme (Section 4).

CHAPTER III

This chapter described the structure and regulation of nitrilase gene from R. rhodochrous J1.

The author cloned and sequenced the nitrilase gene (nitA) from R. rhodochrous J1. The predicted amino acid sequence from nitA showed similarity with that of the bromoxynil nitrilase from Klebsiella ozaenae. nitA modified in the sequence upstream of the presumed ATG start codon was expressed to about 50% of the total soluble protein in E. coli. The 5,5'-dithiobis(2-nitrobenzoic acid) modification of the nitrilase from R. rhodochrous J1 resulted in inactivation with the loss of one sulphydryl group per enzyme subunit. Mutant enzymes containing Ala or Ser instead of Cys-165 which was only conserved in the Klebsiella nitrilase
did not exhibit nitrilase activity. These findings suggested that Cys-165 played an essential role in the function of the active site (Section 1).

The author investigated the regulation of nitA expression in *R. rhodochrous* J1. The 1.4-kb downstream region from nitA was responsible for the isovaleronitrile-dependent induction of nitrilase synthesis in a *Rhodococcus* host strain. Sequence analysis of the region revealed the existence of a new gene, nitR, which would encode a protein with Mr of 35,100. Its deduced amino acid sequence showed similarity to a positive regulator family including XylS from *P. putida* and Arac from *E. coli*. Deletion of the central and 3' terminal portion of nitR resulted in the complete loss of nitrilase activity. These findings together with the result that nirA modified in the sequence upstream of the ATG start codon was highly expressed under the control of the lac promoter in *E. coli*, even when nitR was absent in the downstream region of nitA, strongly demonstrated that nitR coded for a transcriptional positive regulator in nitA expression. 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 transcriprional 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 bp -52, in induction of nitA transcription (Section 2).

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