A Novel Gene Cluster Including the Rhodococcus rhodochrous J1 nhlBA Genes Encoding a Low Molecular Mass Nitrile Hydratase (L-NHase) Induced by Its Reaction Product*

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The 3.5 kilobases (kb) of the 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 NhChC, for the expression of a high molecular mass nitrile hydratase from R. rhodochrous J1. Assays of NHase activity of transformants carrying nhlD deletion or nhlC deletion mutations suggest a negative regulatory role for nhlD and a positive regulatory role for nhlC in the process of the L-NHase formation. Assays of NHase-correlated amidase activities and Western blot analyses of each Rhodococcus transformant carrying various deletion plasmids, 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 controlling the expression of L-NHase, which is induced by the reaction product (amide) in the “practical microorganism” R. rhodochrous J1.

Nitrile compounds are 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 are also widely used in agriculture. 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, will eventually threaten the environment. Therefore, nitriles are urgent targets from the standpoint of environmental remediation and preservation.

Nitrile hydratase (NHase); EC 4.2.1.84) (1) catalyzes the hydration of a nitrile to the corresponding amide, followed by hydrolysis to the acid plus ammonium by an amidase, whereas nitrilase (2) catalyzes the direct hydrolysis of a nitrile to the corresponding acid plus ammonium (3). Both nitrile-converting enzymes have versatile functions in applied and academic fields and have received increasing general interest: bioremediation, enzymatic production of useful compounds (4), and biosynthesis of a plant hormone (5–8).

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 (4). 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 or cyclohexanecarboxamide in the presence of cobalt ions, H-NHase and L-NHase are selectively induced, respectively (4). 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 biocconversion process for the manufacture of a commodity chemical (4). Furthermore, the industrial production of the vitamin nicotinamide from 3-cyanoypyridine using cells containing L-NHase, which is induced by crotonamide, is due to start next year. Both H- and L-NHases contain cobalt ions as a cofactor in contrast with ferric-NHases from Rhodococcus sp. N-774 (4) and Pseudomonas chlororaphis B23 (9), which had been used for the acrylamide manufacture as 1st and 2nd generation strains, respectively.

We have previously cloned and sequenced both H- and L-NHase genes (nhhBA and nhlBA), respectively, from R. rhodochrous J1 (10). 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 (11), P. chlororaphis B23 (12) and Rhodococcus erythropolis J1 CM 6823 (13). In R. rhodochrous J1, we have found an amidase gene (amDA) 1.9-kb downstream of nhlA (14); however, in Rhodococcus sp. N-774 (11), P. chlororaphis B23 (12), Brevibacterium sp. R312 (15) and Rhodococcus sp. (16), both amidase and NHase genes are found just upstream of 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.

We have examined expression of both nhhBA and nhlBA in

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The abbreviations used are: NHase, nitrile hydratase; bp, base pair(s); kb, kilobases(s); L-NHase, low molecular mass nitrile hydratase; H-NHase, high molecular mass nitrile hydratase; ORF, open reading frame.
Escherichia coli cells under the control of a lac promoter, but the level of NHase activity in the cell-free extracts is much lower than those of H- and L-NHases in R. rhodochrous J1 (10), suggesting that an E. coli RNA polymerase could not recognize the promoters of R. rhodochrous J1 genes or that uncharacterized regulatory genes could be present in this strain. Research suggests that an R. rhodochrous lower than those of H-and L-NHases in the level of NHase activity in the cell-free extracts is much higher.

MATERIALS AND METHODS

Strains and Plasmids—R. rhodochrous J1 was previously isolated from soil (22). E. coli JM109 (23) was the host for pUC plasmid transformation and phage M13mp18/19 propagation. R. rhodochrous ATCC12674 was the host for a Rhodococcus-E. coli shuttle vector plasmid pk4 (17) and its derivatives, and used for nhlBA expression. R. rhodochrous ATCC12674 and pk4 were kindly provided by Prof. Bopp's group (The University of Tokyo, Japan). The plasmid pHJ20L (10) carrying nhlBA of R. rhodochrous J1 in the 9.4-kb Sac fragment on pHU190 (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). [32P]ATP (180 TBq/mmol) and [γ-32P]ATP were from Amer sham (Tokyo, Japan). All other chemicals were used from commercial sources and were of reagent grade.

DNA Manipulation—Total DNA of R. rhodochrous J1 was prepared as described previously (10). DNA manipulation was performed essentially as described by Sambrook et al. (23). The DNA sequence was determined by the dideoxynucleotide chain termination method (24). [α-32P]ATP and Sequenase (U. S. Biochemical Corp.) or [γ-32P]ATP and a Tth sequencing 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 × 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) were mixed with 1 μg of DNA in 1 μl of TE buffer (10 mM-Tris, 1 mM EDTA, pH 8.0) in a 1 mm-gapped electrocuvette (Bio-Rad), and subjected to a 2-kV electric pulse from a Gene Pulser (Bio-Rad) connected to a pulse controller (25-microfarad capacitor; external resistance, 400 Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (17) and incubated for 2 h at 26 °C. They were then spread on MYP medium containing 75 μg of kanamycin ml−1.

Preparation of Cell Extracts and Enzyme Assay—R. rhodochrous ATCC12674 transfomants were grown at 28 °C for 24 h in MYP medium containing CoCl2·6H2O (0.01 glitler) with or without crotonamide (2 glitler), harvested by centrifugation at 4,000 × 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-butyrinic acid, disrupted by sonication for 20 min (39 kHz, Insonator model 201M; Kubota, Tokyo, Japan), and centrifuged at 12,000 × 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 benzamide, 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 of 1 M HCl. The amount of benzamide formed in the reaction mixture was determined as described previously (10). Amidase activity was assayed in a reaction mixture (1 ml) consisting of 10 mM potassium phosphate buffer (pH 7.5), 10 mM benzamidine, 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 of 1 M HCl. The amount of benzamidine formed in the reaction mixture was determined as described previously (14). One unit of these enzymes was defined as the amount catalyzing the formation of 1 μmol of benzamide or benzamidine/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 (25) 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) (14), respectively. Cell extracts prepared by sonication were applied onto an SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane by a standard procedure (23). 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).

RESULTS

Cloning of the 5′ Upstream Region of nhlBA—We cloned the upstream region of nhlBA by the DNA-probing method with a 3.5-kb fragment, which was isolated from pHJ20L (10) 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. Colony hybridization with the probe for screening ampicillin-resistant transformants containing the restriction fragment yielded pHJ20L (10) (Fig. 1). Analysis by restriction endonuclease and by sequencing of the fragment showed that two inserts from pHJ20L and pHJ20L shared a common 1.35-kb SacI-EcoRI region. Plasmid pNLUD30 was constructed by inserting a 6.15-kb EcoRI-Sad fragment from pHJ20L and a 9.4-kb Sac fragment from pHJ20L into the EcoRI-Sad sites of pBluescript SK−.
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-subunit proteins, respectively (10), and amdA encodes an amidase (14). nhlD and nhlC are the newly identified genes described in this paper. The probe used in the experiment are shown by box. Various deletion plasmids are diagramed below the restriction maps. Right panel, NHase activity of whole cells, which were cultivated in the medium with (2 g/liter) or without (−) crotonamide, was detected as described under "Materials and Methods" using benzonitrile as a substrate. +, much; −, trace.

| Plasmid | Nucleotide Activity | Amino Acid Activity |
|---------|---------------------|---------------------|
| pLJK10  | ND                  | ND                  |
| pLJK20  | ND                  | ND                  |
| pLJK30  | ND                  | ND                  |
| pLJK40  | ND                  | ND                  |
| pLJK50  | ND                  | ND                  |
| pLJK60  | ND                  | ND                  |
| pLJK70  | ND                  | ND                  |

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

R. rhodochrous ATCC12674 transformants were cultured in the medium with (+) or without (−) crotonamide (2 g/liter).

- Crotonamide + Crotonamide

units/mg

| Plasmid | pLJK10 | pLJK20 | pLJK30 | pLJK40 | pLJK50 | pLJK60 | pLJK70 |
|---------|--------|--------|--------|--------|--------|--------|--------|
| PK4     | ND     | 0.160  | 0.320  | 0.236  | 0.320  | 0.160  | 0.320  |
| pLJK10  | 18.9   | 47.3   | 41.2   | 17.1   | 49.3   | 25.1   | 17.1   |
| pLJK20  | 27.5   | 43.3   | 45.9   | 17.1   | 49.3   | 25.1   | 17.1   |
| pLJK30  | 25.1   | 43.3   | 45.9   | 17.1   | 49.3   | 25.1   | 17.1   |
| pLJK40  | 8.82   | 27.5   | 43.3   | 17.1   | 49.3   | 25.1   | 17.1   |
| pLJK50  | 25.1   | 43.3   | 45.9   | 17.1   | 49.3   | 25.1   | 17.1   |
| pLJK60  | 25.1   | 43.3   | 45.9   | 17.1   | 49.3   | 25.1   | 17.1   |

The Requirement of nhlC and nhlD for the Amide-dependent Expression of nhlBA—The transformant carrying pLJK30 exhibited the amide-inducible L-NHase expression, whereas the transformant carrying pLJK40, which excludes nhlD, expressed L-NHase constitutively (Fig. 1, Table I). Considering

the downstream region containing amdA encoding the amidase (14) appeared to have no influence on the L-NHase induction.

The L-NHase formation in R. rhodochrous ATCC12674 transformants was examined by SDS-polyacrylamide gel electrophoresis and Western blot analysis (Fig. 2). Immunostaining of the Western blots in this experiment showed two bands of 29 and 26 kDa that corresponded to the accumulated protein detected by Coomassie Brilliant Blue staining. No immunoreactive bands were detected in the R. rhodochrous ATCC12674 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 J1M109 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.

Primary Structure of the Upstream Region of nhlB—The SacI–EcoRI 1.73-kb fragment containing nhlBA was sequenced (10). We further determined the nucleotide sequence of the EcoRV.1-SacI region described above. Fig. 3 depicts the nucleotide and amino acid sequences of two ORFs (nhlC and nhlD) newly found in the region. The presumptive ATG start codon was found for nhlC, but the far less frequent initiation codon TTG was found for nhlD. nhlC and nhlD were preceded by Shine-Dalgarno sequences (SD) located within reasonable distances from the respective presumptive start sites (Fig. 3).

The first ORF named nhlC is 1071 nucleotides long and would encode a protein of 357 amino acids. nhlC showed a significant similarity of amino acid sequence with regulatory genes of nhhC from R. rhodochrous J1 (18) (33.2% identity, 55.4% similarity) and amIC from Pseudomonas aeruginosa (19) (20.2% identity, 41.0% similarity) (Figs. 4A and 7). The AmIC protein (26) is structurally similar to the members of the periplasmic binding protein family, for example BraC from P. aeruginosa (27). NhlC also showed a relatively weak match with BraC (17.2% identity, 43.3% similarity) (Fig. 4A).

The second ORF named nhlD, is 336 nucleotides long, and would encode a protein of 112 amino acids. A computer-aided FASTA search of the SwissProt protein database indicated that nhlD showed similarity to regulatory genes, merR from Streptomyces lividans (28) (24.8% identity, 44.8% similarity), cadC from Staphylococcus aureus (29) (24.7% identity, 43.0% similarity), and arrR from E. coli (30) (24.3% identity, 45.9% similarity). These genes homologous to nhlD are all located upstream of the heavy metal resistance genes (merAB, cadA, and arrBC, respectively) and are supposed to have transcriptional regulatory functions for the resistance genes. Although the identity between NhlD and these homologs is relatively low, they are of similar size (112–125 amino acid residues) and 11 consensus amino acid residues are distributed throughout the sequence (Fig. 4B).

There are extensive noncoding sequences between nhlD and nhlC (357 bp) and nhlC and nhlB (884 bp), possibly indicating regulatory independence of these three genes thus separated.

The Requirement of nhlC and nhlD for the Amide-dependent Expression of nhlBA—The transformant carrying pLJK30 exhibited the amide-inducible L-NHase expression, whereas the transformant carrying pLJK40, which excludes nhlD, expressed L-NHase constitutively (Fig. 1, Table I). Considering

TABLE I

| Plasmid | NHase Activity |
|---------|----------------|
| PK4     | ND             |
| pLJK10  | 0.160          |
| pLJK20  | 0.320          |
| pLJK30  | 0.236          |
| pLJK40  | 28.9           |
| pLJK50  | 40.3           |
| pLJK60  | 28.0           |

a ND, not detected.
that nhlC is present in both pLJK30 and pLJK40, in the presence of amide as an inducer, nhlD functions negatively, whereas nhlC functions positively for the L-NHase expression. To examine the necessity of nhlC for nhlBA 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 unit/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, NhlC inhibited the action of repressor NhlD, leading to the L-NHase expression, while NhlC could not function in the absence of amide in the medium, leading to repression of the L-NHase expression by NhlD.

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 II) 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 I), suggesting that both nhlBA and

Fig. 2. Expression of L-NHase α- and β-subunit proteins in R. rhodochrous transformants. Upper panel, Coomassie Brilliant Blue-stained gel showing electrophoretically separated proteins. R. rhodochrous ATCC12674 was transformed with pLJK4 and pLJK4 derivatives and cultured in the medium with (+) or without (−) crotonamide (2 g/liter). The extra bands corresponding to the α- and β-subunit proteins are indicated by arrows. 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 kDa), and α-lactalbumin (14 kDa). Lower panel, Western blot of a similar gel after immuno-staining with antibodies specific for the R. rhodochrous J1 L-NHase.

Fig. 3. Nucleotide sequences and the deduced amino acid sequences for nhlC and nhlD. The deduced amino acid sequences of the ORFs are shown below the nucleotide sequence in one-letter code. Potential ribosome-binding sequences are marked as SDs, and relevant stop codons are indicated by asterisks. The sequence is numbered from the EcoRV.1 site upstream of nhlD (Fig. 1).
amidA 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 NhlD 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 medium with or absence of cobalt ions and in the presence or absence of crotonamide. Western blot analysis was carried out using anti-L-NHase antiserum (Fig. 6A) and anti-amidase antiserum (Fig. 6B) 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 unit/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 nhlBA by measuring NHase activity.

**DISCUSSION**

Here, we report two ORFs (nhlC and nhlD), which are located upstream nhlB in R. rhodochrous J1. This gene organization is distinct from that of each NHase gene that has so far been reported. nhlC seems to play a positive role for formation of active L-NHase in the presence of amide-inducer. The 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 (31, 32). Formation of the Pseudomonas amidase derived from the amiE gene is positively regulated by AmiR via a transcription anti-termination mechanism (33), 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 (26). L-NHase is also induced by amide compounds (not by nitriles), i.e. acetamide, propionamide, acrylamide, methacrylamide, crotonamide, and cyclohexanecarboxamide, which are products of the nitrile hydration reaction catalyzed by NHase. The finding that nhIC (amiC homolog) is responsible for the amide-inducible expression of nhh enzyme is very interesting, while similarity in amino acid sequence is not observed among amide-degrading enzymes; AmiE (the P. aeruginosa amidae) (34) does not show any similarity to AmdA from R. rhodochrous J1. Assuming that nhIC, as well as AmiC, functions as a sensor protein sensitive to amide compounds in the culture medium, we suggest that nhIC is involved in induction of the L-NHase synthesis in some way. nhC has higher similarity with nhhC than with amiC.

We showed here the coordinate expression of nhhBA and amdA in the experiments including enzyme assays and Western blot analyses using various transformants. We have already reported (14) that L-NHase and the amidase showed similar trends in substrate specificity for aliphatic and aromatic compounds: L-NHase acts on benzamidine (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.

nhID was found to be involved in nhhBA expression in R. rhodochrous ATCC12674. nhID possibly functions as a repressor in the absence of the inducer amide, and this repression would be relieved by the action of nhIC in the presence of amide in the medium. It is interesting to note that nhID is similar to merR, cadC, and arsR, which transcriptionally regulate the heavy metal resistance systems, i.e. detoxification or transport of heavy metals. L-NHase, the expression of which is regulated by nhID, is also associated with a heavy metal; L-NHase contains 1.7 atoms cobalt/mol enzyme,2 and is produced as an active form in R. rhodochrous J1 only in the presence of cobalt ions in the culture medium (4). In the experiments using R. rhodochrous ATCC12674 transformants, moreover, Co2+ is the essential element for the NHase expression in the culture medium. merR, cadC, and arsR are considered to repress respective structural genes conferring heavy metal resistance, and the repression may be relieved by the presence of the heavy metal ions, Hg2+, Cd2+, and AsO3−, respectively (35–37). In contrast, repression of nhhBA expression by nhID appears to be inhibited by nhIC in the presence of the inducer amide, instead of cobalt ions. Furthermore, Western blot analyses and assays for amidase activity demonstrated that the presence of cobalt ions had no effect on the formation of L-NHase and amidase, but the former enzyme requires cobalt ions in its active form. These findings raise the possibility that the repressor nhID might have lost a function as a heavy metal sensor during the course of evolution.

Fig. 7 compares the organization of nhhBA 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 transposable and constitutes a possible insertion sequence (IS1164) together with its flanking inverted repeats (18), and nhhe is similar to the gene (ORF5) found in a potential transposable element R46 (38). 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 nhhBA and nhhCB and nhhhBA, together with the finding that both nhhC and nhhD may play roles in the L- and H-NHase expression, respectively, although nhhD 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.

The role of nitrile-converting enzymes in the biosynthesis of a plant hormone, indole-3-acetic acid, is recently attracting increasing attention (5–7). We have also reported the occurrence of a biosynthetic pathway for indole-3-acetic acid from indole-3-acetonitrile via indole-3-acetamide by the combined action of NHase and amidase in phytopathogenic bacteria Agrobacterium tumefaciens and in leguminous bacteria Rhizobium (8). Studies on nitrile metabolism in Rhodococcus at both protein and gene levels could also provide information about the biosynthesis of indole-3-acetic acid in plant-associated bacteria and plants, and the evolutionary relationships of the former to the latter organisms.

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A Novel Gene Cluster Including the *Rhodococcus rhodochrous* J1 nhlBA Genes Encoding a Low Molecular Mass Nitrile Hydratase (L-NHase) Induced by Its Reaction Product

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