Nitrile Hydratase Gene from *Rhodococcus* sp. N-774 Requirement for Its Downstream Region for Efficient Expression

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For improvement of the production of nitrile hydratase (NHase) from *Rhodococcus* sp. N-774 by recombinant DNA techniques, several plasmids, each of which had a deletion of the upstream or downstream region of the genes encoding the α and β subunits of NHase, were constructed. Enzyme assays of recombinant *R. rhodochrous* and *Escherichia coli* cells showed that a downstream region of the NHase genes was indispensable for the production of active NHase in both cells, but for the production of the active amidase, no genes other than the amidase structural gene were required. The nucleotide sequence of the downstream region contained a single open reading frame (Orf1188) with 396 amino acids. Orf1188 showed similarity in amino acid sequence to P47K, an open reading frame found downstream of the NHase genes from *Pseudomonas chlororaphis* B23, and also to the cobW gene product, which may be involved in cobalamin biosynthesis in *Pseudomonas denitrificans*. Because the distance between the TGA stop codon for the NHase β-subunit and the ATG codon for Orf1188 is only 98 bp, and because production of both Orf1188 and NHase is dependent on a promoter upstream of the amidase gene, these genes appear to be co-transcribed in a polycistrionic manner, forming an operon. By optimization of the culture conditions of *R. rhodochrous* carrying pKRN1H2, which contained the amidase, NHase, and Orf1188 genes, the transformant showed the NHase activity 6-fold higher than that of the original strain, *Rhodococcus* sp. N-774.

Nitrile hydratase (NHase), which catalyzes the hydration of various nitrile compounds to the corresponding amides, is used for industrial production of acrylamide.1–3 Recent screening for microorganisms that have an NHase activity found that the microorganisms also contains an amidase(s) which converts the amide compounds formed by NHase into the corresponding carboxylic acids and ammonia.4 We previously cloned the genes for both α and β subunits of NHases and for amidases from *Rhodococcus* sp. N-774,5,6 *Pseudomonas chlororaphis* B23,7 *Rhodococcus rhodochrous* J1,8,9 and *Rhodococcus erythropolis* JCM6823,10 and showed that the NHase and amidase genes were present, forming a gene cluster. Attempts to produce these NHases in *Escherichia coli* have so far failed. For example, when the NHase genes from *Rhodococcus* sp. N-774 were expressed in *E. coli* under the control of the lac promoter, the NHase proteins accumulated as insoluble proteins with no activity in the cells. Therefore, denaturation-renaturation with urea under alkaline conditions were required to obtain the active NHase.5 On the other hand, the host-vector system for a *Rhodococcus* strain turned out to be useful for efficient production of active NHase proteins in a yield comparable to that produced by the original strain *Rhodococcus* sp. N-774.11

For further improvement of production of the NHase from *Rhodococcus* sp. N-774, which is useful for the production of acrylamide in a higher yield, we constructed several NHase-expression plasmids and examined the production of NHase directed by the plasmids. From this analysis, a downstream region of the NHase genes was found to be required for production of the active NHase both in *R. rhodochrous* and *E. coli*. In this paper, we describe the nucleotide sequence of the downstream region of the NHase genes from *Rhodococcus* sp. N-774 along with overproduction of the NHase by use of a *Rhodococcus* expression system.

Materials and Methods

**Bacterial strains and growth conditions.** *Rhodococcus rhodochrous* ATCC12674 was obtained from the American Type Culture Collection and was cultured at 26.5°C in MYP medium as described previously.9 *E. coli* TG1 [Δlac pro] supE thi hsdS5 FTRD36 proAB lacIq lacZAM15] was grown at 37°C in 2×YT medium.12

**Enzymes and chemicals.** Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, and bacterial alkaline phosphatase were purchased either from Takara Shuzo or Boehringer-Mannheim GmbH. [γ-32P]ATP (15 TBq/mmole) was from Amersham Japan. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Wako Pure Chemicals (Tokyo, Japan).

**Transformation and Plasmid DNA isolation from Rhodococcus.** Plasmid DNA was isolated from *R. rhodococcus* ATCC12674 by the method of Vogt Singer & Finnerty.13 Transformation of *R. rhodochrous* ATCC12674 was done by electroporation as described previously.11

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Abbreviations: NHase, nitrile hydratase; IPTG, Isopropyl-β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PQQ, pyrroloquinoline quinone.
DNA manipulation. Procedures with E. coli were done as described by Maniatis et al. For nucleotide sequencing, specific restriction fragments covering the downstream region of the NHase genes were cloned on appropriate M13 vectors and sequenced by the dideoxynucleotide chain-termination method.

Enzyme assays. The activities of NHase and amidase were measured using whole cells as described previously. Protein was measured by the Coomassie brilliant blue G-250 dye-binding method of Bradford using a dye reagent supplied by Bio-Rad (Richmond, CA, U.S.A.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli and proteins were stained with Coomassie brilliant blue R-250. The NHase was detected by Western blotting with the anti-NHase antiserum against the NHase purified from Rhodococcus sp. N-774 using horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad).

Results

Effects of deletion of the upstream and downstream regions of the NHase gene on the NHase production in Rhodococcus rhodochrous

When methylylamine was added, R. rhodochrous ATCC12674 harboring pK4 produced a small amount of its own NHase protein, which cross-reacted with the antibodies raised against the NHase from Rhodococcus sp. N-774 and showed weak NHase activity (Fig. 1). However, when pKRNH2 with a 8.9-kb PstI–SpfI fragment, which carried the genes for amidase and NHase from Rhodococcus sp. N-774 was introduced, R. rhodochrous ATCC12674 produced large amounts of both amidase and NHase in the presence of 0.1% methacrylamide as an inducer, as described previously (Fig. 1). As a first step for further improvement of the yields of these enzymes, especially of NHase, we constructed the plasmid pAKR325, which contained the whole structural genes for amidase and both subunits of NHase but lacked both an upstream portion of the amidase-coding region and a downstream portion of β-subunit-coding region of pKRNH2 (Fig. 1-C). When R. rhodochrous cells harboring pAKR325 were cultured in the medium with methylylamine, the transformant produced active amidase protein in a large amount as did the transformant carrying pKRNH2, but NHase was not detected in the transformant carrying pAKR325 (Figs. 1-A, B, C), suggesting that a determinant necessary for the production of NHase is present upstream or downstream of the NHase gene.

Requirement of the downstream region of the NHase genes for the NHase production

We next examined which portion, upstream or downstream, of the NHase genes is necessary for the NHase production by using an E. coli expression system. For this purpose, we constructed two other plasmids, pYUK123

Fig. 1. Production of NHase from Rhodococcus sp. N-774 in R. rhodochrous.

(A) SDS-polyacrylamide gel electrophoresis of crude extracts prepared from R. rhodochrous ATCC 12674 transformants, stained with Coomassie brilliant blue. Molecular mass standards used were phosphorylase β (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and a-lactalbumin (14.4 kDa). Lanes 1 and 3, crude extract prepared from Rhodococcus sp. N-774 cultured without methylylamine (MA), as a control; 2 and 3, same as lane 1 but prepared from R. rhodochrous ATCC12674 harboring pk4 cultured without and with MA, respectively; 4 and 5, same as lane 2 but prepared from R. rhodochrous ATCC12674 harboring pKRNH2 cultured without and with MA, respectively; 6 and 7, same as lane 2 but prepared from R. rhodochrous ATCC12674 harboring pAKR325 cultured without and with MA, respectively.

(B) Western blotting with anti-NHase serum. Lanes: 1 to 8, same as panel (A).

(C) Restriction maps of DNA fragments cloned into pk4 plasmid and enzyme activities directed by the recombinant plasmids.

Arrows indicate the direction of transcription. Abbreviations: a, gene of NHase subunit; β, gene of NHase subunit; S, SpfI; B, BamHI; H, HindIII; Sa, SalI; P, PstI.
and pANH305, with upstream and downstream deletions, respectively, and analyzed the NHase production, by an immunological method, and the enzyme activities of the cells. *E. coli* cells harboring pANH305 with the amidase and NHase genes as well as the upstream region showed amidase activity but not NHase activity (Fig. 2C). On the other hand, *E. coli* transformant carrying pYUK123, with the 4.3-kb *HindIII*-SphI fragment containing both NHase-subunit genes and their downstream portion, produced NHase mostly in a soluble fraction and had significant NHase activity (Fig. 2). These results clearly indicate that the downstream region of the NHase genes has a crucial role in the production of active NHase enzyme.

**Nucleotide sequence of the downstream region of the NHase β-subunit gene**

To identify a putative determinant required for the production of the NHase, the nucleotide sequence of the downstream region was analyzed (Fig. 3). A single open reading frame (Orf1188) of 1188 bp encoding a protein of 396 amino acids (Mr 43,441) was found. A potential ribosome-binding sequence (SD), GGAAGGA, was present 11 nucleotides upstream of the putative translational initiation codon (ATG). The distance between the TGA stop codon of the β-subunit of NHase and the initiation codon, ATG, of this open reading frame is only 98 bp, suggesting the possibility that these genes are transcribed as a single operon. Orf1188 showed amino acid sequence similarity (44%) to the open reading frame found downstream of the NHase genes of *P. chlororaphis* B23 (P47K).7) To identify the function of these putative proteins, we searched for proteins showing amino acid sequence similarity to these proteins in NRBB protein data bank by the method of Lipmann and Pearson.19) This analysis showed that Orf1188 and P47K had significant similarities in amino acid sequence (28.9% and 25.9%, respectively) to the CobW protein from *Pseudomonas denitrificans* in the NH2-terminal portion (Fig. 4).20) CobW is the product of the *cobW* gene which is contained in a gene cluster for biosynthesis of cobalamin, a cobalt-containing coenzyme, although its function in biosynthesis is not known.

**Overproduction of the NHase in *R. rhodochrous ATCC12674***

As described above, the downstream region encoding
Orf1188 was required for production of active NHase. We next tried to enhance the yield of the NHase by controlling culture conditions of *R. rhodochrous* ATCC12674 harboring pKNH2, which had the downstream region as well as the amidase and NHase genes. When an overnight culture of *R. rhodochrous* cells harboring pKNH2 was transferred to fresh medium at a ratio of 1:100, and cultured aerobically in the presence of 0.2% methacrylamide, NHase activity of 155 units per mg dry cell was detected after 24h of cultivation (Table I). However, prolonged culture resulted in a decrease in the NHase activity. Addition of the inducer at higher concentrations (0.3 and 0.4%) inhibited the growth of the transformant (data not shown). On the other hand, when the full growth culture of *R. rhodochrous* cells harboring pKNH2 was inoculated into fresh medium at a higher ratio (1:10), growth of the cells in the medium containing methacrylamide up to 0.3% was observed. After 20h of cultivation in the presence of 0.3% methacrylamide, the transformants had NHase activity of 518 units (Table II), which was 6 times higher than that produced by the original strain, *Rhodococcus* sp. N-774 (83.4 units/mg dry cell). In addition, overproduction of the amidase was also observed in the transformant (2.0 units/mg dry cell) compared with the original strain *Rhodococcus* sp. N-774 (0.3 units/mg dry cell). When the amount of the NHase and amidase proteins were analyzed by densitometry of SDS-polyacrylamide gel of the crude extract after Coomassie brilliant blue staining, the amidase and NHase proteins were estimated to occupy 23% and 40% of the total soluble protein, respectively (Fig. 5). A protein of approximately 45 kDa in size, which is similar to that of Orf1188, was also inducibly produced only in the presence of methacrylamide.

Fig. 3. Nucleotide Sequence of the Downstream Region of NHase β-Subunit-coding Region and Deduced Amino Acid Sequences.
Activation of Nitrile Hydratase

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>MA (%)</th>
<th>Specific activities (units/mg dry cell)</th>
<th>12</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>48</th>
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</thead>
<tbody>
<tr>
<td>pK4</td>
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<td>0.60</td>
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<tr>
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<td>8.16</td>
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<tr>
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<td>139</td>
<td>155</td>
<td>103</td>
<td>39.3</td>
<td>16.8</td>
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</table>

MA: methacrylamide.
Cultivation time represents the time after 1% inoculation of the overnight culture to a fresh medium with or without 0.2% methacrylamide.

Table II. Effects of Methacrylamide Concentration on NHase Production of R. rhodochrous Carrying pKRNH2.

<table>
<thead>
<tr>
<th>Plasmids</th>
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<th>Growth (OD630)</th>
<th>Specific activities (units/mg dry cell)</th>
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</thead>
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<td></td>
<td>0.3</td>
<td>5.1</td>
<td>518</td>
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</table>

MA: methacrylamide.
The NHase activity was measured with whole cells cultured for 20h after 10% inoculation of the overnight culture to a fresh medium.

Discussion

This study showed that the Orf1188 gene was necessary for production of the active NHase in both E. coli and Rhodococcus. Since NHase and amidase were inducibly produced by the addition of methacrylamide in R. rhodochrous ATCC12674, or IPTG in E. coli, transcription of the NHase genes may depend on a promoter just upstream of the amidase gene in Rhodococcus sp. N-774 (unpublished data), or on the lac promoter in the E. coli system. In addition, NHase proteins were produced even in the absence of Orf1188, although it was accumulated as inclusion bodies (Fig. 2 (B)). Therefore, we assume that Orf1188 activates the NHase posttranslationally. The NHases from
Rhodococcus sp. N-774, P. chlororaphis B23 and Brevibacterium sp. R312 contain ferric ions and pyrroloquinoline quinone (PQQ) as prosthetic groups \(^{21,22}\) (I. Watanabe, unpublished results). Therefore, it is possible that Orf1188 may play a role in incorporating a ferric ion or PQQ into the cell or into the enzyme. However, preliminary observations seem to deny this possibility, because ferric ion and PQQ added to the E. coli transformant producing the N-cleaved form of Orf1188, have no effects on the production or enzyme activity of the N-cleaved form. \(^{7}\) On the other hand, the fact that Orf1188 and P47K show significant similarity in amino acid sequence to CobW, which is probably involved in the cobalamin biosynthesis in P. denitrificans, may give a clue to the role of Orf1188. Amino acid sequence similarity of Orf1188 and P47K to CobW was found only in the N-terminal portions of these proteins. The most striking similarity was observed in Pro7-Thr21, Glu62-Thr77, Leu86-Ile104, and Arg150-Ala186. It is noteworthy that the homologous regions (Pro7-Thr21 and Leu86-Ile104) contain each of the two consensus motifs found in ATP-binding protein (Fig. 6). \(^{23}\) This observation suggests that these proteins require ATP for their functions, which indicates us of molecular chaperones serving for several proteins to take a correct folding. \(^{24,25}\) To clarify the role of Orf1188 in the NHase activation, further study is obviously required.

*R. rhodochrous* harboring pKRNH2 produced large amounts of amidase and NHase (23 and 40%, respectively). However, Orf1188 was produced only at a low level (Fig. 5), although the Orf1188 genes may be transcribed in a polycistronic manner together with the amidase and NHase genes, forming an single operon, because the distance between the TGA stop codon of the beta-subunit of NHase and the initiation codon, ATG, of this open reading frame is relatively short, and no possibly promoter sequence is found in this region. An inverted repeat sequence capable of forming a strong hairpin structure (\( \Delta G = -165.5 \text{kJ/mol} \)) is found in the intervening region between the genes encoding the beta-subunit of NHase and Orf1188. Therefore, we speculate that the possible secondary structure of mRNA may decrease the elongation of mRNA toward the Orf1188 gene downstream. Detailed analyses will be required for discovering how the gene expression of the NHase operon is regulated.

**References**

Activation of Nitrile Hydratase