Transcriptional Regulation of the Nitrile Hydratase Gene Cluster in *Pseudomonas chlororaphis* B23[▽]

Toshihide Sakashita,[†] Yoshiteru Hashimoto,[†] Ken-Ichi Oinuma, and Michihiko Kobayashi*

Institute of Applied Biochemistry, and Graduate School of Life and Environmental Sciences, The University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Received 13 January 2008/Accepted 2 April 2008

An enormous amount of nitrile hydratase (NHase) is inducibly produced by *Pseudomonas chlororaphis* B23 after addition of methacrylamide as the sole nitrogen source to a medium. The expression pattern of the *P. chlororaphis* B23 NHase gene cluster in response to addition of methacrylamide to the medium was investigated. Recently, we reported that the NHase gene cluster comprises seven genes (*oxdA*, *amiA*, *nhpA*, *nhpB*, *nhpC*, *nhpS*, and *acsA*). Sequence analysis of the 1.5-kb region upstream of the *oxdA* gene revealed the presence of a 936-bp open reading frame (designated *nhpR*), which should encode a protein with a molecular mass of 35,098. The deduced amino acid sequence of the *nhpR* product showed similarity to the sequences of transcriptional regulators belonging to the XylS/AraC family. Although the transcription of the eight genes (*nhpR*, *oxdA*, *amiA*, *nhpABC*, *nhpS*, and *acsA*) in the NHase gene cluster was induced significantly in the *P. chlororaphis* B23 wild-type strain after addition of methacrylamide to the medium, transcription of these genes in the *nhpR* disruptant was not induced, demonstrating that *nhpR* codes for a positive transcriptional regulator in the NHase gene cluster. A reverse transcription-PCR experiment revealed that five genes (*oxdA*, *amiA*, *nhpA*, *nhpB*, and *nhpC*) are cotranscribed, as are two other genes (*nhpS* and *acsA*). The transcription start sites for *nhpR*, *oxdA*, *nhpA*, and *nhpS* were mapped by primer extension analysis, and putative -12 and -24 σ^{54} -type promoter binding sites were identified. NhpR was found to be the first transcriptional regulator of NHase belonging to the XylS/AraC family.

We have extensively studied the biological metabolism of toxic compounds which have a triple bond between their carbon and nitrogen atoms, such as nitriles ($R-C\equiv N$) (22) and isonitriles ($R-N\equiv C$) (5, 8, 9). The microbial degradation of nitriles proceeds through two different enzymatic pathways (21): (i) nitrilase catalyzes the hydrolysis of nitriles into acids [$R-C(=O)-OH$] and ammonia (19, 25), and (ii) nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides [$R-C(=O)-NH_2$] (1, 24), which are subsequently hydrolyzed to acids and ammonia by amidase (18). These enzymes have received much attention in applied fields (13, 14, 48), as well as academic fields (2, 4, 36, 38). One of the fruits of our application-oriented nitrile studies is the current industrial production of acrylamide and nicotinamide using the NHase of an actinomycete, *Rhodococcus rhodochrous* J1 (22, 46). Acrylamide is one of the most important commodities, and it is in great demand (200,000 tons per year worldwide) as a flocculant, a component of synthetic fibers, a soil conditioner, and a petroleum recovery agent. The industrial production of acrylamide using the ferric NHase of *Rhodococcus* sp. strain N-774, the first-generation strain, started in 1985 (12, 45). This biotransformation of acrylonitrile to acrylamide by a microbial NHase was the first application of biotechnology 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. On the other hand, the NHase of *Pseudomonas chlororaphis* B23 (33), which was previously used as a catalyst for acrylamide manufacture (42, 46), is now used for the production of 5-cyanovaleramide, a herbicide intermediate, at the industrial level (10). Strong induction of NHase formation was observed after addition of methacrylamide as the sole nitrogen source (47). A substrate of an enzyme often acts as an inducer; however, the reaction product of NHase, methacrylamide, serves as a strong inducer of the NHase of *P. chlororaphis* B23. Because the amount of NHase produced comprises more than 50% of the total soluble proteins in *P. chlororaphis* B23 when this strain is cultured in the presence of methacrylamide (34), there must be a very interesting regulation mechanism for enzyme expression. We previously cloned the NHase gene from this strain and discovered the existence of a gene cluster that includes the aldoxime dehydratase (29, 37), NHase, and amidase (35) genes. Very recently, we also discovered that the acyl coenzyme A (acyl-CoA) synthetase gene (*acsA*) is an additional member of this gene cluster and that aldoxime dehydratase, NHase, amidase, and acyl-CoA synthetase sequentially cooperate with one another in vivo for utilization butyraldoxime as a carbon and nitrogen source (11). Although we have clarified the overall “nitrile pathway” (aldoxime $\rightarrow$ nitrile $\rightarrow$ amide $\rightarrow$ acid $\rightarrow$ acyl-CoA) at the protein and gene levels, the mechanism that regulates NHase gene cluster expression in *P. chlororaphis* B23 has not been clarified.

In this study, we investigated transcriptional regulation of the NHase gene cluster in *P. chlororaphis* B23. We found that *nhpR* located in the region upstream of the aldoxime dehydratase gene encodes a transcriptional activator that controls NHase gene cluster expression. The proposed transcriptional

* Corresponding author. Mailing address: Institute of Applied Biochemistry, and Graduate School of Life and Environmental Sciences, The University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan. Phone: (81)-29-853-4679. Fax: (81)-29-853-4605. E-mail: sec-mmb@agbi.tsukuba.ac.jp..

[†] T.S. and Y.H. contributed equally to this work.

[▽] Published ahead of print on 11 April 2008.

activation mechanism for the NHase gene cluster provides an explanation for the induction of nitrile degradation and synthesis enzymes in response to addition of methacrylamide to the growth medium.

MATERIALS AND METHODS

Plasmids, strains, and media. Plasmid pPCN4 comprising the 6.5-kb SphI-SalI fragment of pUC19 (35) was used as a probe to clone the region upstream of the aldolase dehydratase gene in *P. chlororaphis* B23. *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA) was used as the host for pUC (49) and pSTV29 (Takara Bio, Tokyo, Japan). *E. coli* transformants were grown in 2× YT medium (31). The minimum medium for *P. chlororaphis* B23 was composed of 1% sucrose, 0.5% (NH₄)₂SO₄, 0.05% KH₂PO₄, 0.05% K₂HPO₄, 0.05% MgSO₄ · 7H₂O, and 0.001% FeSO₄ · 7H₂O (pH 7.0). Unless otherwise stated, sucrose and (NH₄)₂SO₄ were used as the carbon and nitrogen sources, respectively.

DNA manipulations. Restriction endonucleases, DNA polymerase, and T4 DNA ligase were purchased from Toyobo Co., Ltd. (Osaka, Japan). Nucleotides were sequenced by the dideoxy chain termination method using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). Unless otherwise stated, DNA manipulations were performed essentially as described by Maniatis et al. (31).

Disruption of the chromosomal *nhpR* gene. To disrupt the *nhpR* gene on the *P. chlororaphis* B23 chromosome, a 2.9-kb DNA region containing the complete *nhpR* gene between the HindIII and XhoI sites was cloned into the HindIII and SalI sites of pSTV29, which is a chloramphenicol-resistant vector and cannot replicate in *P. chlororaphis* B23. The coding sequence of the kanamycin resistance gene was amplified by PCR with pUC-4K (43) as the template. In order to disrupt the HindIII and XhoI sites in the kanamycin resistance gene, site-directed mutagenesis was carried out by means of an overlap extension PCR protocol without any change in the amino acid sequence (15, 39). The primers used for amplification of the kanamycin resistance gene were Km-F (5'-ATCGGTGCGGCCCTCTTCGC-3'), Km-R (5'-CGAGTGAGTAATCCGTGGGGT-3'), HindIII-F (5'-TCCGGTGAGAATGCAACAGTTTATGCATT-3'), HindIII-R (5'-TCTGAAAGAAATGCATAAAGTGTGGCCAT-3'), XhoI-F (5'-TGGAAATTTAATCGCGGCTGGAACAAGACG-3'), and XhoI-R (5'-CAACGGGAAACGTCTTGTTCAGGCCGCGA-3'). The amplified kanamycin resistance gene (containing no HindIII and XhoI sites) was digested with HincII and then inserted into the Ball site in the *nhpR* gene on pSTV29. The resulting plasmid was introduced into *P. chlororaphis* B23 by electroporation to generate a *P. chlororaphis* B23 *nhpR* disruptant. Transformants were selected on 2× YT agar plates containing kanamycin (50 µg/ml) and the same plates containing chloramphenicol (30 µg/ml). Double crossovers were identified as Km^r and Cm^s. Transformants were subjected to PCR and Southern hybridization analyses in order to examine insertion of the kanamycin resistance gene into the *nhpR* gene on the *P. chlororaphis* B23 chromosome.

Enzyme assay. Aldoxime dehydratase activity was measured anaerobically under reduced conditions (standard assay B), as described previously (37). One unit of aldoxime dehydratase activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol butyronitrile/min from butyraldoxime under the standard assay conditions. NHase activity was assayed by the method described previously (33). One unit of NHase activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol propionamide from propionitrile under the standard assay conditions. Protein concentrations were determined as described by Bradford (3). Specific activity is expressed in U/mg of protein.

RNA experiments. For the isolation of RNA from *P. chlororaphis* B23, cells were cultivated in 10 ml of medium with or without 0.5% methacrylamide at 28°C. Total RNA was isolated with an RNeasy mini kit (Qiagen, Hilden, Germany).

Primer extension analysis. Primer extension experiments were performed using SuperScript III reverse transcriptase (Invitrogen) by the method of Maniatis et al. (31). The primers used for detection of the start sites of the *nhpR*, *oxdA*, *nhpA*, and *nhpS* genes were 5'-AGTGCCAGCGTGTGCTTTTC-3', 5'-TGTGCAAAGGCCAGGAAGCG-3', 5'-TCGAAGGTGTGCGAGTCGTG-3', and 5'-AGTCGCCGACACAGCTGTGTC-3', respectively. The primers were labeled with ³²P at the 5' end with T4 polynucleotide kinase (Takara Bio).

RT-PCR. For cDNA synthesis, total RNA was incubated for 200 min at 50°C with SuperScript III reverse transcriptase (Invitrogen). Control reactions to assess the level of DNA contamination in the RNA samples were carried out without the reverse transcriptase. Primers RT1, RT2, and RT3 were used for the reverse transcription (RT) reaction, and the F1-R1, F2-R2, and F3-R2 primer pairs were used for successive PCR amplification. The sequences of the primers were as follows: RT1, 5'-GCGTGGGGCGTGCCGATAAG-3'; RT2, 5'-ACAG

GCAGGCCTTGCGCATC-3'; RT3, 5'-TTGCGCAGAATGAACCGTTG-3'; F1, 5'-CCCATGCATCGACAACGTTTC-3'; F2, 5'-AAAATGGAAGCTACCGATAC-3'; F3, 5'-GCTTGCCGACAGACTGGACC-3'; R1, 5'-ATAAGGCCTCGGTGTCAAAG-3'; and R2, 5'-TGGGCAGATGGTTCGACAAAC-3'. The thermal cycling conditions were as follows: 1 min at 94°C, followed by 30 cycles of 10 s at 98°C and 15 min at 68°C with TaKaRa LA *Taq* (Takara Bio).

Real-time PCR. For cDNA synthesis, total RNA was incubated for 50 min at 50°C with SuperScript III reverse transcriptase (Invitrogen) in the presence of random hexamer primers. Control reactions to assess the level of DNA contamination in the RNA samples were carried out without the reverse transcriptase. Real-time PCR was performed using a thermal cycler Dice real-time system (Takara Bio) with SYBR Premix EX *Taq* (Takara Bio). Transcripts of *nhpR*, *oxdA*, *amiA*, *nhpABC*, *nhpS*, and *acsA* were quantified with the following primers: *nhpR*-F (5'-ACGAGAAGGTGCGAGCAAAGC-3'), *nhpR*-R (5'-ACACGGCAATGGTCTCGAC-3'), *oxdA*-F (5'-ACGCATCTCAAATGCCACG-3'), *oxdA*-R (5'-TACTGCACGCCGAGATAACC-3'), *amiA*-F (5'-ACAGGACATCACCGGGCATC-3'), *amiA*-R (5'-GTCCACCGAGGCTTCAAACG-3'), *nhpA*-F (5'-TCAAGAGCAAGAACTCATC-3'), *nhpA*-R (5'-TTCCGTCTTGAGCAGCAGC-3'), *nhpB*-F (5'-TGCACCCGACCTCAGAGCAG-3'), *nhpB*-R (5'-CGTCACCCCATAGATCTTTC-3'), *nhpC*-F (5'-GGACGAATATCGGTTGCAGG-3'), *nhpC*-R (5'-GCACTCAGCAACAATGGTC-3'), *nhpS*-F (5'-GACACAGGAAGTCACCCAAC-3'), *nhpS*-R (5'-GCAGCGGTTCCATTCACCTC-3'), *acsA*-F (5'-GATTATCTGCAGAGCGCCAC-3'), and *acsA*-R (5'-CATGGCCATCCTGCGCTTCG-3'). The transcript level of each gene was normalized to that of the internal control, the ribosomal *rpsL* gene. The PCR primers used for quantifying *rpsL* transcripts were *rpsL*-F (5'-AAACCGTTGGTCAGACGCAC-3') and *rpsL*-R (5'-ACGTCGTGGCGTATGCATC-3'). The thermal cycling conditions were as follows: 10 s at 95°C, followed by 45 cycles of 5 s at 95°C and 30 s at 60°C. The data acquisition step was performed at 60°C, and a final melting curve analysis was used to ensure amplification of a single product. This experiment was carried out three times independently, and each RNA sample was analyzed at least in duplicate. All experiments gave similar results.

The *oxdA*, *amiA*, and *nhpA* transcripts were quantified by RT followed by absolute real-time PCR using a thermal cycler Dice real-time system with SYBR Premix EX *Taq*. For cDNA synthesis, total RNA was incubated for 50 min at 50°C with SuperScript III reverse transcriptase (Invitrogen). Primer *nhpC*-R was used for the RT reaction, and transcripts of *oxdA*, *amiA*, and *nhpA* were quantified with the following primers: *oxdA*-F, *oxdA*-R, *amiA*2-F (5'-TACCTCGACCAGGTTTTCAG-3'), *amiA*2-R (5'-TAGGCGTCGAAACTCGGTTG-3'), *nhpA*-F, and *nhpA*-R.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ/GenBank database under accession number AB374936.

RESULTS

Cloning and nucleotide sequence of the 5' region upstream of the aldoxime dehydratase gene. In previous studies (11, 35, 37), we found that seven genes, which encode aldoxime dehydratase, amidase, the α- and β-subunits of NHase, *NhpC*, *NhpS*, and acyl-CoA synthetase in that order, are located close to each other. To clarify the overall gene organization involved in the nitrile metabolic pathway of *P. chlororaphis* B23, gene walking upstream from the position of *oxdA* was performed. A 5.2-kb PstI-PstI fragment (accession number AB374936) was cloned into pUC18, and the resultant plasmid was designated pPCN11.

We determined the sequence of the region upstream of the *oxdA* gene and found an open reading frame (ORF) in the orientation opposite that of the structural genes of aldoxime dehydratase, amidase, the α- and β-subunits of NHase, *NhpC*, *NhpS*, and acyl-CoA synthetase (Fig. 1A). This ORF was 936 nucleotides long and encoded a 311-amino-acid protein (molecular mass, 35,098 Da). The amino acid sequence of the gene product is similar to the amino acid sequences of regulatory proteins of the XylS/AraC family (44), including NitR, which is the positive regulator of nitrile metabolism in *R. rhodochrous* J1 (25) (26% identity), alkylbenzoate metabolism regulatory

FIG. 1. (A) Organization of the NHase gene cluster, including *nhpR*, in the *P. chlororaphis* B23 genome. Large arrows indicate ORFs, and the translation products and designations of the genes are indicated above the arrows. The oligonucleotides used for the RT-PCR experiment are indicated by small arrows at the bottom. (B) Mapping of the 5' ends of the *nhpR*, *oxdA*, *nhpA*, and *nhpS* transcripts. Primer extension analysis involving total RNA isolated from the *P. chlororaphis* B23 wild-type strain cultured in the presence (lane 1) or absence (lane 2) of methacrylamide was carried out. Primer-extended products were electrophoresed in parallel with sequence ladders generated with the same primer. The positions of the transcription start site are indicated by arrows. (C) Analysis of NHase gene cluster transcription by RT-PCR. Lane 1, amplification with RT1, F1, and R1 (5.8 kb); lane 2, amplification with RT2, F1, and R1; lane 3, amplification with RT3, F3, and R2 (2.9 kb); lane 4, amplification with RT3, F2, and R2; lane M, molecular mass markers.

protein XylS1 in *Pseudomonas putida* (7) (23% identity), and the *cad* gene regulator CadR in *Bradyrhizobium* sp. strain HW13 (17) (22%) (Fig. 2). Accordingly, the ORF was designated *nhpR*.

NhpR is a transcriptional regulator for the NHase gene cluster. Since prokaryotic regulators are frequently located adjacent to the genes under their control (32), we hypothesized that *nhpR* encoded a regulator of the NHase gene cluster. In order to assess the significance of NhpR-associated changes in the expression of genes involved in the metabolism of nitriles, we constructed an *nhpR* disruptant of *P. chlororaphis* B23 as described in Materials and Methods. Both the wild-type strain and the resulting *nhpR* disruptant grew in minimum medium (Fig. 3A). The wild-type strain was able to grow in minimum medium containing methacrylamide instead of ammonium sulfate as a nitrogen source, and the formation of NHase was highly enhanced after addition of methacrylamide to the medium. Although the wild-type strain culture was found to have a significant, high optical density at 600 nm, the *nhpR* disruptant did not grow on methacrylamide instead of ammonium sulfate as the sole nitrogen source (Fig. 3B). Considering that the gene products of the NHase gene cluster are responsible for the degradation of methacrylamide, these findings indicated that NhpR should be an activator of the genes in the NHase gene cluster.

To confirm the activation of the gene products derived from the NHase gene cluster by NhpR, the *nhpR* disruptant was further analyzed to determine its ability to produce aldoxime dehydratase and NHase after addition of methacrylamide as an

inducer to the minimum medium. Although aldoxime dehydratase activity and NHase activity were detected for the wild-type strain, neither activity was detected for the *nhpR* disruptant even in the presence of methacrylamide as an inducer (Table 1), suggesting that NhpR is an activator for the expression of the gene products of the NHase gene cluster.

Determination of the transcription start sites. To find the transcription start site of each gene in the NHase gene cluster, primer extension analysis was carried out using RNA prepared from *P. chlororaphis* B23 cells grown in the minimum medium with ammonium sulfate or methacrylamide as a nitrogen source. A radiolabeled primer was annealed to the RNA sequence near the 5' end of the coding sequence of each gene in the NHase gene cluster, and the primers were then extended with reverse transcriptase. Three transcription initiation sites were identified in the regions upstream of *oxdA*, *nhpA*, and *nhpS* only with RNA prepared from cells grown in the presence of methacrylamide as the sole nitrogen source in the medium (Fig. 1B). Although a transcription initiation site of *nhpR* was identified from RNA prepared in the absence of methacrylamide, slight transcriptional activation of *nhpR* was observed with RNA prepared in the presence of methacrylamide. No initiation site was identified upstream of *amiA*, *nhpB*, *nhpC*, or *acsA*. A single initiation site for *nhpR* transcription is located 328 bp upstream from the ATG initiation codon of *nhpR*; a single initiation site for *oxdA* transcription is located 233 bp upstream from the ATG initiation codon of *oxdA*; a single initiation site for *nhpA* transcription is located 34 bp upstream from the ATG initiation codon of *nhpA*; and a

FIG. 2. Alignment of the amino acid sequence of NhpR from *P. chlororaphis* B23 with homologous sequences. Highlighting indicates identical sequences, and dashes indicate gaps introduced to maximize the alignment. The helix-turn-helix motif (amino acids 228 to 247 of NhpR) is enclosed in a box. Designations: NitR, a regulator of nitrilase from *R. rhodochrous* J1 (25); CadR, a regulator of the *cad* gene from *Bradyrhizobium* sp. strain HW13 (17); and XylS1, a regulator involved in alkylbenzoate metabolism from *P. putida* (7).

FIG. 3. Growth curves for the *P. chlororaphis* B23 wild-type strain (◆) and *nhpR* disruptant (□) at 28°C in the minimum medium with ammonium sulfate (A) or methacrylamide (B) as the sole nitrogen source. Growth was measured by determining the optical density at 600 nm (OD₆₀₀).

single initiation site for *nhpS* transcription is located 276 bp upstream from the ATG initiation codon of *nhpS*. Around 24 bp upstream of each transcription start site, possible σ^{54} -dependent sequences, -24(TGGGGTG) and -12(ATCAG) for *nhpR*, -24(TTCCGCC) and -12(TTTCA) for *oxdA*, -24(A GACCGG) and -12(TTGCA) for *nhpA*, and -24(TCAT CGA) and -12(TCGCA) for *nhpS*, were found.

Transcription units of the NHase gene cluster. To establish whether adjacent genes in the NHase gene cluster are cotranscribed, we conducted RT-PCR with RNA prepared from *P. chlororaphis* B23 grown in the minimum medium with methacrylamide instead of ammonium sulfate as the sole nitrogen source. When the RT primer (RT1) that hybridizes to the 3' end of *nhpC* and PCR primers F1 (5' of *oxdA*) and R1 (3' of

TABLE 1. OxdA activity and NHase activity in cell extracts of the *P. chlororaphis* B23 wild-type strain and the *nhpR* disruptant cultured in the presence or absence of methacrylamide

Strain	Methacrylamide	OxdA activity (U/mg)	NHase activity (U/mg)
B23 wild type	–	ND ^a	ND
	+	17.6	23.2
<i>nhpR</i> disruptant	–	ND	ND
	+	ND	ND

^a ND, not detected.

FIG. 4. Analysis of expression of the seven genes in the NHase gene cluster and *nhpR* by real-time PCR. The expression levels in the *P. chlororaphis* B23 wild-type strain and *nhpR* disruptant were determined. Each strain was cultured in the minimum medium with methacrylamide or ammonium sulfate as the nitrogen source. The results were normalized to the transcription of *rpsL*. Differences for the wild-type strain and the *nhpR* disruptant with and without methacrylamide were calculated based on the transcription in the wild-type strain without methacrylamide, which was defined as 1.0.

nhpC) were used, a PCR product of the expected size (5.8 kb) was obtained. In contrast, when the RT primer (RT2) that hybridizes to the 5' end of *nhpS* and the same PCR primers (F1 and R1) were used, no PCR product was obtained. Furthermore, when the RT primer (RT3) that hybridizes to the 3' end of *acsA* and PCR primers F3 (5' of *nhpS*) and R2 (3' of *acsA*) were used, a PCR product of the expected size (2.9 kb) was obtained, although when the same RT primer (RT3) and PCR primers F2 (3' of *nhpC*) and R2 (3' of *acsA*) were used, no PCR product was obtained (Fig. 1C). No amplification product was obtained when reverse transcriptase was omitted from the reaction mixture (data not shown). These results demonstrated that five of the genes (*oxdA*, *amiA*, *nhpA*, *nhpB*, and *nhpC*) and two of the genes (*nhpS* and *acsA*) are transcribed as two major polycistronic transcriptional units. Considering the transcription start site upstream of *nhpA*, three genes (*nhpABC*) would be transcribed as a third polycistronic transcriptional unit.

Gene transcription analysis by quantitative real-time PCR.

In order to determine whether the seven genes are under the control of NhpR in the presence of methacrylamide, real-time PCR was performed. RNAs were prepared from the *P. chlororaphis* B23 wild-type strain and *P. chlororaphis* B23 *nhpR* disruptant grown in minimum medium (containing ammonium sulfate as a nitrogen source) with or without methacrylamide as an inducer. As a result, addition of methacrylamide to the medium led to significant increases in the transcription of seven genes (*oxdA*, *amiA*, *nhpABC*, *nhpS*, and *acsA*) in the wild-type strain. On the other hand, when the *nhpR* disruptant was grown in the presence of methacrylamide (with ammo-

nium sulfate as a nitrogen source), no increase in the transcription of the seven genes was observed (Fig. 4). This difference in the real-time PCR data suggests that NhpR triggers the transcription of the seven genes in response to the addition of methacrylamide to the medium. Similar to the transcription of the seven genes, the transcription of the *nhpR* gene was also inducibly triggered by NhpR with methacrylamide (Fig. 4), demonstrating that *nhpR* autoactivates its own transcription as well.

NHase was produced at a high level in *P. chlororaphis* B23 grown in the medium with methacrylamide. Therefore, we compared the level of mRNA transcribed from the *nhpA* promoter with the level of mRNA transcribed from the *oxdA* promoter. To determine the absolute transcription ratio for the *oxdA* and *nhpA* promoters, we carried out real-time PCR with RNA prepared from *P. chlororaphis* B23 cells grown in the minimum medium with methacrylamide as an inducer. After the RT reaction had been performed with the RT primer (*nhpC*-R) that hybridizes to the 3' end of *nhpC*, the PCR was conducted to compare the levels of transcription of *oxdA*, *amiA*, and *nhpA*. As a result, the transcription level of *oxdA* was found to be almost the same as that of *amiA*. This finding is consistent with the finding that no promoter was identified upstream of *amiA* and with the finding that *oxdA* and *amiA* are cotranscribed as the same mRNA unit. On the other hand, the levels of *nhpA* transcribed from the two promoters upstream of *oxdA* and *nhpA* were about three times higher than the levels of *oxdA* and *amiA* transcribed from the promoter upstream of *oxdA* (data not shown).

DISCUSSION

We were interested in the discovery of a regulatory protein that plays a key role in control of the expression of the NHase gene cluster in *P. chlororaphis* B23 for the following reasons: (i) the induction level of useful NHase is very high (34); and (ii) although a substrate of an inducible enzyme often acts as an inducer in the regulation mechanism, methacrylamide, which is the reaction product of NHase, acts as a strong inducer of NHase in *P. chlororaphis* B23 (47).

Sequence analysis of the region upstream of the aldoxime dehydratase gene revealed the presence of the *nhpR* gene exhibiting sequence similarity to a gene encoding a positive regulator belonging to the XylS/AraC family, which includes NitR involved in nitrile metabolism in *R. rhodochrous* J1 (25). Like NHase in *P. chlororaphis* B23, the nitrilase in *R. rhodochrous* J1 is strongly induced after addition of an inducer to the medium (20). In contrast to the finding that methacrylamide (which is a reaction product) induces NHase in *P. chlororaphis* B23, isovaleronitrile (which is a reaction substrate) induces nitrilase in *R. rhodochrous* J1. Although *nitR* is in the region downstream of the nitrilase gene (*nitA*) in the same direction (25), *nhpR* is in the region upstream of the NHase gene cluster in the direction opposite that of the structural genes in the NHase gene cluster (Fig. 1A). Very recently, an ORF which exhibits sequence similarity to the ORF encoding an AraC family protein was found to be located in the region upstream of a cluster including the aldoxime dehydratase and NHase genes in *Pseudomonas* sp. strain K-9 (16). However, the function of this ORF has not been determined. The XylS/AraC family, the members of which are positive regulators involved in the metabolism of carbon sources and in pathogenesis (41), is characterized by sequence similarity within the carboxyl terminus, which is the region containing a helix-turn-helix DNA-binding motif (6, 40). Consistent with the sequence similarity with the XylS/AraC family, analysis of the deduced amino sequence of NhpR revealed that it has a helix-turn-helix DNA-binding motif at its carboxyl terminus (Fig. 2). Within this family, for regulators recognizing chemical signals (inducers), the nonconserved N-terminal region is presumed to be responsible for binding to an activator molecule (6).

In *P. chlororaphis* B23, the chromosomal *nhpR* mutation led to an inability to grow in the medium with methacrylamide as the sole nitrogen source (Fig. 3) and to an inability to activate the transcription of the eight genes (*nhpR*, *oxdA*, *amiA*, *nhpABC*, *nhpS*, and *acsA*) in the NHase gene cluster in the minimum medium containing ammonium sulfate as a nitrogen source with methacrylamide as an inducer (Fig. 4). These results indicated that the *nhpR* gene product is a transcriptional activator of the NHase gene cluster. Consistent with this finding, the *nhpR* mutation resulted in complete loss of aldoxime dehydratase and NHase activities (Table 1). Because the genes in the NHase gene cluster are close together, it was speculated that the genes in this cluster constitute an operon. To experimentally verify this hypothesis, we determined the transcription start sites for these genes. Using total RNA from *P. chlororaphis* B23, we performed a primer extension analysis of the region upstream of each gene in the NHase gene cluster. Interestingly, four transcription start sites appeared to be upstream of *nhpR*, *oxdA*, *nhpA*, and *nhpS* (Fig. 1B). The promoter regions

upstream of the transcription start sites of *nhpR*, *oxdA*, *nhpA*, and *nhpS* contained putative σ^{54} -dependent sequences, -24(TGGG GTG)/-12(ATCAG), -24(TTCCGCC)/-12(TTTCA), -24(A GACCGG)/-12(TTGCA), and -24(TCATCGA)/-12(TCG CA), respectively. σ^{54} is a regulatory factor needed for expression of the genes whose products function in the assimilation of nitrogen. For example, σ^{54} is required for transcription of genes whose products are needed for biological nitrogen fixation (e.g., genes encoding amino acid transport components and degradative enzymes) (30). Although the four σ^{54} -dependent promoters that we identified upstream of *nhpR*, *oxdA*, *nhpA*, and *nhpS* are not significantly similar to the consensus σ^{54} -dependent promoter sequences -24(TGGCACC)/-12(TTGCA), it is reasonable to conclude that σ^{54} -dependent promoter sequences are in the NHase gene cluster because aldoxime dehydratase, amidase, and NHase are involved in nitrile metabolism (aldoxime $\rightarrow$ nitrile $\rightarrow$ amide $\rightarrow$ acid + ammonia) for supplying a nitrogen source (11). To the best of our knowledge, this is the first report that a σ^{54} -dependent regulatory system is involved in nitrile metabolism.

To understand the putative operon structure, we conducted Northern blot analysis using RNA prepared from *P. chlororaphis* B23 grown in the minimum medium with methacrylamide as the sole nitrogen source. Hybridization with a probe for each gene resulted in no clear specific signal, probably due to degradation of the mRNA. Therefore, we conducted RT-PCR. As shown in Fig. 1C, we observed amplified 5.8- and 2.9-kb fragments corresponding to the lengths of *oxdA-nhpC* and *nhpS-acsA*. This suggested that *oxdA-nhpC* and *nhpS-acsA* constitute operons. Together with the two transcriptional start sites in the *oxdA-nhpC* operon, the *nhpABC* genes would be transcribed from two promoters. It is very interesting that the transcription of *nhpABC* from these two promoters is controlled by *nhpR* in response to the addition of methacrylamide. Because nitriles are generally very toxic due to their cyano functional groups, a high level of NHase expression could be needed for the immediate catabolism of nitriles in *P. chlororaphis* B23. Furthermore, because the reaction product of NHase acts as a strong inducer of the NHase gene cluster, a large amount of NHase can cause strong induction of the genes in the NHase gene cluster.

Since an enormous amount of NHase (corresponding to more than 50% of the total soluble protein) is produced in *P. chlororaphis* B23 grown with methacrylamide (34), a strong inducible promoter for the expression of NHase was predicted to be located upstream of *nhpA*. Contrary to our speculation, it was demonstrated that the level of transcription of the NHase gene was almost three times the levels of transcription of *oxdA* and *amiA*, indicating that the transcription ratio for the promoter region upstream of *nhpA* is about twice that for the promoter region upstream of *oxdA*. It is presumed that the high level of NHase expression is due to the fact that the regulation of NHase gene expression occurs at the posttranscriptional level or to stabilization of the protein (e.g., through protection from proteolytic degradation).

In this study, we initially demonstrated that *nhpR* upstream of the NHase gene cluster positively regulates the expression of four enzymes derived from the eight genes (*oxdA*, *amiA*, *nhpA*, *nhpB*, *nhpC*, *nhpS*, *acsA*, and *nhpR*) comprising the NHase gene cluster after addition of methacrylamide to the minimum medium as an inducer (Fig. 4 and 5). This regulation mecha-

FIG. 5. Proposed model for transcription of the NHase gene cluster caused by NhpR in *P. chlororaphis* B23. mRNA transcribed from the cluster is indicated by arrows at the bottom. +, stimulation of transcription.

nism and the organization of the gene cluster in this strain are different from the regulation mechanism and the organization of both gene clusters encoding the NHase (26, 27, 28) and nitrilase (23, 25) in *R. rhodochrous* J1. We previously reported that four enzymes (aldoxime dehydratase, NHase, amidase, and acyl-CoA synthetase) encoded in the NHase gene cluster are inducibly expressed when *P. chlororaphis* B23 is grown with butyraldioxime as the sole carbon and nitrogen source (11). Although we investigated the regulatory mechanism involving methacrylamide in this research, the regulatory mechanism involving butyraldioxime is assumed to be the same as that involving methacrylamide since butyraldioxime can be degraded to butyramide (which is a product of the NHase reaction together with methacrylamide) in this strain. Until now, the question of whether NhpR directly binds to sequences upstream of *oxdA*, *nhpA*, and *nhpS* had not been addressed. In order to purify NhpR, we constructed expression plasmids for *nhpR*. Although *E. coli* was transformed with each of the resultant plasmids, NhpR expressed in *E. coli* was produced as inclusion bodies under any conditions (data not shown). Thus, further analyses to obtain an overview of the regulatory system for the nitrile pathway are in progress.

ACKNOWLEDGMENTS

This work was supported in part by the 21st Century COE Program (<http://www.tara.tsukuba.ac.jp/~coe21/>), by a grant-in-aid for scientific research, and by a grant-in-aid for young scientists from the Ministry of Education, Culture, Sports, Science, and Technology.

We thank Hideaki Maseda (The University of Tokushima) for useful discussions.

REFERENCES

- Asano, Y., Y. Tani, and H. Yamada. 1980. A new enzyme "nitrile hydratase" which degrades acetonitrile in combination with amidase. *Agric. Biol. Chem.* **44**:2251–2252.
- Bartling, D., M. Seedorf, C. R. Schmidt, and W. E. Weiler. 1994. Molecular characterization of two cloned nitrilases from *Arabidopsis thaliana*: key enzymes in biosynthesis of the plant hormone indole-3-acetic acid. *Proc. Natl. Acad. Sci. USA* **91**:6021–6025.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Endo, I., M. Odaka, and M. Yohda. 1999. An enzyme controlled by light: the molecular mechanism of photoreactivity in nitrile hydratase. *Trends Biotechnol.* **17**:244–248.
- Fukatsu, H., Y. Hashimoto, M. Goda, H. Higashibata, and M. Kobayashi. 2004. Amine-synthesizing enzyme, N-substituted formamide deformylase: screening, purification, characterization and gene cloning. *Proc. Natl. Acad. Sci. USA* **101**:13726–13731.
- Gallegos, M. T., C. Michan, and J. L. Ramos. 1993. The XylS/AraC family of regulators. *Nucleic Acids Res.* **21**:807–810.
- Gallegos, M. T., A. P. Williams, and L. J. Ramos. 1997. Transcriptional control of the multiple catabolic pathways encoded on the TOL plasmid pWW53 of *Pseudomonas putida* MT53. *J. Bacteriol.* **179**:5024–5029.
- Goda, M., Y. Hashimoto, S. Shimizu, and M. Kobayashi. 2001. Discovery of a novel enzyme, isonitrile hydratase, involved in nitrogen-carbon triple bond cleavage. *J. Biol. Chem.* **276**:23480–23485.
- Goda, M., Y. Hashimoto, M. Takase, S. Herai, Y. Iwahara, H. Higashibata, and M. Kobayashi. 2002. Isonitrile hydratase from *Pseudomonas putida* N19-2. *J. Biol. Chem.* **277**:45860–45865.
- Hann, C. E., A. Eisenberg, K. S. Fager, E. N. Perkins, G. F. Gallagher, M. S. Cooper, E. J. Gavan, B. Stieglitz, M. S. Hennessey, and R. DiCosimo. 1999. 5-Cyanovaleramide production using immobilized *Pseudomonas chlororaphis* B23. *Bioorg. Med. Chem.* **7**:2239–2245.
- Hashimoto, Y., H. Hosaka, K.-I. Oinuma, M. Goda, H. Higashibata, and M. Kobayashi. 2005. Nitrile pathway involving acyl-CoA synthetase: overall metabolic gene organization, and purification and characterization of the enzyme. *J. Biol. Chem.* **280**:8660–8667.
- Hashimoto, Y., M. Nishiyama, S. Horinouchi, and T. Beppu. 1994. Nitrile hydratase gene from *Rhodococcus* sp. N-774 requirement for its downstream region for efficient expression. *Biosci. Biotechnol. Biochem.* **58**:1859–1865.
- Hashimoto, Y., M. Nishiyama, F. Yu, I. Watanabe, S. Horinouchi, and T. Beppu. 1992. Development of a host-vector system in a *Rhodococcus* strain and its use for expression of the cloned nitrile hydratase gene cluster. *J. Gen. Microbiol.* **138**:1003–1010.
- Herai, S., Y. Hashimoto, H. Higashibata, H. Maseda, H. Ikeda, S. Omura, and M. Kobayashi. 2004. Hyper-inducible expression system for streptomycetes. *Proc. Natl. Acad. Sci. USA* **101**:14031–14035.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using polymerase chain reaction. *Gene* **77**:51–59.
- Kato, Y., and Y. Asano. 2006. Molecular and enzymatic analysis of the "aldoxime-nitrile pathway" in the glutaronitrile degrader *Pseudomonas* sp. K-9. *Appl. Microbiol. Biotechnol.* **70**:92–101.
- Kitagawa, W., S. Takami, K. Miyauchi, E. Masai, Y. Kamagata, T. M. James, and M. Fukuda. 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment. *J. Bacteriol.* **184**:509–518.
- Kobayashi, M., Y. Fujiwara, M. Goda, H. Komeda, and S. Shimizu. 1997. Identification of active sites in amidase: evolutionary relationship between amide bond- and peptide bond-cleaving enzymes. *Proc. Natl. Acad. Sci. USA* **94**:11986–11991.
- Kobayashi, M., H. Izui, T. Nagasawa, and H. Yamada. 1993. Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues. *Proc. Natl. Acad. Sci. USA* **90**:247–251.
- Kobayashi, M., T. Nagasawa, and H. Yamada. 1989. Nitrilase of *Rhodococcus rhodochrous* J1. Purification and characterization. *Eur. J. Biochem.* **182**:349–356.
- Kobayashi, M., T. Nagasawa, and H. Yamada. 1992. Enzymatic synthesis of acrylamide: a success story not yet over. *Trends Biotechnol.* **10**:402–408.
- Kobayashi, M., and S. Shimizu. 1998. Metalloenzyme nitrile hydratase: structure, regulation, and application to biotechnology. *Nat. Biotechnol.* **16**:733–736.
- Kobayashi, M., and S. Shimizu. 2000. Nitrile hydrolases. *Curr. Opin. Chem. Biol.* **4**:95–102.
- Kobayashi, M., T. Suzuki, T. Fujita, M. Masuda, and S. Shimizu. 1995. Occurrence of enzymes involved in biosynthesis of indole-3-acetic acid from indole-3-acetonitrile in plant-associated bacteria, *Agrobacterium* and *Rhizobium*. *Proc. Natl. Acad. Sci. USA* **92**:714–718.
- Komeda, H., Y. Hori, M. Kobayashi, and S. Shimizu. 1996. Transcriptional regulation of the *Rhodococcus rhodochrous* J1 *nitA* gene encoding a nitrilase. *Proc. Natl. Acad. Sci. USA* **93**:10572–10577.
- Komeda, H., M. Kobayashi, and S. Shimizu. 1996. 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. *J. Biol. Chem.* **271**:15796–15802.
- Komeda, H., M. Kobayashi, and S. Shimizu. 1996. Characterization of the gene cluster of high-molecular-mass nitrile hydratase (H-NHase) induced by its reaction product in *Rhodococcus rhodochrous* J1. *Proc. Natl. Acad. Sci. USA* **93**:4267–4272.
- Komeda, H., M. Kobayashi, and S. Shimizu. 1997. A novel transporter in cobalt uptake. *Proc. Natl. Acad. Sci. USA* **94**:36–41.
- Konishi, K., K. Ishida, K.-I. Oinuma, T. Ohta, Y. Hashimoto, H. Higashibata, T. Kitagawa, and M. Kobayashi. 2004. Identification of crucial histidines involved in carbon-nitrogen triple bond synthesis by aldoxime dehydratase. *J. Biol. Chem.* **279**:47619–47625.
- Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss. 1989. Expression of sigma 54 (*nutA*)-dependent genes is probably united by a common mechanism. *Microbiol. Rev.* **53**:367–379.
- Maniatis, T., F. E. Fritsch, and J. Sambrook. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Moriwaki, N., K. Kimbara, and F. Kawai. 2006. Dual regulation of a polyethylene glycol degradative operon by AraC-type and GalR-type regulators in *Shingopyxis macrogoltabida* strain 103. *Microbiology* **152**:452–457.
- Nagasawa, T., H. Nanba, K. Ryuno, K. Takeuchi, and H. Yamada. 1987.

- Nitrile hydratase of *Pseudomonas chlororaphis* B23. Purification and characterization. Eur. J. Biochem. **162**:691–698.
34. Nagasawa, T., K. Ryuno, and H. Yamada. 1989. Superiority of *Pseudomonas chlororaphis* B23 nitrile hydratase as a catalyst for the enzymatic production of acrylamide. Experientia **45**:1066–1070.
35. Nishiyama, M., S. Horinouchi, M. Kobayashi, T. Nagasawa, H. Yamada, and T. Beppu. 1991. Cloning and characterization of genes responsible for metabolism of nitrile compounds from *Pseudomonas chlororaphis* B23. J. Bacteriol. **173**:2465–2472.
36. Normanly, J., P. Grisafi, R. G. Fink, and B. Bartel. 1997. *Arabidopsis* mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. Plant Cell **10**:1781–1790.
37. Oinuma, K.-I., Y. Hashimoto, K. Konishi, M. Goda, T. Noguchi, H. Higashibata, and M. Kobayashi. 2003. Novel aldoxime dehydratase involved in carbon-nitrogen triple bond synthesis of *Pseudomonas chlororaphis* B23. J. Biol. Chem. **278**:29600–29608.
38. Pekarsky, Y., M. Campiglio, Z. Siprashvili, T. Druck, Y. Sedkov, S. Tillib, A. Draganescu, P. Wermuth, H. J. Rothman, K. Huebner, M. A. Buchberg, A. Mazo, C. Brenner, and M. C. Croce. 1998. Nitrilase and Fhit homologs are encoded as fusion proteins in *Drosophila melanogaster* and *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **95**:8744–8749.
39. Pogulis, R. J., A. N. Vallejo, and L. R. Pease. 1996. In vitro recombination and mutagenesis by overlap extension PCR methods. Mol. Biol. **57**:167–176.
40. Ramos, L. J., F. Rojo, L. Zhou, and K. N. Timmis. 1990. A family of positive regulators related to the *Pseudomonas putida* TOL plasmid XylS and the *Escherichia coli* AraC activators. Nucleic Acids Res. **18**:2149–2152.
41. Rosenberg, E. Y., D. Bertenthal, M. L. Nilles, K. P. Bertrand, and H. Nikaido. 2003. Bile salts and fatty acids induce the expression of the *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. Mol. Microbiol. **48**:1609–1619.
42. Ryuno, K., T. Nagasawa, and H. Yamada. 1988. Isolation of advantageous mutants of *Pseudomonas chlororaphis* B23 for the enzymatic production of acrylamide. Agric. Biol. Chem. **52**:1813–1816.
43. Taylor, L. A., and R. E. Rose. 1988. A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. Nucleic Acids Res. **16**:358.
44. Tobes, R., and L. J. Ramos. 2002. AraC-XylS database: a family of positive transcriptional regulators in bacteria. Nucleic Acids Res. **30**:318–321.
45. Watanabe, I., Y. Satoh, and K. Enomoto. 1987. Screening, isolation and taxonomical properties of microorganisms having acrylonitrile-hydrating activity. Agric. Biol. Chem. **51**:3193–3199.
46. Yamada, H., and M. Kobayashi. 1996. Nitrile hydratase and its application to industrial production of acrylamide. Biosci. Biotechnol. Biochem. **60**:1391–1400.
47. Yamada, H., K. Ryuno, T. Nagasawa, K. Enomoto, and I. Watanabe. 1986. Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase. Agric. Biol. Chem. **50**:2859–2865.
48. Yamada, H., S. Shimizu, and M. Kobayashi. 2001. Hydratases involved in nitrile conversion: screening, characterization and application. Chem. Rec. **1**:152–161.
49. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.