Transcriptional Regulation of the Nitrile Hydratase Gene Cluster in Pseudomonas chlororaphis B23

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Transcriptional regulation of the nitrile hydratase gene cluster in *Pseudomonas chlororaphis* B23

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ABSTRACT

An enormous amount of nitrile hydratase (NHase) is inducibly produced by *Pseudomonas chlororaphis* B23 on the addition of methacrylamide as a sole nitrogen source to the medium. The expression pattern of the *P. chlororaphis* B23 NHase gene cluster in response to the 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 existence of an open reading frame (named *nhpR*) of 936 bp, which should encode a protein with a molecular mass of 35,098. The deduced amino acid sequence of *nhpR* showed similarity to those of transcriptional regulators belonging to the XylS/AraC family. Although the transcription of the eight genes (*nhpR* itself, *oxdA, amiA, nhpABC, nhpS* and *acsA*) in the NHase gene cluster was induced significantly in the *P. chlororaphis* B23 wild-type strain on the addition of methacrylamide to the medium, transcription of these genes in the *nhpR*-disruptant was not induced, demonstrating that *nhpR* codes for a transcriptional positive regulator in the NHase gene cluster. A reverse transcription (RT)-PCR experiment revealed that five (*oxdA, amiA, nhpA, nhpB* and *nhpC*) and two (*nhpS* and *acsA*) genes are cotranscribed, respectively. The transcription start sites for *nhpR, oxdA*, *nhpA* and *nhpS* were mapped by primer extension analysis, and putative -12, -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.

INTRODUCTION

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≡N] (22) and isonitriles [R-N≡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 ones (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, being in great demand (200,000 tons per year worldwide) as a flocculant, a component of synthetic fibers, a soil conditioner, and a petroleum-recovering agent. The industrial production of acrylamide using the ferric NHase of *Rhodococcus* sp. N-774, the first generation strain, started in 1985 (12, 45). This biotransformation of acrylonitrile to acrylamide by a microbial NHase was the first case of biotechnology being 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. On the other hand, the NHase of *Pseudomonas chlororaphis* B23 (33), which was previously used as a catalyst for acrylamide manufacture (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 on addition of methacrylamide as a 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 the enzyme expression. We have already cloned the NHase gene from this strain, and discovered the existence of a gene cluster including the aldoxime dehydratase (29, 37), NHase and amidase (35) genes. Very recently, we also discovered that the 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* to utilize butyraldoxime as a carbon and nitrogen source (11). Although we have clarified the overall "nitrile pathway" (aldoxime → nitrile → amide → acid → 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 activation mechanism for the NHase gene cluster provides an explanation for the induction of nitrile-degradative and -synthetic enzymes in response to the addition of methacrylamide to the growth medium.

MATERIALS AND METHODS

Plasmids, strains, and media

Plasmid pPCN4 comprising the 6.5-kb SphI-SalI fragment on pUC19 (35) was used as a probe to clone the region upstream of the aldoxime 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% of sucrose, 0.5% of $(NH_4)_2SO_4$, 0.05% of KH_2PO_4, 0.05% of K_2HPO_4, 0.05% of $MgSO_4 \cdot 7H_2O$ and 0.001% of $FeSO_4 \cdot 7H_2O$ (pH 7.0). Unless otherwise stated, sucrose and $(NH_4)_2SO_4$ were used as 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 terminating 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 a 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'-ATCGGTGCGGGCCTCTTCGC-3'), Km-R (5'-CGAGTGAGTAATCCGTGGGGT-3'), HindIII-F (5'-TCCGGTGAGAATGGCAACAGTTTATGCATT-3'), HindIII-R (5'-TCTGGAAAGAAATGCATAAACTGTTGCCAT-3'), XhoI-F (5'-TGGAATTTAATCGCGGCCTGGAACAAGACG-3'), and XhoI-R (5'-CAACGGGAAACGTCTTGTTCCAGGCCGCGA-3'). The amplified kanamycin resistance gene (containing no HindIII and XhoI sites) was digested with HincII and then inserted into the BalI 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 according to Bradford (3). Specific activity is expressed as units/mg of protein.

RNA experiments

For the isolation of RNA from *P. chroloraphis* B23, cells were cultivated in 10 ml of medium with or without 0.5% of 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'-AGTGCCAGCGTTGTCTTTTC-3', 5'-TGTGCAAAGGCCAGGAAGCG-3', 5'-TCGAAGGTGTCGCAGTCGTG-3' and 5'-AGTCGCCGACAACGCTGTCC-3', respectively. The primers were labeled with [^{32}P] 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 RT reaction, and pairs of primers, F1-R1, F2-R2 and F3-R2, were used for successive PCR amplification. The sequences were as follows: RT1 (5'-GCGTGGGGCGTGCCGATAAG-3'), RT2 (5'-ACAGGCAGGCCTTGCGCATC-3'), RT3 (5'-TTGCGCAGAATGAACCGTTG-3'), F1 (5'-CCCATGCATCGACAACGTTC-3'), F2 (5'-AAAATGGAAGCTACCGATAC-3'), F3 (5'-GCTTGCCGACAGACTGGACC-3'), R1 (5'-ATAAGGCCTCGGTGTCAAAG-3'), and R2 (5'-TGGGCAGATGGTCGACAAAC-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'-ACGAGAAGGTCGAGCAAAGC-3'), nhpR-R (5'-ACACGGCAATGGTCCTCGAC-3'), oxdA-F (5'-ACGCATCTCAAATGCCCACG-3'), oxdA-R (5'-TACTGCACGCCGAGATAACC-3'), amiA-F (5'-ACAGGACATCACCGGGCATC-3'), amiA–R (5'-GTCCACCGAGGCTTCAAACG-3'), nhpA-F (5'-TCAAGAGCAAGGAACTCATC-3'), nhpA-R (5'-TTCCGTCCTTGAGCAGCAGC-3'), nhpB-F (5'-TGCACCGCACCTCAGAGCAG-3'), nhpB-R (5'-CGTCACCCCATAGATCTTTC-3'), nhpC-F (5'-GGACGAATATCGGTTGCAGG-3'), nhpC-R (5'-GCACTCAGCAAACAATGGTC-3'), nhpS-F (5'-GACACAGGAAGTCACCCAAC-3'), nhpS-R (5'-GCAGCGGTTCCATTCACCTC-3'), and acsA-F (5'-GATTATCTGCAGAGCGCCAC-3'), acsA-R (5'-CATGGCCATCCTGCGCTTCG-3'). The transcript level of each gene was normalized as 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'-ACGTCGTGGCGTATGCACTC-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, with final melting-curve analysis 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, nhpA* transcripts were quantified by reverse transcription (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, amiA2-F (5'-TACCCTCGACCAGGTTTTAG-3'), amiA2-R (5'-TAGGCGTCGAAACTCGGTTG-3'), nhpA-F, and nhpA-R.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper appear 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 the close location of seven genes, which encode aldoxime dehydratase, amidase, the α- and β-subunits of NHase, NhpC, NhpS and acyl-CoA synthetase, in that order. 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 named pPCN11.

We determined the sequence of the region upstream of the *oxdA* gene and newly found an open reading frame (ORF) in the opposite orientation to the structural genes of aldoxime dehydratase, amidase, the α- and β-subunits of NHase, NhpC, NhpS and acyl-CoA synthetase (Fig. 1A). The ORF was 936 nucleotides long, and encoded a protein of 311 amino acids (35,098 Da). The gene product is similar in amino acid sequence to regulatory proteins of the XylS/AraC family (44) including positive regulator NitR of nitrile-metabolism in *Rhodococcus rhodochrous* J1 (25) (26% identity), alkylbenzoate metabolism regulatory protein XylS1 in *Pseudomonas putida* (7) (23% identity), and *cad* gene regulator CadR in *Bradyrhizobium* sp. HW13 (17) (22%) (Fig. 2). Accordingly, the ORF was designated as *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 a *nhpR*-disruptant of *P. chlororaphis* B23 as described under Materials and Methods. Both the wild-type strain and the resulting *nhpR*-disruptant grow in minimum medium (Fig. 3A). The wild-type strain was able to grow in the minimum medium containing methacrylamide instead of ammonium sulfate as a nitrogen source, and the formation of NHase was highly enhanced on the addition of methacrylamide to the medium. Although the wild-type strain was found to exhibit a significantly high OD_{600} value, the *nhpR*-disruptant did not grow on methacrylamide instead of ammonium sulfate as a 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 as to the ability to produce aldoxime dehydratase and NHase on the 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 I), suggesting that NhpR is an activator for the expression of the gene products derived from 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 sites of transcription initiation were identified in the regions upstream of *oxdA*, *nhpA* and *nhpS* only from RNA prepared in the presence of methacrylamide as a 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 from 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 exists 328 bp upstream from the ATG initiation codon of *nhpR*. A single initiation site for *oxdA* transcription exists 233 bp upstream from the ATG initiation codon of *oxdA*. A single initiation site for *nhpA* transcription is located 34 bp upstream from that of *nhpA*. A single initiation site for *nhpS* transcription exists 276 bp upstream from that 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(AGACCGG) and –12(TTGCA) for *nhpA*, and –24(TCATCGA) 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 a sole nitrogen source. When the RT primer (RT1) that hybridizes to the 3' end of *nhpC* and PCR primer pairs (F1 [5' of *oxdA*] and R1 [3' of *nhpC*]) were used, a PCR product of the expected size (5.8 kb) was obtained. On the contrary, when the RT primer (RT2) that hybridizes to the 5' end of *nhpS* and the same PCR primer pairs (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 primer pairs (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 primer pairs (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 (i.e., *oxdA, amiA, nhpA, nhpB* and *nhpC*) and two (i.e., *nhpS* and *acsA)* of the genes are transcribed as two major polycistronic transcriptional units, respectively. Considering the transcription start site upstream of *nhpA*, three genes (i.e., *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 the 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 ammonium 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 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 that 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 reaction was conducted to compare the levels of transcription among *oxdA*, *amiA* and *nhpA*. As a result, the transcription level of *oxdA* was found to be almost the same as that of *amiA*. This is consistent with the findings that no promoter was identified upstream of *amiA*, and that both *oxdA* and *amiA* are cotranscribed as the same mRNA unit. On the other hand, the

transcription levels of *nhpA* transcribed from the two promoters upstream of *oxdA* and *nhpA* were about three times higher than those 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 existence of the *nhpR* gene exhibiting sequence similarity to a positive regulator belonging to the XylS/AraC family including NitR involved in the nitrile metabolism in *R. rhodochrous* J1 (25). Like NHase in *P. chlororaphis* B23, the nitrilase in *R. rhodochrous* J1 is strongly induced on the addition of an inducer to the medium (20). In contrast with the phenomenon 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* exists in the region downstream of the nitrilase gene (*nitA*) in the same direction (25), *nhpR* exists in the region upstream of the NHase gene cluster in the opposite direction to the structural genes in the NHase gene cluster (Fig. 1A). Very recently, an ORF, which exhibits sequence similarity to that of 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. K-9 (16). However, the function of this ORF has never 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 non-conserved 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 both to grow in the medium with methacrylamide as a sole nitrogen source (Fig. 3), and to activate the transcription of the eight genes (*nhpR* itself, *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 the complete loss of aldoxime dehydratase and NHase activities (Table I). Because of the close gene organization of the NHase gene cluster, the genes in this cluster were speculated to constitute an operon. To experimentally verify this possibility, we determined the transcription start sites for these genes. Using total RNA from *P. chlororaphis* B23, we performed primer extension analysis of upstream of each gene in the NHase gene cluster. Interestingly, four transcription start sites appeared to exist 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(TGGGGTG) / –12(ATCAG), –24(TTCCGCC) / –12(TTTCA), –24(AGACCGG) / –12(TTGCA), and –24(TCATCGA) / –12(TCGCA), 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 the 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 consensus σ^{54}-dependent promoter sequences –24(TGGCACG) / –12(TTGCA), it is reasonable for σ^{54}-dependent promoter sequences to exist in the NHase gene cluster because aldoxime dehydratase, amidase and NHase are involved in nitrile metabolism (aldoxime → nitrile → amide → acid + ammonia) for the supply of 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 a sole nitrogen source. Hybridization with a probe against 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 kb 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, respectively. 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 expression level of NHase could possibly 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 compared to those of *oxdA* and *amiA*, indicating that the transcription ratio for the promoter region upstream of *nhpA* is about twice compared with that for the promoter region upstream of *oxdA*. It is presumed that the high expression level of NHase is due to the phenomenon that the regulation of NHase gene expression occurs at the posttranscriptional level or 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* itself) comprising the NHase gene cluster on the addition of methacrylamide to the minimum medium as an inducer (Fig. 4). This regulation mechanism and the organization of the gene cluster in this strain are different from those of the both gene clusters of the NHase (26,27,28) and nitrilase (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 butyraldoxime as a sole carbon and nitrogen source (11). Although we investigated the regulatory mechanism involving methacrylamide in this research, the regulatory mechanism involving butyraldoxime is assumed to be the same as that involving methacrylamide since butyraldoxime can be degraded to butyramide (which is a product of the NHase reaction as well as methacrylamide) in this strain. Until now, the question of whether NhpR directly binds to upstream sequences upstream of *oxdA, nhpA* and *nhpS* has not yet 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.

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FIGURE LEGENDS

Fig. 1. (A) Gene organization of the NHase gene cluster including *nhpR* on the *P. chlororaphis* B23 genome. Bold arrows indicate open reading frames, and the translation products and names of the genes are given above the arrows. The oligonucleotides used for the RT-PCR experiment are also indicated by small arrows at the bottom. (B) 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; and lane M, molecular mass markers. (C) 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 position of the transcription start site is indicated by an arrow.

Fig. 2. Alignment of the amino acid sequences of NhpR from *P. chlororaphis* B23 with homologous sequences. Residues in black boxes indicate identical sequences; dashes denote gaps introduced to maximize the alignment. The helix-turn-helix motif (amino acids 228-247 of NhpR) is enclosed by a box. Abbreviations (refs); NitR, a regulator of nitrilase from *R. rhodochrous* J1 (25); CadR, a regulator of the cad gene from *Bradyrhizobium* sp. HW13 (17); and XylS1, a regulator involved in alkylbenzoate

metabolism from *P. putida* (7).

Fig. 3. Growth curves of 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 a sole nitrogen source. Growth was measured by determining the optical density at 600 nm (OD_{600}).

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 and *nhpR*-disruptant were determined. Each strain was cultured in the minimum medium with methacrylamide or ammonium sulfate as a nitrogen source. The results were normalized as to the expression of *rpsL*. Differences from the B23 wild-type/*nhpR*-disruptant, and with and without methacrylamide were calculated based on the B23 wild-type without methacrylamide expression, which was defined as 1.0.

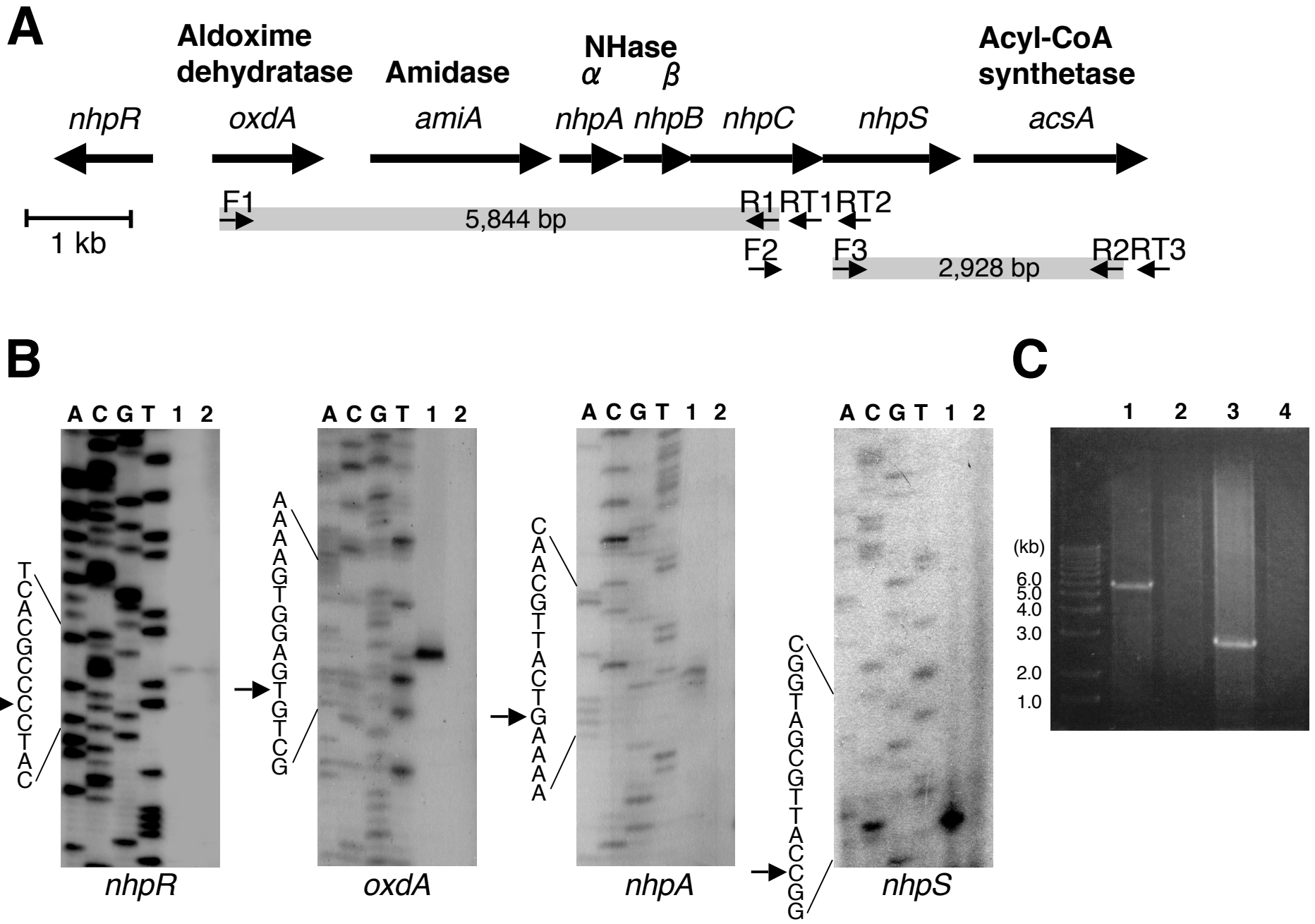
Fig. 5. Proposed model for transcription of the NHase gene cluster caused by NhpR in *P. chroloraphis* B23. mRNA transcribed from the cluster is denoted by arrows at the bottom. +, positive stimulation of transcription.

Table I. OxdA activity and NHase activity in cell-free extracts of the *P. chlororaphis* B23 wild-type strain and the *nhpR*-disruptant were measured. Each strain was cultured in the presence or absence of methacrylamide.

	Methacrylamide	OxdA activity (units/mg)	NHase activity (units/mg)
B23 wild-type strain	-	ND	ND
	+	17.6	23.2
nhpR-disruptant	-	ND	ND
	+	ND	ND

ND, not detected.

Figure 1


A
Aldoxime dehydratase
Amidase
NHase
α β
Acyl-CoA synthetase
nhpR
oxdA
amiA
nhpA nhpB
nhpC
nhpS
acsA
1 kb
F1
5,844 bp
R1RT1RT2
F2
F3
2,928 bp
R2RT3
B
A C G T 1 2
TCACGCCCCTAC
AAAAGTGGAGTGTCG
CAACGTTACTGAAAA
CGGTAGCGTTACCGG
nhpR
oxdA
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5.0
4.0
3.0
2.0
1.0

Figure 2

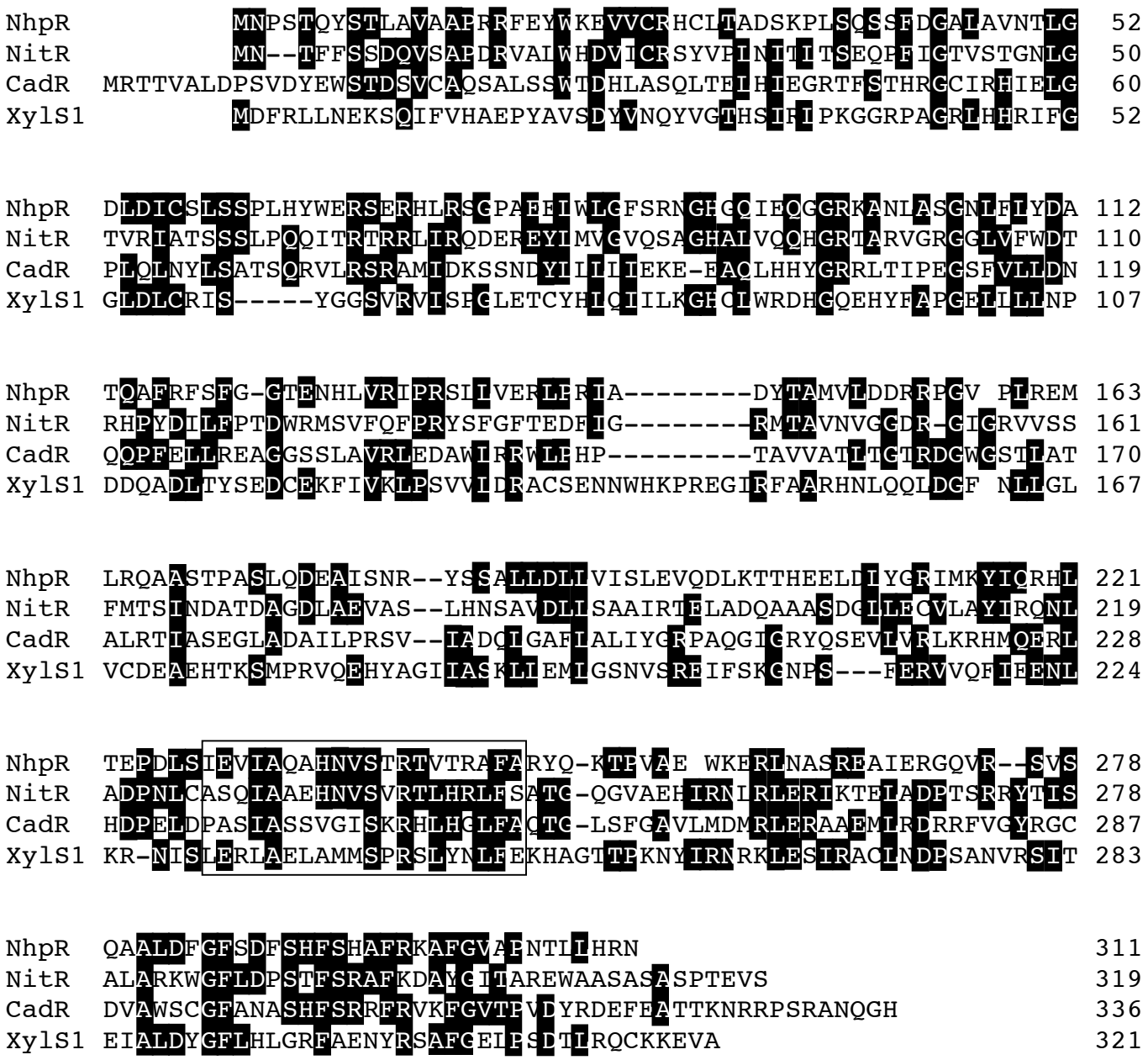
NhpR MNPSTQYSTLAVAAFRRFEYWKEVVCRHCLTADSKPLSQSSFDGALAVNTLG 52
NitR MN--TFFSSDQVSAFDRVALWHDVICRSYVPINITITSEQFFIGTVSTGNLG 50
CadR MRTTVALDPSVDYEWSTDSVCAQSALSSWTDHLASQLTEIHIEGRTFSTHRGCIRHIELG 60
XylS1 MDFRLLNEKSQIFVHAEPYAVSDYVNQYVGTHSIRIPKGGRPAGRLHHRIFG 52
NhpR DLDICSLSSPLHYWERSERHLRSGPAFEIWLGFSRNGHGQIEQGGRKANLASGNLFIYDA 112
NitR TVRIATSSSLPQQITRTRRLIRQDEREYIMVGVQSAGHALVQQHGRTARVGRGGLVFWDT 110
CadR PLQLNYLSATSQRVLRSRAMIDKSSNDYILLIIEKE-EAQLHHYGRRLTIPEGSFVLLDN 119
XylS1 GLDLCRIS-----YGGSVRVISPGLETCYHIQIILKGHCLWRDHGQEHYFAPGEILLLNP 107
NhpR TQAFRFSFG-GTENHLVRIPRSILVERLERIA--------DYTAMVLDDRRPGV PLREM 163
NitR RHPYDILFPTDWRMSVFQFPRYSFGFTEDFIG--------RMTAVNVGGDR-GIGRVVSS 161
CadR QQPFELLREAGGSSLAVRLEDAWIRRWLEHP---------TAVVATLTGTRDGWGSTLAT 170
XylS1 DDQADLTYSEDCEKFIVKLPSVVIDRACSENNWHKPREGIRFAARHNLQQLDGF NLLGL 167
NhpR LRQAASTPASLQDEAISNR--YSSALLDLLVISLEVQDLKTTHEELLIYGRIMKYICRHL 221
NitR FMTSINDATDAGDLAEVAS--LHNSAVDLLSAAIRTELADQAAASDGLLECVLAYIRQNL 219
CadR ALRTIASEGLADAILPRSV--IADQIGAFLALIYGRPAQGICRYQSEVLVRLKRHMQERL 228
XylS1 VCDEAEHTKSMPRVQEHYAGIIASKLIEMLGSNVSREIFSKCNPS---FERVVQFIEENL 224
NhpR TEPDLSIEVIAQAHNVSTRTVTRAFARYQ-KTPVAE WKERLNASREAIERGQVR--SVS 278
NitR ADPNLCASQIAAEHNVSVRTLHRLFSATG-QGVAEHIRNLRLERIKTELADETSRRYTIS 278
CadR HDPELDPASIASSVGISKRHLHGLFAQTG-LSFGAVLMDMRLERAAEMLRDRRFVGYRGC 287
XylS1 KR-NISLERLAELAMMSPRSLYNLFEKHAGTTPKNYIRNRKLESIRACLNDPSANVRSIT 283
NhpR QAALDFGFSDFSHFSHAFRKAFGVAPNTLLHRN 311
NitR ALARKWGFLDPSTFSRAFKDAYGITAREWAASASASPTEVS 319
CadR DVAWSCGFANASHFSRRFRVKFGVTPVDYRDEFEATTKNRRPSRANQGH 336
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Figure 3

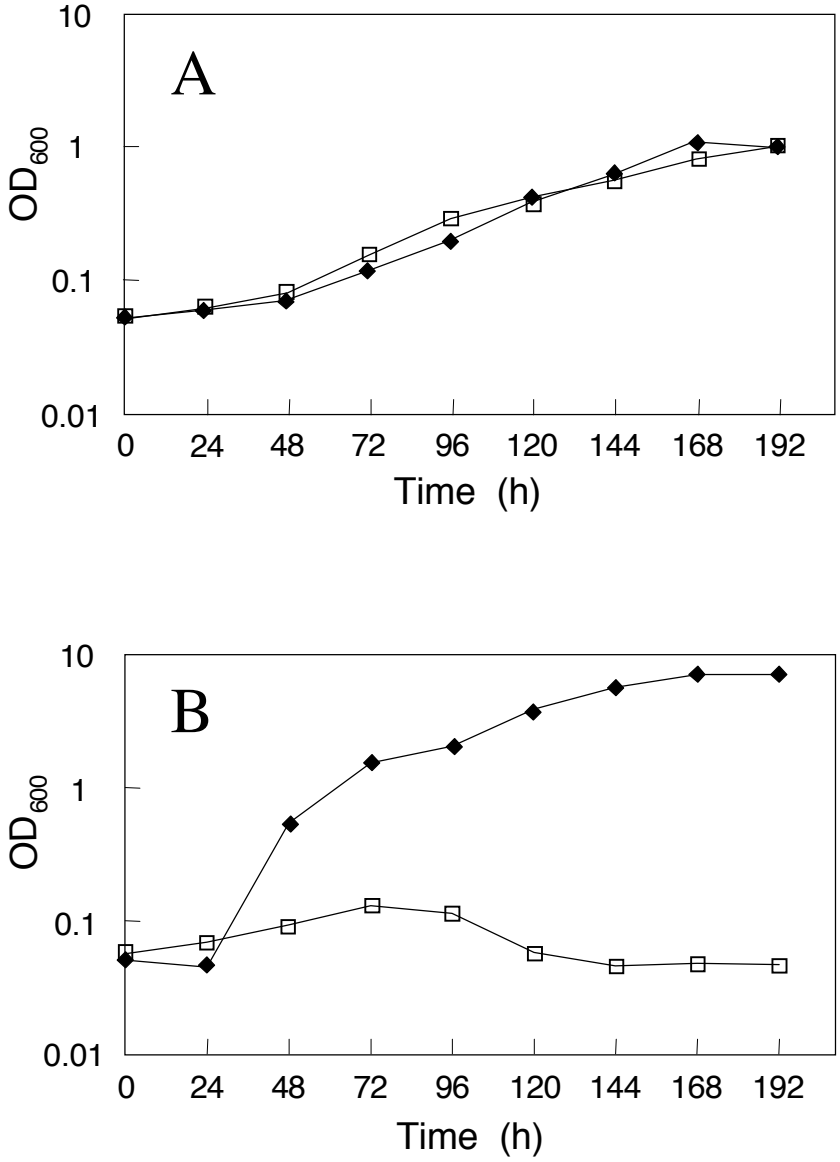
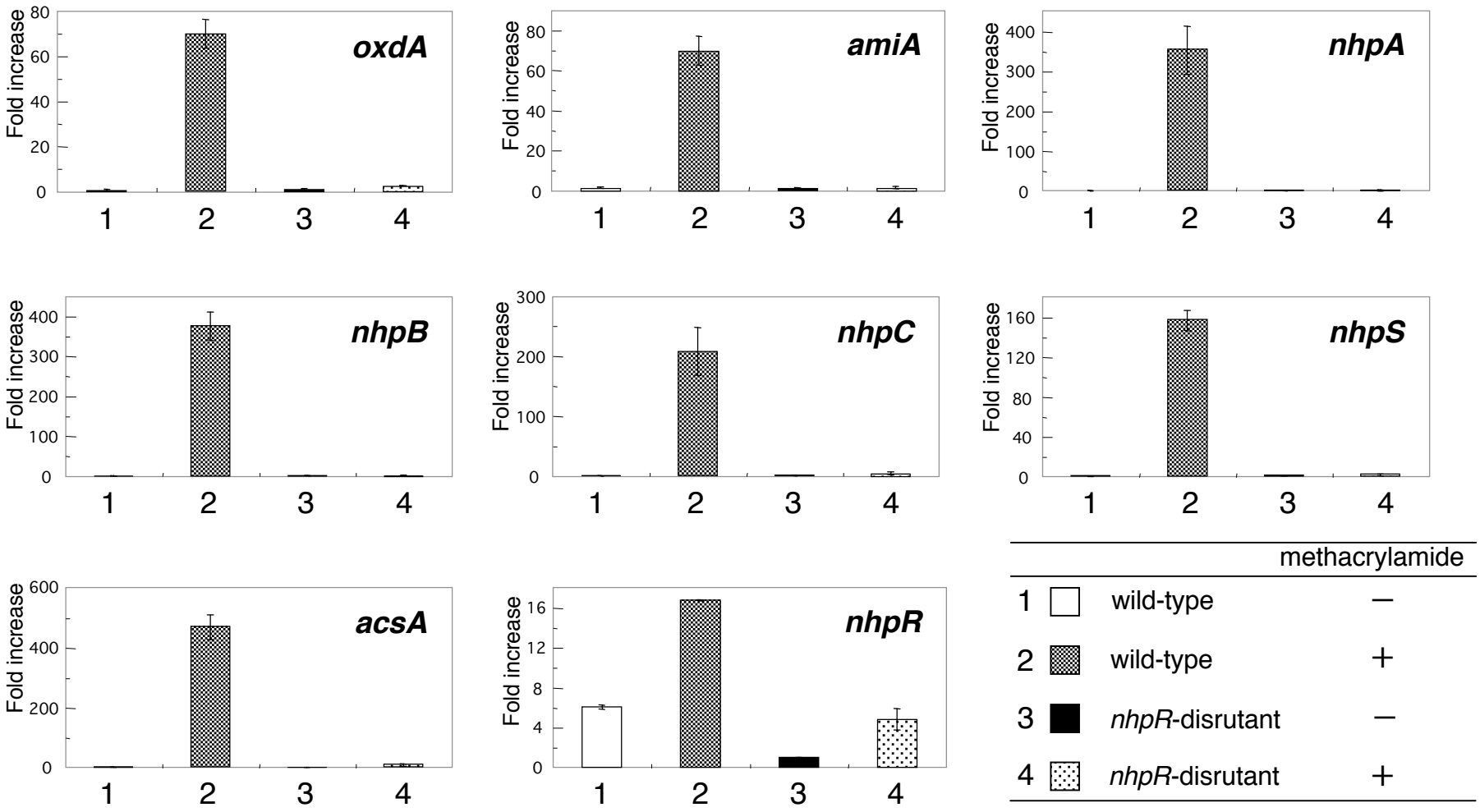


Figure 4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amiA
nhpA
nhpB
nhpC
nhpS
acsA
nhpR
Fold increase
1
2
3
4
methacrylamide
1 wild-type −
2 wild-type +
3 nhpR-disrutant −
4 nhpR-disrutant +

Figure 5