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The Naphthalene Catabolic (*nag*) Genes of *Polaromonas naphthalenivorans* CJ2: Evolutionary Implications for Two Gene Clusters and Novel Regulatory Control

Che Ok Jeon,[1,2] Minjeong Park,[2] Hyun-Su Ro,[2,3] Woojun Park,[4] and Eugene L. Madsen[1]*

Department of Microbiology, Cornell University, Ithaca, New York 14853-8101[1]; Division of Environmental Biotechnology, EBNCRC & PMBBRC, Gyeongsang National University, 900 Gazwa-dong, JinJu, GyeongNam 660-701, Korea[2]; Department of Microbiology and Research Institute of Life Science, Gyeongsang National University, 900 Gazwa-dong, JinJu, GyeongNam 660-701, Korea[3]; and Division of Environmental Science and Ecological Engineering, Korea University 1, Anam-DongGa, Seungbuk-Ku, Seoul 136-701, Korea[4]

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***Polaromonas naphthalenivorans* CJ2, found to be responsible for the degradation of naphthalene in situ at a coal tar waste-contaminated site (C.-O. Jeon et al., Proc. Natl. Acad. Sci. USA 100:13591–13596, 2003), is able to grow on mineral salts agar media with naphthalene as the sole carbon source. Beginning from a 484-bp *nagAc*-like region, we used a genome walking strategy to sequence genes encoding the entire naphthalene degradation pathway and additional flanking regions. We found that the naphthalene catabolic genes in *P. naphthalenivorans* CJ2 were divided into one large and one small gene cluster, separated by an unknown distance. The large gene cluster (*nagRAaGHAbAcAdBFCQEDJI′ORF1tnpA*) is bounded by a LysR-type regulator (*nagR*). The small cluster (*nagR2ORF2I″KL*) is bounded by a MarR-type regulator (*nagR2*). The catabolic genes of *P. naphthalenivorans* CJ2 were homologous to many of those of *Ralstonia* U2, which uses the gentisate pathway to convert naphthalene to central metabolites. However, three open reading frames (*nagY*, *nagM*, and *nagN*), present in *Ralstonia* U2, were absent. Also, *P. naphthalenivorans* carries two copies of gentisate dioxygenase (*nagI*) with 77.4% DNA sequence identity to one another and 82% amino acid identity to their homologue in *Ralstonia* sp. strain U2. Investigation of the operons using reverse transcription PCR showed that each cluster was controlled independently by its respective promoter. Insertional inactivation and lacZ reporter assays showed that *nagR2* is a negative regulator and that expression of the small cluster is not induced by naphthalene, salicylate, or gentisate. Association of two putative *Azoarcus*-related transposases with the large cluster and one *Azoarcus*-related putative salicylate 5-hydroxylase gene (*ORF2*) in the small cluster suggests that mobile genetic elements were likely involved in creating the novel arrangement of catabolic and regulatory genes in *P. naphthalenivorans*.**

The degradation of naphthalene has been studied extensively in two *Pseudomonas* species that carry the archetypal catabolic plasmids, NAH7 (in *P. putida* G7) and pDTG1 (in *P. putida* NCIB9816-4) (6, 11, 41). In both strains, the *nah* dissimilatory genes are organized into two operons: one coding for the enzymes involved in the conversion of naphthalene to salicylate (naphthalene degradation upper pathway) and another coding for the conversion of salicylate to pyruvate and acetyl coenzyme A via meta-cleavage (naphthalene degradation lower pathway) (2, 42, 42, 54). In contrast to the *nah* naphthalene catabolic pathway in *Pseudomonas* species, the *nag* genes of *Ralstonia* sp. strain U2 encode the alternative gentisate pathway, which converts naphthalene to fumarate and pyruvate via salicylate and gentisate (14). The *nag* genes are organized in a single operon (56, 57). Despite the contrasts in structural genes and their arrangements, the *nah* and *nag* systems exhibit strikingly similar regulation. Transcription of *nag* and both *nah* operons is controlled by a single Lys-R-type regulatory protein (NagR and NahR, respectively). These proteins act as positive regulators for both pathways, and salicylate functions as an inducer (23, 34, 37, 39).

Detailed genetic and/or biochemical information on naphthalene metabolism have been obtained via studies of many bacteria: e.g., *Burkholderia* sp. strain DNT (45), *Burkholderia* sp. strain RP007 (27), *Comamonas testosteroni* GZ39 (16), *Comamonas* sp. strain JS765 (29), *Cycloclasticus* sp. strain A5 (25), and *Sphingomonas* sp. strain CHY-1 (8). As this information accrues, we have the opportunity to gain understanding about how these catabolic operons evolve. For instance, a recent study by Kulakov et al. (26) has shown that genetic rearrangements of *nar* genes have been important in the evolution of naphthalene metabolism in *Rhodococcus*. In the present study, we examine *Polaromonas naphthalenivorans* CJ2, a bacterium found to be responsible for the field biodegradation of naphthalene at a coal tar waste-contaminated site (21, 22). Here we report DNA sequences showing that the *nag* genes of *P. naphthalenivorans* CJ2 are similar to those of *Ralstonia* sp. strain U2, but they are divided into two clusters and exhibit one duplication and several deletions. Furthermore, regulatory control of the two clusters from strain CJ2 is novel.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. All bacterial strains, vectors and plasmids used in the present study are listed in Table 1. *P. naphthalenivorans* CJ2 was grown at 20°C and maintained on mineral salts basal medium (MSB) (43) with either naphthalene vapor or a 0.5% (wt/vol) suspension of crystals

* Corresponding author. Mailing address: Department of Microbiology, Cornell University, Ithaca, NY 14853-8101. Phone: (607) 255-3086. Fax: (607) 255-3904. E-mail: elm3@cornell.edu.

TABLE 1. Bacterial strains, plasmids, and PCR primers used in this study[a]

Name of strain, plasmid, or primer	Description or sequence	Length (bp)	Source or reference(s)
Strains			
P. naphthalenivorans CJ2	Naphthalene degrader		21, 22
Strain CJN110	*P. naphthalenovorans* CJ2 Δ*nagR*::*kan* LacZ$^+$		This study
Strain CJM110	*P. naphthalenovorans* CJ2 Δ*R2*::*kan* LacZ$^+$		This study
Strain CJM112	*P. naphthalenovorans* CJ2 *R2-ORF2*::LacZ$^+$ fusion		This study
E. coli SY327 λ*pir*	λ*pir* Δ(*lac pro*) *argE*(Am) *recA56 nalA* Rif(λ*pir*); carries π protein for R6Kγ *ori*		24
E. coli S17-1 λ*pir*	*trp* Sm^r *recA thi pro hsdM*$^+$ RP4-2-Tc::Mu::Km Tn*7* λ*pir*; *hsdR* mutant		24
INV-αF′	Cloning host, F′ *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169* λ$^-$		Invitrogen
Plasmids or vector			
pVIK110	*lacZY* for translational fusions, R6KoriV, suicide vector; Km^r		24
pCJN110	Internal *nagR* fragment (derived from pVIK112); Km^r		This study
pCJM110	Internal *R2* fragment (derived from pVIK112); Km^r		This study
pVIK112	*lacZY* for transcriptional fusions, R6KoriV, suicide vector; Km^r		24
pCJM112	*R2-ORF2*::LacZ$^+$ fusion (derived from pVIK112); Km^r		This study
pCR2.1-TOPO[a]	Km^r Amp^r; cloning vector		Invitrogen
PCR primers			
RT1-F	5′-ACATCCTGGGCTTCTACGCC-3′	20	
RT2-F	5′-AATGGCCTCGTCCTACTACA-3′	20	
RT2-R	5′-CAGATCGATGCCTACTGTTG-3′	20	
RT3-F	5′-CTGGAGTCGCAGATTGTGAA-3′	20	
RT3-R	5′-ATTGCTCCCTGCTTTGTTCT-3′	20	
RT4-F	5′-CTGTTGGTGCAGGCTGTTAC-3′	20	
RT4-R	5′-GGCTTTGGCTTGGGCATTGA-3′	20	
RT5-F	5′-GCTTGTTCAGCAGTTCCATG-3′	20	
RT5-R	5′-ACATTGACCAGATGCCTGGA-3′	20	
RT6-F	5′-GCTGCTGTCGCTGACGAAC-3′	19	
RT6-R	5′-TCACATTGACCAGATGCCTG-3′	20	
RT7-F	5′-TCAGTTCCTGGACCCCTTCC-3′	20	
RT7-R	5′-GTCGAGGTCAGGAATTCGAC-3′	20	
RT8-F	5′-AACTGCCACCTGGTGCACTA-3′	20	
RT8-R	5′-CAGCACATCGAGCCAGATCA-3′	20	
RT9-F	5′-GATGGTGTTCCATGTGATCG-3′	20	
RT9-R	5′-TGGCAACCACCAGCTCCATT-3′	20	
RT10-F	5′-TGGGACTTGGGCAAGGACGT-3′	20	
RT10-R	5′-CATCTGCGGGTTGACGGCTT-3′	20	
RT11-F	5′-ACACCAGCGCTGTTGCCAGG-3′	20	
RT11-R	5′-TGATGGTCTCGAAAGCGAAG-3′	20	
RT12-F	5′-GTGTCCCAGCTCTTCACCTC-3′	20	
RT12-R	5′-GAGCAGTCGAAAACAGACGT-3′	20	
RT13-F	5′-CCCAGCGCATGCTGCACTTC-3′	20	
RT13-R	5′-GTGAGCTTGTTCAGATCGAA-3′	20	
nrc-F	5′-CCC<u>TCTAGAGA</u>TCAAGCAAGCCATCCACT-3′	29	
nrc-R	5′-CCC<u>GTCGAC</u>CATTCACCACACGCAACTCT-3′	29	
mrc-F	5′-CCC<u>TCTAGA</u>CTGAGTTCGTTGTTCGTGCT-3′	29	
mrc-R	5′-CCC<u>GTCGAC</u>AGTACGCCATCCTCCAGT-3′	27	
sc-F	5′-CCG<u>GAATTC</u>GCATGTGGTGCGGATTGTCG-3′	29	
sc-R	5′-CGC<u>TCTAGA</u>CCAGCACCTTGACGGAAA-3′	27	
onrc-F	5′-AGTGGCGATGAGATCGGTAG-3′	20	
omrc-F	5′-GGAGCGGGAGTGAAGGCAG-3′	19	
lacZ-R	5′-CGCCAAGACTGTTACCCATC-3′	20	
nahAc114-F	5′-CTGGC(T/A)(T/A)TT(T/C)CTCAC(T/C)CAT-3′	19	
nahAc595-R	5′-TC(C/G)GC(G/A)GGTG(T/C)CTTCCAGTTG-3′	21	

[a] Nomenclature: o, outer; F, forward; R, reverse; pro, promoter. Restriction enzyme sites (for future study) are underlined. Km^r, kanamycin resistance; Amp^r, ampicillin resistance.

(MSB-N) or 0.2% (wt/vol) pyruvate (MSB-P) as the sole carbon source. Other culture media were prepared according to the procedures described previously (20, 44). All *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium in a shaking incubator (100 rpm). When required, the appropriate antibiotics and reagents were added to the media: X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 30 μg/ml), kanamycin (40 μg/ml), and salicylate (2.5 mM). Growth was monitored by measuring the optical density of the cultures at a wavelength of 600 nm after naphthalene crystals had settled. Spontaneous rifampin-resistant *P. naphthalenivorans* CJ2 was selected on R2A plates containing a rifampin gradient (0 to 200 μg/ml) (12, 44).

Plasmid characterization and Southern hybridization. Large plasmids were isolated from *P. naphthalenivorans* CJ2, *Pseudomonas putida* NCIB 9816-4, and *Pseudomonas putida* G7 according to a method described previously (44). Plasmid DNAs were separated on a 0.7% agarose gel in Tris-acetate-EDTA. The voltage was 30 V until the dye entered the gel, and then it was increased to 60 V. For Southern hybridizations of the isolated plasmid, *nahAc* probes were gener-

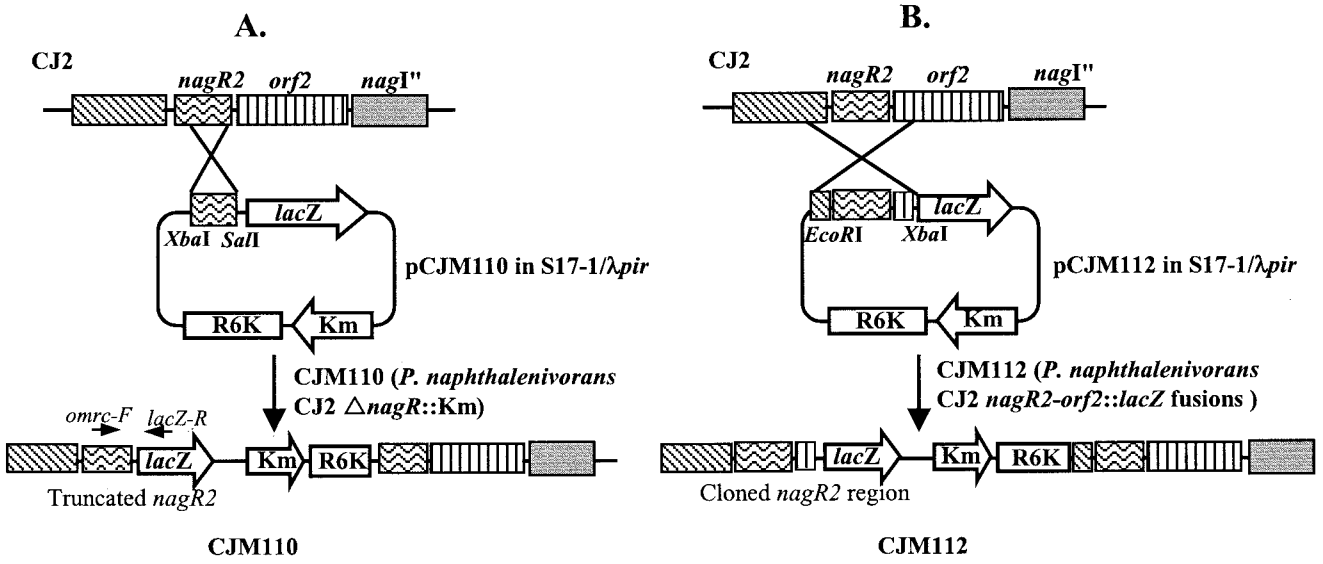


FIG. 1. Construction of mutants CJM110 and CJM112 via Campbell-type homologous recombination in the small cluster of *P. naphthalenivorans* strain CJ2 naphthalene degradation genes. (A) The mutant CJM110 was designed to create a polar knock out of regulator *nagR2*. (B) The mutant CJM112 reported transcriptional activity in the small cluster of the naphthalene degradation pathway.

ated via PCR from strains CJ2, NCIB 9816-4, and G7 using *nahAc*-based degenerate primers (Table 1) as previously described (52). The probes were mixed 1:1:1. The primers were 5′ end labeled using [γ-^{32}P]dATP and a T4 polynucleotide kinase (Invitrogen). Southern hybridizations were carried out according to the instructions of standard protocol (36). To seek evidence for a naphthalene-degrading plasmid, curing assays were performed by periodically subculturing the bacterium in the absence of naphthalene (on R2A medium [44]), by exposing growing cultures to ethidium bromide (7), and by cultivation on 1-chloronaphthalene (49). Genomic DNA isolation, digestion with restriction enzymes, electrophoresis, transfer onto nylon membranes, and Southern hybridizations were done according to standard protocols (36).

Nucleotide sequence determination and sequence analysis. Nucleotide sequences were determined by the "genome walking" strategy using GenomeWalker Kit (Clontech) from a conserved, PCR-amplifiable (484 bp) region of the naphthalene dioxygenase (*nahAc*) gene as the starting point. Our first effort at genome walking allowed sequencing to proceed for approximately 25 kb, and the similarity to the operon of *Ralstonia* sp. strain U2 was noted. For unknown reasons, extending the sequence beyond *nagI* was unsuccessful. Then, using primers designed from the naphthalene operon of *Ralstonia* sp. strain U2, we found that *P. naphthalenivorans* CJ2 carried another copy of gentisate dioxygenase (*nagI*). Sequences of genes adjacent to both copies were obtained and were analyzed with the Lasergene software package (DNASTAR). BLASTX was used for the deduced amino acid identity search, and BLASTN was used for the nucleotide identity search (1). Transcription promoters and termination sequences of the *nag* gene clusters were analyzed by using web-based programs (http://www.softberry.com/; http://www.fruitfly.org/seq.tools/promoters.html.

RT-PCR. Cells were grown on MSB-N agar plates. Total RNA was prepared from colonies by using RNeasy minicolumns (QIAGEN). The RNA was treated to remove any genomic DNA contamination by incubation with 1 U of RNase-free DNase I (Promega) and 1 U of RNasin (Promega) in 40 mM Tris-HCl (pH 7.9) containing 10 mM NaCl, 10 mM $CaCl_2$, and 6 mM $MgSO_4$ for 30 min at 37°C. The RNA preparation was cleaned by passage through an RNase minicolumn. Next, a reverse transcriptase-PCR (RT-PCR) reaction was carried out by using SuperScript II RT (Invitrogen/Life Technologies, Carlsbad, Calif.). The intergenic regions between the *nag* genes were amplified by using primer pairs of RT1-RT13 (Table 1). To confirm that cDNA synthesis occurred and that RNA preparation was free of genomic DNA, a negative control RT-PCR was performed with *Taq* polymerase (omitting the RT).

Insertional inactivation of *nagR* and *nagR2* by homologous recombination. Strains with mutations in regulatory genes (*nagR* and *nagR2*) were prepared by using the suicide translational fusion vector, pVIK110 (containing the R6K oriV region so that it cannot replicate in the absence of the λ*pir* replication system of *E. coli* SY327 λ*pir*; Table 1). Campbell-type homologous recombination with *nagR* and *nagR2* was achieved by PCR amplification of 396 and 322 bp, respectively, internal to *nagR* and *nagR2*. The primer pairs NRC-F/NRC-R and MRC-F/MRC-R) (Table 1) generated fragments with XbaI and SalI cohesive ends that were subcloned into the XbaI-SalI cloning site of pVIK110, creating plasmids pCJN110 and pCJM110, respectively. The pCJN110 and pCJM110 plasmids were introduced by electroporation into *E. coli* S17-1 λ*pir* that has the *tra* region of RP4. Then, pCJN110 and pCJM110 were conjugated into rifampin-resistant *P. naphthalenivorans* CJ2 by mating on R2A agar media at 25°C for 16 h, as previously described (35). The transconjugants (strain CJN110 [*P. naphthalenivorans* CJ2 Δ*nagR*::*kan*] and strain CJM110 [*P. naphthalenivorans* CJ2 Δ*R2*::*kan*]) were selected on R2A plates containing kanamycin (40 μg/ml) and rifampin (200 μg/ml) at 20°C. Confirmation of the transconjugants was conducted by using PCR. Figure 1A shows the schematic diagram for creating strain CJM110 via homologous recombination.

Growth tests of regulatory mutant strains CJN110 and CJM110 and Northern blot analysis. Cells of strains CJN110, CJM110, and CJ2, grown in MSB-P medium for 24 h at 20°C were inoculated (8% [vol/vol]) into MSB-N media with 0.5% naphthalene crystals. Growth was monitored by measuring the optical density at 600 nm of the cultures.

To measure mRNA expression of *ORF2* in mutant CJM110 and wild-type CJ2, total RNA was isolated from exponentially growing cells in MSB-N media by using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. The RNA concentration was measured by determining the absorbance at 260 nm. A total of 100 μg of total RNA per sample was transferred to nylon membranes (Schleicher & Schuell, Keene, NH) by using a Slot blotter (Schleicher & Schuell). Northern hybridization was carried out by using standard protocol (36). Random-primed DNA labeling with digoxigenin-dUTP was applied to PCR products (478 bp) amplified by using the primers *ORF2*-226-F and *ORF2*-703R (Table 2). Detection of the labeled DNA by enzyme immunoassay on nylon membranes was performed with a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences) according to the manufacturer's instructions.

Construction of *nagR2-ORF2*::*lacZ* fusions and β-galactosidase assays. Figure 1B shows how the reporter plasmid was constructed. Suicide transcriptional fusion vector pVIK112 (containing a stop codon immediately downstream of multicloning sites and the R6KoriV region so that it cannot replicate without the λ*pir* replication system) was maintained in *E. coli* SY327 λ*pir* (Table 2). Using the procedures described above, pCJM112 was prepared (Fig. 1B). The *R2-ORF2*::*lacZ* fusion was achieved by introducing pCJM112 into *E. coli* S17-1 λ*pir* and subsequent conjugation into strain CJ2 as described above.

For β-galactosidase assays, cultures were grown overnight in 5 ml of minimal medium containing 0.2% (wt/vol) sodium pyruvate with or without naphthalene, salicylate, and gentisate. Cells were lysed with chloroform and sodium dodecyl

TABLE 2. *P. naphthalenivorans* strain CJ2 genes and gene products

Cluster and gene	Putative function	Length (aa)[a]	Most similar gene product(s) (species, accession no.) or reference	% Identity (aa)
Large gene cluster				
nagR	Transcription regulator (LysR type)	301 (−)	LysR-like regulatory protein (*Ralstonia* sp. strain U2, AAG13636)	80
			LysR-type regulatory protein (*Burkholderia* sp. strain DNT, AAP70493)	79
nagAa	Ferredoxin reductase	328 (+)	Ferredoxin reductase (*Ralstonia* sp. strain U2, AAD12606)	75
			DntAa (*Burkholderia cepacia* R34, AAL50024)	75
nagG	Salicylate-5-hydroxylase, large oxygenase component	420 (+)	Salicylate-5-hydroxylase large oxygenase component (*Ralstonia* sp. strain U2, AAD12607)	88
			ORF2 (*Burkholderia* sp. strain DNT, AAB09764)	84
nagH	Salicylate-5-hydroxylase, small oxygenase component	161 (+)	Salicylate-5-hydroxylase small oxygenase component (*Ralstonia* sp. strain U2, AAD12608)	86
			ORFX (*Burkholderia* sp. DNT, AAD15560)	86
nagAb	Ferredoxin	112 (+)	Ferredoxin (*Ralstonia* sp. strain U2, AAD12609)	78
			DntAb (*Burkholderia cepacia* R34, AAL50022)	78
nagAc	Naphthalene dioxygenase, large dioxygenase component	447 (+)	Large dioxygenase subunit (*Comamonas testosterone* H, AAF72976)	95
			Large dioxygenase component (*Ralstonia* sp. strain U2, AAD12610)	95
nagAd	Naphthalene dioxygenase, small oxygenase component	194 (+)	2,4-DNT dioxygenase; DntAd (*Burkholderia* sp. DNT, AAS09912)	88
			Dioxygenase small subunit (*Comamonas testosterone* H, AAF72977)	88
nagB	*cis*-Naphthalene dihydrodiol dehydrogenase	259 (+)	*cis*-Naphthalene dihydrodiol dehydrogenase (*Comamonas testosterone* H, AAF72978)	86
			cis-Naphthalene dihydrodiol dehydrogenase (*Ralstonia* sp. strain U2, AAD12612)	86
nagF	Salicylaldehyde dehydrogenase	483 (+)	Salicylaldehyde dehydrogenase (*Ralstonia* sp. strain U2, AAD12613)	88
			Dehydrogenase (*Pseudomonas aeruginosa* Pak1, BAA12243)	84
nagC	1,2-Dihydroxynaphthalene dehydrogenase	302 (+)	1,2-Dihydroxynaphthalene dioxygenase (*Ralstonia* sp. strain U2, AAD12614)	94
			1,2-Dihydroxynaphthalene dioxygenase (*Pseudomonas putida* NCIB 9816-4)	88
nagQ	Putative aldolase	212 (+)	Putative aldolase (*Ralstonia* sp. strain U2, AAD12615)	91
			Dibenzothiophene oxidation protein (*Pseudomonas* sp. strain ND6, NP_943091)	71
nagE	*trans-o*-Hydroxybenzylidenepyruvate hydratase-aldolase	345 (+)	*trans-o*-Hydroxybenzylidenepyruvate hydratase-aldolase (*Ralstonia* sp. strain U2, AAD12616)	96
			trans-o-Hydroxybenzylidenepyruvate hydratase-aldolase (*Pseudomonas putida* NCIB 9816-4, NP_863078)	87
nagD	2-Hydroxychromene-2-carboxylate isomerase	197 (+)	2-Hydroxychromene carboxylate isomerase (*Ralstonia* sp. strain U2, AAD12617)	88
			2-Hydroxychromene-2-carboxylate dehydrogenase (*Pseudomonas stutzeri* AN10, AAD02142)	68
nagJ	GST homolog (glutathione *S*-transferase-like protein)	201 (+)	Glutathione *S*-transferase-like protein (*Ralstonia* sp. strain U2, AAD12618)	93
			Putative glutathione *S*-transferase (*Pseudomonas putida* NCIB 9816-4, NP_863083)	76
nagI′	Gentisate 1,2-dioxygenase	334 (+)	Gentisate 1,2-dioxygenase (*Ralstonia* sp. strain U2, AAD12619)	82
			Putative 1,2-dioxygenase (*Salmonella enterica* serovar Typhimurium LT2, NP_461123)	32
ORF1	Unknown	289 (+)	2-Keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway) (*Mesorhizobium* sp. strain BNC1, ZP_00195913)	44
			2-Hydroxyhepta-2,4-diene-1,7-dioate isomerase (*Bacillus halodurans* C-125, NP_242871)	41
tnpA	Putative transposase	344 (+)	Transposase and inactivated derivatives (*Azotobacter vinelandii*, ZP_00089601)	49
			Probable transposase (*Pseudomonas aeruginosa* PAO1, NP_249136)	49
None	Putative terminator		Rho-independent terminator	
Small gene cluster				
R2	Putative transcriptional regulator (MarR type)	161 (−)	Transcriptional regulatory protein (*Bradyrhizobium japonicum* USDA110, NP_766748)	42
			Transcriptional regulators (*Burkholderia cepacia* R1808, ZP_00213511)	44
ORF2	Unknown	400 (+)	Putative salicylate 5-hydroxylase (*Azoarcus* sp. strain EbN1)	89
			2-Polyprenyl 6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases (*Ralstonia eutropha* JMP134)	60
nagI″	Gentisate 1,2-dioxygenase	351 (+)	Gentisate 1,2-dioxygenase (*Ralstonia* sp. strain U2, AAD12619)	82
			Putative 1,2-dioxygenase (*Salmonella enterica* serovar Typhimurium LT2, NP_461123)	38
nagK	Fumarylpyruvate hydrolase	192 (+)	Fumarylpyruvate hydrolase (*Ralstonia* sp. strain U2, AAD12620)	80
			Unnamed protein product (*Sphingomonas* sp. strain RW1, CAA73583)	59
nagL	Maleylpyruvate isomerase	212 (+)	Maleylpyruvate isomerase (*Ralstonia* sp. strain U2, AAD12621)	69
			Glutathione *S*-transferase (*Ralstonia eutropha* JMP134, ZP_00171533)	55

[a] aa, amino acids. The orientation of coding strands are indicated in parentheses.

sulfate, and β-galactosidase activities were determined by using standard methods (30).

Nucleotide sequence accession numbers. The nucleotide sequences of *P. naphthalenivorans CJ2* have been deposited in GenBank under accession no. DQ167474 for the large gene cluster and accession no. DQ167475 for the small gene cluster.

RESULTS

Localization of the nag gene clusters on the chromosome DNA. To examine whether the naphthalene catabolic genes reside on the chromosome or on a plasmid, the plasmids of strain CJ2 were subjected to electrophoresis and then transferred to a membrane which was probed with a genetically identical 488-bp ^{32}P-labeled *nahAc* PCR product. The result showed that at least five plasmids in strain CJ2, but none of the plasmids, hybridized with the probe tested (Fig. 2), although positive-control plasmids from other naphthalene-degrading pseudomonads hybridized as expected. Instability of the Nah$^+$

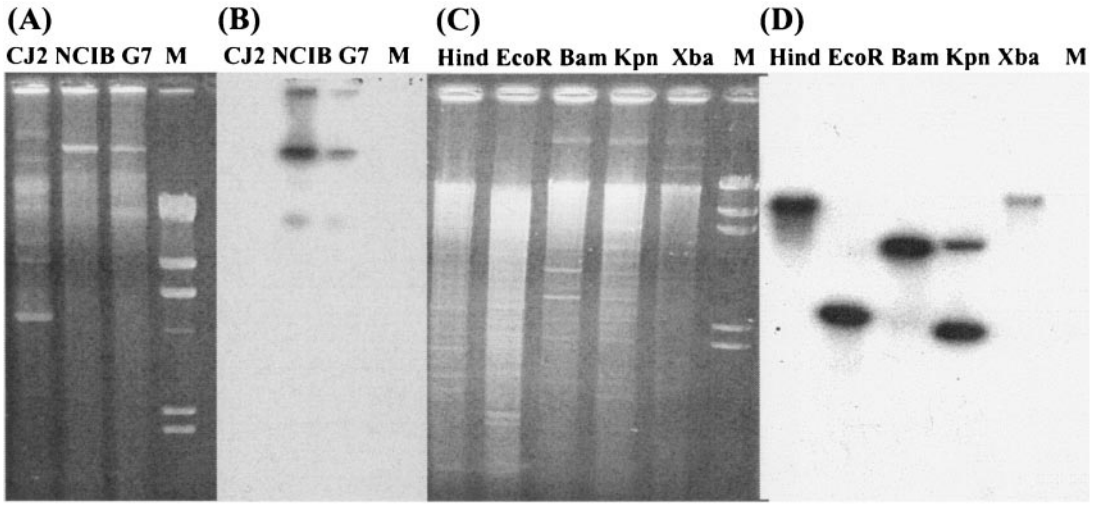


FIG. 2. Localization of naphthalene degradation genes in *P. naphthalenivorans* strain CJ2. (A) Plasmids retrieved from wild-type CJ2 and two positive-control pseudomonads carrying 80-kb naphthalene catabolic plasmids, *P. putida* NCIB 9816-4 and *P. putida* G7; (B) Southern hybridization of plasmids with *nahAc* probes; (C) the genomic DNA from strain CJ2 digested with HindIII, EcoRI, KpnI, and XbaI; (D) Southern hybridization of the digested genomic DNA with *nahAc* probes; M, molecular size marker, λ-DNA digested with HindIII.

phenotype, often associated with curable catabolic plasmids, was examined as an additional way to detect a naphthalene catabolic plasmid. Strain CJ2 was not cured of a plasmid by traditional ethidium bromide or chloro-naphthalene treatment or by periodic subculturing in the absence of naphthalene. Southern blots of total genomic DNA digested with five different restriction enzymes probed positively for *nahAc* (Fig. 2C and D). Thus, despite the common occurrence of naphthalene catabolic genes on plasmids, our results indicated that the naphthalene degradation pathway in strain CJ2 is chromosomally encoded. These findings resemble those from *P. stutzeri* AN 10 (4).

Sequence analysis of the naphthalene degradation genes. The nucleotide sequence of the gene clusters encoding the naphthalene-degradation pathway was determined by using a genome walking strategy. Twenty-one open reading frames (ORFs) related to the naphthalene catabolic genes were deduced; these were divided into two clusters *nagRAaGHAbAc AdBFCQEDJI′ORF1tnpA* and *nagR2ORF2I″KL* (large cluster; 25,748 bp, 56.64% G+C content; small cluster, 14,217 bp, 64.37% G+C content) (Fig. 3A). For each gene, the putative function, position, predicted product size, and significant matches to the predicted gene product are summarized (Table 2).

Analysis of the DNA sequences showed that the naphthalene catabolic operon structure of strain CJ2 was similar to that of *Ralstonia* sp. strain U2, which metabolizes naphthalene via the gentisate pathway, encoded by the *nag* operon. The putative two-component salicylate 5-hydroxylase of the large cluster (*nagG* [with the Rieske-type iron-sulfur center] and small subunit, *nagH*) is homologous to that of strain U2, which function together with *nagAaAb* gene products (14). Surprisingly, strain CJ2's naphthalene catabolic genes were not contiguous; in fact, efforts at long PCR were unable to span the gap between the two clusters. Furthermore, in the large cluster three ORFs (*nagY* [putatively involved in chemotaxis], *nagM*, and *nagN*), present in *Ralstonia* sp. strain U2, were absent.

Examination of the DNA sequence between *nagR* and *nagAa* revealed that the promoter of strain CJ2 had a high degree of identity to that of *Ralstonia* sp. strain U2, especially near the putative AreR binding region and the putative −35 and −10 boxes (Fig. 4A). Upstream of this promoter between bases −76 and −59 is a symmetrical dyad motif TTCAN6TGAT (Fig. 4B) characteristic of the LysR family which has been identified as important for NahR and NagR function (23, 39). BLAST searches examining the downstream region of the large gene cluster revealed two putative transposase-related ORFs, *tnpA′* and *istB* (Fig. 3A).

The small cluster of *P. naphthalenivorans* CJ2 featured a MarR-type transcriptional regulator (*nagR2*), a putative gentisate 1,2-dioxygenase (*nagI″*), and a putative salicylate 5-hydroxylase (*ORF2*). The gentisate dioxygenases (*nagI′* and *nagI″*) of strain CJ2 exhibited 77.4% DNA sequence identity to one another and 82% amino acid identity to the analogous protein of *Ralstonia* sp. strain U2 (Table 2). However, the *ORF2* gene product of the small gene cluster was most closely related to the putative salicylate 5-hydroxylase of *Azoarcus* sp. strain EbN1 with 89% amino acid identity (Table 2). This salicylate 5-hydroxylaase, unlike *nagGH* of strain U2, is a single-component enzyme without a Rieske iron-sulfur center. Surprisingly, the MarR-type transcriptional regulator (*nagR2*), divergently transcribed from *ORF2* of the small cluster, was closely related to a *Bradyrhizobium* regulatory gene and not to typical catabolic regulators of pseudomonads (Table 2).

Expression of *nag* genes. RT-PCR was used to amplify mRNA purified from strain CJ2 cells grown on MSB agar with naphthalene vapor. The primer sets used were specific for the downstream end (*nagI′* and beyond) in the large *nag* cluster and spanned the entire small *nag* cluster (Fig. 3A). Because the large operon closely resembled that of *Ralstonia* sp. strain U2, we presumed that expression began at *nagAa* and extended into *nagI′*. The amplified products were analyzed by agarose gel electrophoresis (Fig. 3B). The presence of amplified DNA

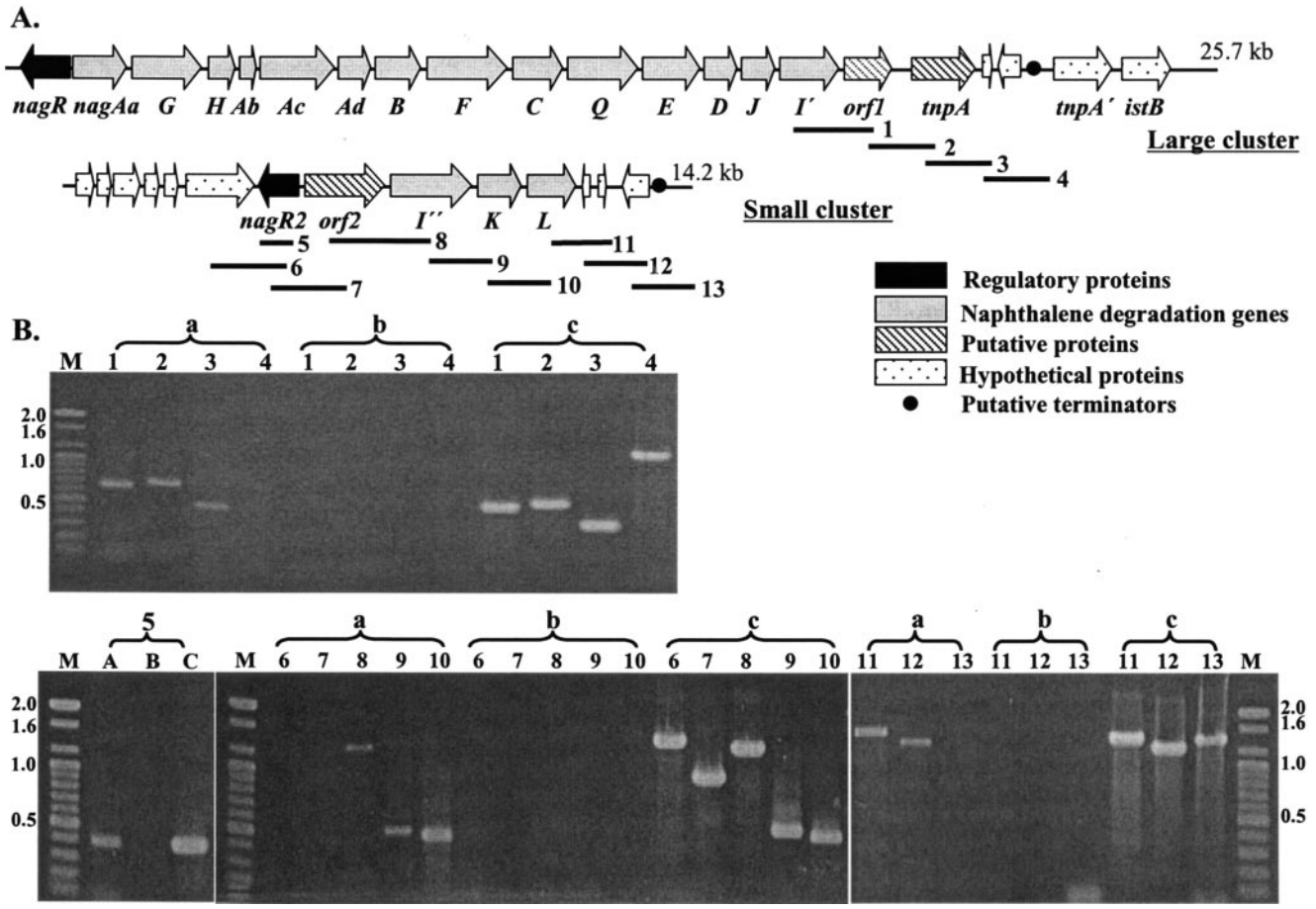


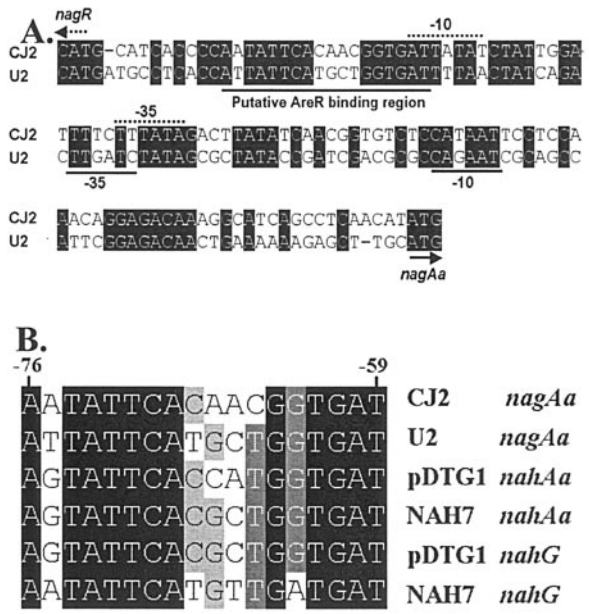
FIG. 3. Physical maps of naphthalene degradation genes from *P. naphthalenivorans* strain CJ2 and of RT-PCR analysis of expressed genes. (A) Gene order in large and small naphthalene catabolic clusters. Bold solid lines show locations of 13 primer pairs used in the RT-PCR assays. (B) Agarose gel electrophoresis of 13 RT-PCR products amplified from strain CJ2 grown on naphthalene. Numbers refer to the locations of PCR fragments shown in Fig. 3A. Lowercase letters refer to amplification conditions: a, RT-PCR products from total RNA; b, PCR products from total RNA without RT; c, PCR products from genomic DNA. M, molecular size marker (100-bp ladder).

fragments obtained with each primer pair suggests that contiguous genes in each cluster were transcribed on the same message. No amplification product was obtained when RT was omitted from the reaction mixture. The data in Fig. 3 show that transcription extended two ORFs beyond *nagI′* (fragments 1 to 3) but not beyond to fragment 4. In the second gene cluster, transcripts were found from "*nagR2* through *ORF2* to *nagI*"*KL* (fragments 5, 8, 9, 10, 11, and 12; Fig. 3B). Thus, we infer that there were termination sequences at the end of each cluster: regions beyond the termination sequences were not transcribed.

Genetic and phenotypic characterization of the *nagR* and *nagR2* genes. To verify the putative role of *nagR* (LysR-type) and *R2* (MarR-type) regulators in *P. naphthalenivorans* CJ2, these regulatory genes were disrupted by using Campbell-type single-crossover homologous recombination. The sought genotypes of transconjugants were verified by using outer primer pairs, *onrc*-F/*lac*Z-R, omrc-F/*lac*Z-R (data not shown). When fewer than 3% (vol/vol) of cells were inoculated, very poor growth was observed from CJ2, as well as the two mutants. However, a heavier inoculum (8% [vol/vol]) allowed growth. The cell density of mutant CJN110, mutant CJM110, and wild-type CJ2, grown on 0.5% naphthalene crystals, was monitored by measuring the optical density at 600 nm of the cultures (Fig. 5). The *nagR* mutant strain, CJN110, showed a serious growth defect: this result was consistent with the previous reports (23, 38, 55). However, the second regulatory (*nagR2*) mutant strain, CJM110, grew faster than the wild type (Fig. 5). This is consistent with the known role of *marR* genes to function as repressors. Although a light brown color from the medium of the wild-type CJ2 was observed after 30 h, the color of the *nagR2* mutant medium (CJM110) became dark black after 45 h. Therefore, the growth (optical density) of strain CJM110 could not be measured beyond 45 h.

Northern blot analysis was conducted to confirm the transcription of the second gene cluster in wild-type strain CJ2 and the mutant strain CJM110. Consistent with the growth of the strains, Northern blot data confirmed that transcription of the second cluster gene expression in strain CJM110 was higher than that of the wild type (date not shown).

Expression of *R2-ORF2*::*lacZ* report construct in strain CJ2. The LysR-type regulatory genes associated with naphthalene catabolism are known to be induced by naphthalene, salicylate, and gentisate. To examine the influence of these compounds



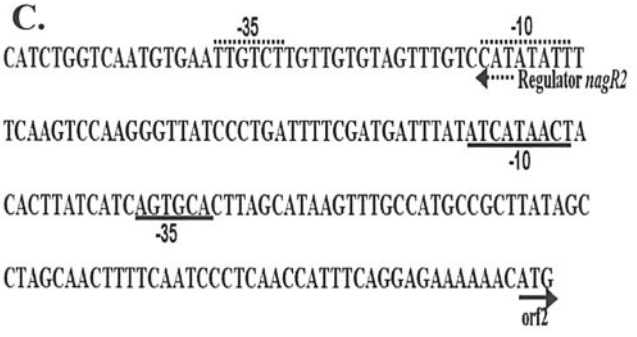


FIG. 4. Comparative sequence analysis of regulatory promoters. (A) Promoters and NagR binding regions of the *nag* promoter region from strains U2 and CJ2. The arrows indicate the start of translation. The putative −35 and −10 motifs and the putative NagR binding motif are underlined. (B) Alignment of the conserved upstream regions controlled by NahR and NagR regulators. (C) The putative promoter region of *nagR2 ORF2* in the small cluster of naphthalene degradation genes in strain CJ2. Conserved regions are enclosed in boxes.

on expression of *nagR2*, we constructed *nagR2-ORF2*::*lacZ* fusion in strain CJ2. Strain CJM112 was incubated in the presence of naphthalene, salicylate, or gentisase and assayed for β-galactosidase expression (Fig. 6). None of the compounds tested caused detectable induction of β-galactosidase. Given the function of *nagR2* as a repressor, we would expect this gene's physiological cues to be distinctive from LysR-type activators.

DISCUSSION

Although metabolism of naphthalene via gentisate has long been biochemically described (see, for example, references 13 and 18), *Ralstonia* sp. strain U2 was the first genetically characterized bacterium utilizing the gentisate pathway from naphthalene to central metabolites (14, 56). In strain U2, 18 structural genes occur in a single operon under regulatory control of the LysR-type gene, *nagR*.

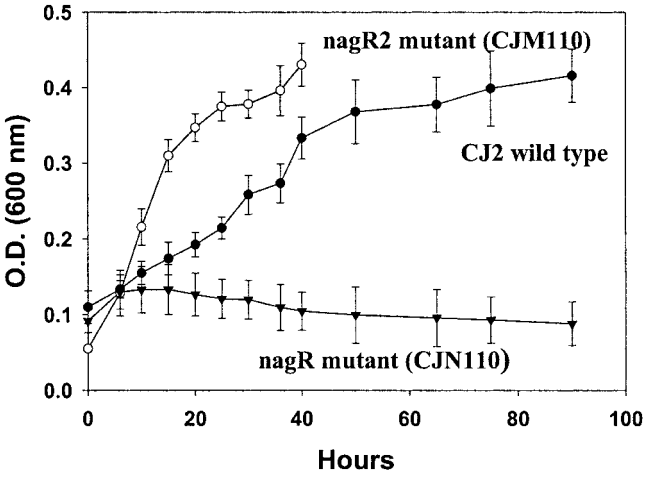


FIG. 5. Characterization of regulatory mutants. (A) Growth of wild-type strain CJ2, the *nagR* mutant CJN110, and *nagR2* mutant CJM110 in MSB-N liquid culture. The growth of each strain was monitored by measuring the optical density (O.D.) at 600 nm of the cultures. The mutant strain CJM110 caused the medium to darken after 45 h. For location of *nagR* and *nagR2*, see Fig. 3.

In the present investigation, the nucleotide sequences in *P. naphthalenivorans* CJ2 were determined for a new complete naphthalene metabolic cluster of genes homologous to those of *Ralstonia* sp. strain U2. Four observations suggest very similar biochemical reactions for naphthalene metabolism in strains U2 and CJ2: (i) the high degree of similarity observed between homologous catabolic genes (Fig. 3 and Table 2); (ii) similar organization of the *nag* promoter region (Fig. 4); (iii) the phenotype of a *nagR* mutant (strain CJN110; Fig. 5) matches that of other naphthalene degraders with mutations in LysR-type regulatory genes (33, 38); and (iv) the expression pattern of a portion of the genes in strain CJ2 resembles that of other naphthalene degraders (Fig. 3).

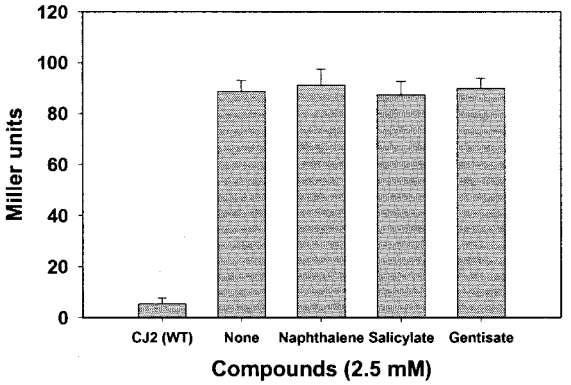


FIG. 6. Reporter assays examining transcriptional activation of *nagR2* in the small gene cluster of *P. naphthalenivorans* strain CJ2. Shown are β-galactosidase activities (Miller units) from the *nagR2-ORF2*::*lacZ* transcriptional fusion in cells grown in MSB liquid media containing naphthalene crystals, salicylate, or gentisate (2.5 mM). Mean values for three independent cultures are shown with standard deviation.

However, the naphthalene catabolic operons in strains U2 and CJ2 also show striking contrasts. The naphthalene catabolic genes of *P. naphthalenivorans* CJ2 were split into large and small clusters (*nagRAaGHAbAcAdBFCQEDJI′ORF1tnpA* and *nagR2ORF2I″KL*). Accompanying this operon division was duplicated functionality of two genes [salicylate-5-hydroxylase (*nagGH* and *ORF2*) and gentisate 1,2-dioxygenase (*nag I′ and nag I″*)] and added MarR-type regulation to the small gene cluster. Furthermore, strain U2's three ORFs (*nagY* [putatively involved in chemotaxis] and *nagM* and *nagN* [the latter two have no known function) (56) were absent in strain CJ2. Operon rearrangements and regulation are discussed below.

The presence of two additional genes (*ORF2* and *nagI*) and two regulators may be physiologically advantageous to the host. It has been suggested that routine gene regulatory mechanisms allow cells to adjust their metabolism within a modest range of conditions. When extreme conditions (such as competition for resources) cannot be accommodated by existing genetic systems, adaptation may be manifest as an increase in gene copy number or alterations in regulatory systems (15, 47). *P. naphthalenivorans* strain CJ2 was discovered by using a field-based stable isotope probing (SIP) procedure in naphthalene-contaminated freshwater sediment (21). Because ecological fitness is implicit in SIP-based identification of active microorganisms, it would be expected that the genetic basis of the likely fitness determinant (metabolizing naphthalene in situ in sediments at a coal tar contaminated site) might be novel. Thus, a distinctive naphthalene operon structure for ecologically fit strain CJ2 was not unexpected. However, it is not yet possible to attribute ecological fitness to a specific constellation of genetic traits because contaminated field sediments feature unknown selective pressures (complex populations and uncharacterized physiological conditions). Recent reports of abundant *nag*-related genes being expressed in contaminated freshwater sediments (10, 21, 52) do provide a strong suggestion that microorganisms carrying *nag* genes may be broadly distributed and ecologically important.

Transcriptional activation plays a prominent role in regulatory control of catabolic pathways in pseudomonads. As summarized by van der Meer (47), XyIS (a member of the AraC family of activator proteins) activates transcription in the TOL system after binding to the effector molecule, benzoate. Furthermore, all LysR-type regulators (]e.g., NahR [naphthalene], CatR/CatM [catechol/benzoate], TfdS/TfdR [2,4-dichlorophenoxyacetic acid], TcbR [trichlorobenzene], ClcR [chlorocatechol], and PcpR [pentachlorophenol]) exert positive control. In contrast, the MarR family of transcriptional regulators comprises a subset of winged helix DNA-binding proteins that act as repressors (50). In *E. coli*, the *marRAB* operon is a regulatory locus that controls multiple antibiotic resistance (46). However, in other bacteria (e.g., *Deinococcus radiodurans*, *Acinetobacter* spp., and *Pseudomonas* spp.) *marR*-type regulators, or related homologous domains, control catabolic functions such as dissimilation of hydroxycinnamic acid (D. Parke and N. Ornston, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. 2003, abstr. K-112, p. 103, 2003) and nitrobenzene (32). Indeed, the MarR family is prominent for its "phenolic-sensing capabilities" involved in "environmental, surveillance of aromatic compounds" (50). Here we reported finding the MarR-type regulator, *nagR2*, in the small gene cluster of strain CJ2. The phenotypic impact of *nagR2* was demonstrated when a *nagR2* mutant showed accelerated growth on naphthalene, confirming that the NagR2 acts as a negative regulator (Fig. 5). Moreover, a *nagR2-ORF2*::*lacZ* fusion experiment showed that the expression of second cluster was not induced by LysR-type inducers (naphthalene, salicylate, or gentisate) (Fig. 6). Thus, it can be concluded that the regulation of the small gene cluster in strain CJ2 is novel: the archetypal positive LysR-type control has been replaced by negative MarR-type control. Additional experiments are required to explain potential physiological advantages (if any) of the replacement.

The "modular" or "mosaic" nature of catabolic operons is well recognized (5, 28, 31, 47, 48, 51). It is clear that variations in catabolic capabilities in prokaryotes have evolved and continue to evolve via a series of acquisitions and rearrangements at DNA scales, ranging from a few nucleotides (e.g., transition or inversion) to many kilobases (e.g., gene transfer, duplication, or deletion) (47). Documenting the mechanism of operon development is not facile. We must rely upon retrospective inspection of nucleotide sequences for traits that include G+C content, codon usage, identity of noncoding homologous DNA regions, and remnants of transposons or IS elements (see, for example, reference 5). These sometimes allow historical steps in the development of operons and entire plasmids to be inferred (9, 17, 26). As demonstrated by Fuenmayor et al. (14), cloning and expression assays can also be insightful and have led to the suggestion that the *nagGH* (salicylate mono-oxygenase) functionality in strain U2 developed by insertion of *nagGH* within the otherwise continuous functional cluster *nagAaAbAcAd*.

Several clues about the potential origin of strain CJ2's peculiar operon structure have emerged. Three observations (below) support the hypothesis that horizontal gene transfer occurred from *Azoarcus* to strain CJ2.

(i) Gene insertion has occurred. In *Ralstonia* sp. strain U2 (which carries a contiguous complete *nag* operon), the order of genes is *nagAaGHAbAcAdBFCQEDJIKLMN*. Relative to strain U2, strain CJ2's small operon features a two-gene insertion (*nagR2* and *ORF2*) at the 5′ end of a duplicated *nagI′*.

(ii) Gene sequence similarities suggest transferred genes originated in *Azoarcus*. BLAST searching and nucleotide analyses of three loci show *Azoarcus* sp. strain EbN1 to be the closest match to genes in strain CJ2's catabolic operons. The *ORF2* gene (putative salicylate 5-hydroxylase) in the second gene cluster exhibited 89% amino acid identity to its homologue in *Azoarcus* sp. strain EbN1. Also, in the region just beyond the putative terminator in the large gene cluster (Fig. 4A), putative transposase-related ORFs, *tnpA′* and *istB*, exhibited 56 and 65% amino acid identity, respectively, with their homologues in *Azoarcus* sp. strain EbN1.

(iii) MarR in *Azoarcus*. Schuehle et al. (40) have shown that the genes encoding aerobic metabolism of 2-aminobenzoate by *Azoarcus evansii* include a MarR-type regulator. Thus, there is precedent for MarR control of catabolism in *Azoarcus*.

The circumstantial evidence presented above suggests that that the two-gene insert, *nagR2ORF2*, and other operon rearrangements in strain CJ2 may have originated in a *Azoarcus*-like host. The mechanism of horizontal gene transfer between the two cell lines and the specific mobile genetic elements involved remain unclear. Such horizontal transfer of aromatic

degradation and regulatory genes (3, 19, 53) may have been enhanced in the naphthalene-contaminated environment from which strain CJ2 was isolated.

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