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The Naphthalene Catabolic (*nag*) Genes of *Ralstonia* sp. Strain U2 Are an Operon That Is Regulated by NagR, a LysR-Type Transcriptional Regulator

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In *Ralstonia* sp. strain U2, the *nag* catabolic genes, which encode the enzymes for the pathway that catabolizes naphthalene via the alternative ring cleavage gentisate pathway, are transcribed as an operon under the same promoter. *nagR*, which encodes a LysR-type transcriptional regulator, is divergently transcribed compared to the *nag* catabolic genes. A 4-bp frameshift deletion in *nagR* demonstrated that NagR is required for expression of the *nag* operon. The transcriptional start of the *nag* operon was mapped, and a putative −10, −35 σ^{70}-type promoter binding site was identified. Further upstream, a site proximal to the promoter was identified as a site that has bases which have been found to be conserved in the activator-binding motif of other naphthalene pathways. Transcriptional fusion studies demonstrated that NagR regulates the expression of the *nag* operon positively in the presence of salicylate and to a lesser extent in the presence of 2-nitrobenzoate. Mutation of the LysR-type activator-binding motif in the *nag* promoter-proximal region resulted in a loss of inducibility of a *lacZ* reporter gene transcriptionally fused to *nagAa*, the first gene of the operon. However, other mutations in the region increased the effectiveness of salicylate as an inducer.

The *nag* genes of *Ralstonia* sp. strain U2 code for the enzymes of the alternative pathway for the catabolism of naphthalene, which converts naphthalene to fumarate and pyruvate via salicylate (2-hydroxybenzoate) and gentisate (2,5-dihydroxybenzoate), and they are organized in a continuous sequence of adjacent genes (8, 28, 29). This is in contrast to the classical naphthalene pathway enzymes, which are encoded on two separate operons, the upper-pathway operon (genes *nahAa* to *nahF*) for the conversion of naphthalene to salicylate and the lower-pathway (or meta-pathway) operon (genes *nahG* to *nahM*) for the conversion of salicylate to acetyl coenzyme A and pyruvate via catechol (5, 27).

Transcriptional control of the classical naphthalene pathway is regulated by NahR, a regulator protein belonging to the LysR-type family of transcriptional regulators (23). NahR is responsible for the regulation of both *nah* operons (21, 22), and the gene that encodes it is located upstream of and is transcribed divergently from *nahG*, the first gene of the meta-pathway operon (27). This gene arrangement has been found in several different classical naphthalene genes cloned from different bacteria (3, 4, 19, 21; GenBank accession number AF491037). The *nag* pathway in *Ralstonia* sp. strain U2 also contains a putative regulator gene, *nagR*, which has high sequence similarity to *nahR*. In contrast to *nahR*, this gene is located upstream of and is divergently transcribed from *nagAa* (28).

In the *nah* system, NahR is expressed constitutively at low levels (20) and binds to promoter-proximal target DNA, regardless of the presence of salicylate. Transcription is activated when the inducer, salicylate (1, 27), binds to NahR and activates both the *nah* upper- and lower-pathway operons by relieving DNA bending at the promoter sites (10, 24). Binding of NahR to its target DNA also appears to autoregulate its own expression by repressing transcription of *nahR* that is transcribed divergently from *nahG* (24). The DNA to which the regulator protein binds is conserved in various naphthalene degradation pathways in *Pseudomonas* (4, 19, 22) (GenBank accession number AF491307), and a similar motif is found upstream of the salicylate hydroxylase gene (*salA*) in *Acinetobacter* sp. strain ADP1, which is also regulated by a NahR homolog, SalR (13).

In this study we investigated regulation of the *nag* genes in *Ralstonia* sp. strain U2. We found that *nagR* is the regulatory gene that controls *nag* gene expression, and we demonstrated the operonic structure of the *nag* genes and determined the inducers. The location of the NagR-binding motif was also probed by examining the effects of some designed mutations on expression.

MATERIALS AND METHODS

Strains and plasmids. The plasmids and bacterial strains used in this study are listed in Table 1.

Chemicals and media. Aromatic substrates were obtained from Sigma-Aldrich Co. Luria-Bertani (LB) medium (20) was used to cultivate bacteria unless noted otherwise. For growth on minimal medium, single carbon sources were added to the minimal salts medium (2) at the following concentrations: salicylate, 2.5 mM; and succinate, 10 mM. Aromatic compounds for induction experiments were added to a final concentration of 1 mM. Where appropriate, ampicillin was added at a concentration of 100 μg/ml, kanamycin and spectinomycin were added at a concentration of 50 μg/ml, and tetracycline was added at a concentration of 25 μg/ml.

DNA manipulation. Unless indicated otherwise, standard methods for DNA manipulation were used (20). Plasmid DNA was prepared from *Escherichia coli* strains by using CONCERT rapid plasmid miniprep systems (Gibco BRL). DNA fragments were recovered from agarose gels by using Qiaquick columns (Qiagen).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Reference or source
Strains		
Ralstonia sp. strain U2	Wild-type naphthalene degrader containing plasmid pWWU2	7
P. putida PaW340	Trp^- Str^r plasmid-free derivative of *P. putida* mt-2	11
Plasmids		
pUC19	Vector; Ap^r; multiple cloning site in *lacZ*α	27
pKOK6.1	Ap^r; promoterless *lacZ*	15
pRK415	Tc^r; mobilizable broad-host-range vector; origin of replication (*oriV*) and origin of transfer (*oriT*) of RK2	14
pRK415-ZP	pRK415 with *lacZ*-Km cassette from pKOK6.1 inserted into *Pst*I site, with the start of *lacZ* adjacent to the *Sal*I site of pRK415	This study
pWWF24	8.3-kb *Xho*I fragment from strain U2 carrying the naphthalene dioxygenase region cloned in the *Xho*I site of pBluescript II SK(+)	8
pWWF116	pWWF24 with a 4-bp deletion at the *Nsi*I site within *nagR*	This study
pWWF119	pGEM-T Easy with RACE fragment created by using P_{nag} as the template	This study
pWWF120F	1.6-kb wild-type fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites	This study
pWWF120R	Like pWWF120F, but with restriction sites reversed, creating a 1.6-kb fragment in the opposite direction	This study
pWWF121	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having bases −68 to −70 of P_{nag} deleted	This study
pWWF122	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having bases −60 to −62 of P_{nag} deleted	This study
pWWF123	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having bases −64 to −66 of P_{nag} deleted	This study
pWWF124	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having bases −60 to −71 of P_{nag} deleted	This study
pWWF125	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having a C-to-T substitution at base −70 of P_{nag}	This study
pWWF126	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having a G-to-A substitution at base −61 of P_{nag}	This study
pWWF128	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having C-to-T and G-to-A substitutions at bases −51 to −53 of P_{nag}	This study
pWWF129	1.6-kb fragment in pUC18 containing *nagR* and 347 bp of *nagAa* PCR amplified at designed *Xba*I and *Kpn*I restriction sites and having C-to-T and G-to-A substitutions at bases −77 to −79 of P_{nag}	This study
pWWF120F-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF120 inserted into pRK415-ZP	This study
pWWF120R-Z	1.6-kb *Kpn*I/*Xba*I fragment from pWWF120 inserted into pRK415-ZP	This study
pWWF121-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF121 inserted into pRK415-ZP	This study
pWWF122-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF122 inserted into pRK415-ZP	This study
pWWF123-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF123 inserted into pRK415-ZP	This study
pWWF124-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF124 inserted into pRK415-ZP	This study
pWWF125-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF125 inserted into pRK415-ZP	This study
pWWF126-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF126 inserted into pRK415-ZP	This study
pWWF128-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF128 inserted into pRK415-ZP	This study
pWWF129-Z	1.6-kb *Xba*I/*Kpn*I fragment from pWWF129 inserted into pRK415-ZP	This study

RT-PCR. Cells were grown on minimal medium containing salicylate or succinate to a density of about 10^8 cells/ml. Total RNA was prepared from 10 ml of the culture with RNeasy Mini columns (Qiagen), with elution in 50 μl of water. The RNA was treated with DNase I to remove any genomic DNA contamination by incubation with 1 U of RNase-free DNase (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 was cleaned by passage through an RNeasy Mini column prior to use in a reverse transcriptase PCR (RT-PCR). The RT-PCR 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 (Table 2). PCR were performed with 50-μl mixtures containing 0.5 μg of total RNA, 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 50 μM, 1 mM $MgSO_4$, 5 U of avian myeloblastosis virus RT, and 5 U of *Tfl* DNA polymerase in the reaction buffer supplied by the manufacturer. After reverse transcription at 48°C for 1 h, the reaction mixtures were heated to 94°C for 2 min and subjected to 40 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 68°C. Negative control reactions to eliminate the possibility that residual DNA was amplified were performed in the same way, except that the RT was omitted from the reaction mixtures.

Mapping the *nagAa* transcription start site. The start of transcription of the *nagAa* gene was mapped by using a kit for rapid amplification of cDNA ends (RACE) as recommended by the manufacturer (5′ RACE System; Invitrogen Life Technologies).

Total RNA was prepared from *Ralstonia* sp. strain U2 by using a Qiagen RNeasy Mini kit. DNA contamination was removed by digestion with 10 U of RQ1 RNase-free DNase (Promega) for 30 min at 37°C. Total RNA (2 μg) in a 20-μl reaction sample was reverse transcribed by using the manufacturer's in-

TABLE 2. Primers used in this study

Primer	Sequence (5′ → 3′)	Relevant information
Primers used in RT-PCR[a]		
nagRT-1	ATCAGCGGCCCCCTCGGAACGGCTTATCT	2.802 to 4.970 kb
nagRT-2	GTGCGCACGATCACGTCTCGGTAATAGC	
nagRT-3	CGCCACGGCAACGCCAAGGACTTCTTCT	3.596 to 5.786 kb
nagRT-4	GAGCCAAAGCCCCAGCCGTGGTAACTGC	
nagRT-5	TGCAGAGGCCCCCACGCTTGTCGATTATCT	5.916 to 7.915 kb
nagRT-6	CCGCCGCCGGCCGCATAGGAAG	
nagRT-7	CGGCGGCGGCGGCTCTTGCTACAT	7.907 to 10.230 kb
nagRT-8	GTGGCCCAGGCCCTGATCACCGGTTAGAAA	
nagRT-9	GCCTGCGGCCAAGCGCCTCAATCATCT	10.359 to 12.977 kb
nagRT-10	TCGCTCCCTGGCTGCCGGGTAATACAGTCC	
nagRT-11	CGGGTGAATGCCGGACTGTATTAC	12.936 to 14.966 kb
nagRT-12	GCCGGGCTTGAGCATCAGGGCGTAGAAG	
nagRT-13	GAAACGGGTGACGACGCCGAGAACATCC	14.907 to 15.827 kb
nagRT-14	CAGGTCGCCGGGTTGCAAGTGGTAGTAGGT	
nagRT-15	GCGGCCGCTACAGCTTTGGCGACACA	16.401 to 18.452 kb
nagRT-16	CGGGGTAGTCTGCACCGAACTTTTGCTCTG	
Primers used for overlap extension mutagenesis[b]		
121-3	CATTATTGCTGGTGATTTTAACTATCA	Delete positions −68 to −70
121-4	AATCACCAGCAATAATGGTGAGGCATCATG	
122-3	CATTATTCATGCTGGTTTTAACTATC	Delete positions −60 to −62
122-4	GTTAAAACCAGCATGAATAATGGTGAG	
123-3	CATTATTCATGGTGATTTTAACTATC	Delete positions −64 to −66
123-4	GTTAAAATCACCATGAATAATGGTGAGGCA	
124-3	CATTATTTTTAACTATCAGACTTGA	Delete positions −60 to −71
124-4	GTCTGATAGTTAAAAATAATGGTGAGGCA	
125-3	CATTATTTATGCTGGTGATTTTAACTAT	C-to-T substitution at position −70
125-4	GTTAAAATCACCAGCATAAATAATGGTGAGGCA	
126-3	CATTATTCATGCTGGTAATTTTAACTATCA	G-to-A substitution at position −61
126-4	GTTAAAATTACCAGCATGAATAATGGTGAG	
128-3	GTGATTTTAATGATCAGACTTGATCTATAGCG	CTA-to-TGC substitution at position −51 to −53
128-4	TCAAGTCTGATCATTAAAATCACCAGCATG	
129-3	GATGCCTCGTTATTATTCATGCTGGTGA	ACC-to-GTT substitution at position −77 to −79
129-4	GAATAATAACGAGGCATCATGGATCTGCGCGA	

[a] The region of the *nag* operon amplified is indicated relative to the gene map shown in Fig. 1 and GenBank accession number AF036940.
[b] The positions mutated are the positions upstream of the start of transcription (Fig. 2A).

structions and a *nagAa*-specific primer, RACEAa-1 (5′-GGGCGGACGCATT CGGGCGTGAAC-3′), which anneals 428 bp downstream of the *nagAa* translational start site. A homopolymeric tail was added to the 3′ end of the synthesized cDNA (corresponding to the 5′ end of *nagAa* mRNA that was reverse transcribed in the reaction described above) by using terminal transferase and dCTP by incubation at 37°C for 10 min as described in the RACE kit protocol.

The dC-tailed cDNA was PCR amplified by using the abridged anchor primer provided with the kit (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGG IIG-3′) and another *nagAa*-specific primer, RACEnagAa-2 (5′-CAGGGCTGA ACTCAAGCGGTTTGGCCAG-3′), which anneals 385 bp downstream of the *nagAa* translational start site. The PCR product obtained was then PCR amplified with primer RACEnagAa-2 and the abridged universal amplification primer provided with the kit (5′-GGCCACGCGTCGACTAGTAC-3′) in order to eliminate any nonspecific PCR products from the first reaction. The amplified products were purified by using a Qiaquick PCR clean-up column (Qiagen) and were cloned into the pGEM-T Easy vector (Promega). Sequences of the cloned 5′ RACE inserts were determined by MWG-Biotech AG (Ebersberg, Germany).

Preparation of cell extracts. Cells were harvested by centrifugation, washed with 100 mM phosphate buffer (pH 7.4), and stored as pellets at −20°C. Cell extracts were prepared by suspending frozen pellets in ice-cold 100 mM phosphate buffer (pH 7.4), disrupting them with a French pressure cell (SLM Instruments, Inc., Urbana, Ill.), and centrifuging them at 120,000 × *g* for 30 min at 4°C. Each supernatant was stored frozen as 1-ml aliquots at 20°C. The total protein concentrations of the cell extracts were determined by using a Microprotein PR kit (Sigma Diagnostics, Inc.).

Construction of *nagR-nagAa*::*lacZ* fusions. The promoterless *lacZ*-Km^r cassette from pKOK6.1 (15) was inserted into the multiple cloning site of pRK415 by digestion with *Pst*I (which excised the cassette on a single fragment), creating pRK415-ZP. The orientation of the cassette was such that the *Kpn*I site and an *Xba*I site were upstream of the *lacZ* translational start.

A region of DNA which included all of *nagR* and the first 347 bp of *nagAa* was PCR amplified by using Proofstart DNA polymerase (Qiagen) according to the manufacturer's instructions together with primers Nag-kpn (5′-GGCAGTTCG GTACCGCTGCCTACGCACAAGAC-3′) and Nag-xba (5′-AGGCGCCTCTA GACATGGGTGGCTTCGTC-3′), which contain engineered *Kpn*I and *Xba*I restriction endonuclease sites (underlined), respectively. For construction of *nagR-nagAa*::*lacZ* fusions in which the region upstream of the *nag* promoter contained a mutation in the *lysR*-type activator-binding motif, complementary overlapping primers were designed that incorporated mutations in the region (Table 2). These primers were used to amplify a region that included all of *nagR* and the first 347 bp of *nagAa* by overlap extension PCR (9); Nag-kpn and Nag-xba were used as the flanking primers. The amplified fragment was incubated at 72°C for 10 min in the presence of *Taq* DNA polymerase, polymerase buffer, and 0.5 mM dATP and was ligated into the pGEM-T Easy TA cloning vector (Promega Inc.). Subsequently, the wild type and the mutated amplified fragments were subcloned from pGEM-T Easy into pRK415-ZP on a *Kpn*I/*Xba*I fragment. This cloning created a *nagR-nagAa*::*lacZ* gene fusion in pRK413, in which the transcription of *nagAa* runs into *lacZ*. The amplified fragments cloned into pGEM-T Easy were sequenced to confirm that the mutation designed had been created and that no PCR errors occurred during amplification.

Triparental mating for transfer of pRK415 constructs into PaW340. The donor, the recipient, and *E. coli* HB101 carrying pRK2103 as a helper plasmid were grown in LB medium until they reached an optical density at 600 nm of 0.6. Then 500-μl portions of the three cultures were mixed and centrifuged, and the pellets were washed in minimal medium. The pellets were finally resuspended in 50 μl of minimal medium and dispensed onto a sterile nylon membrane (Bio-Rad) laid on the surface of an LB medium plate. Following incubation overnight

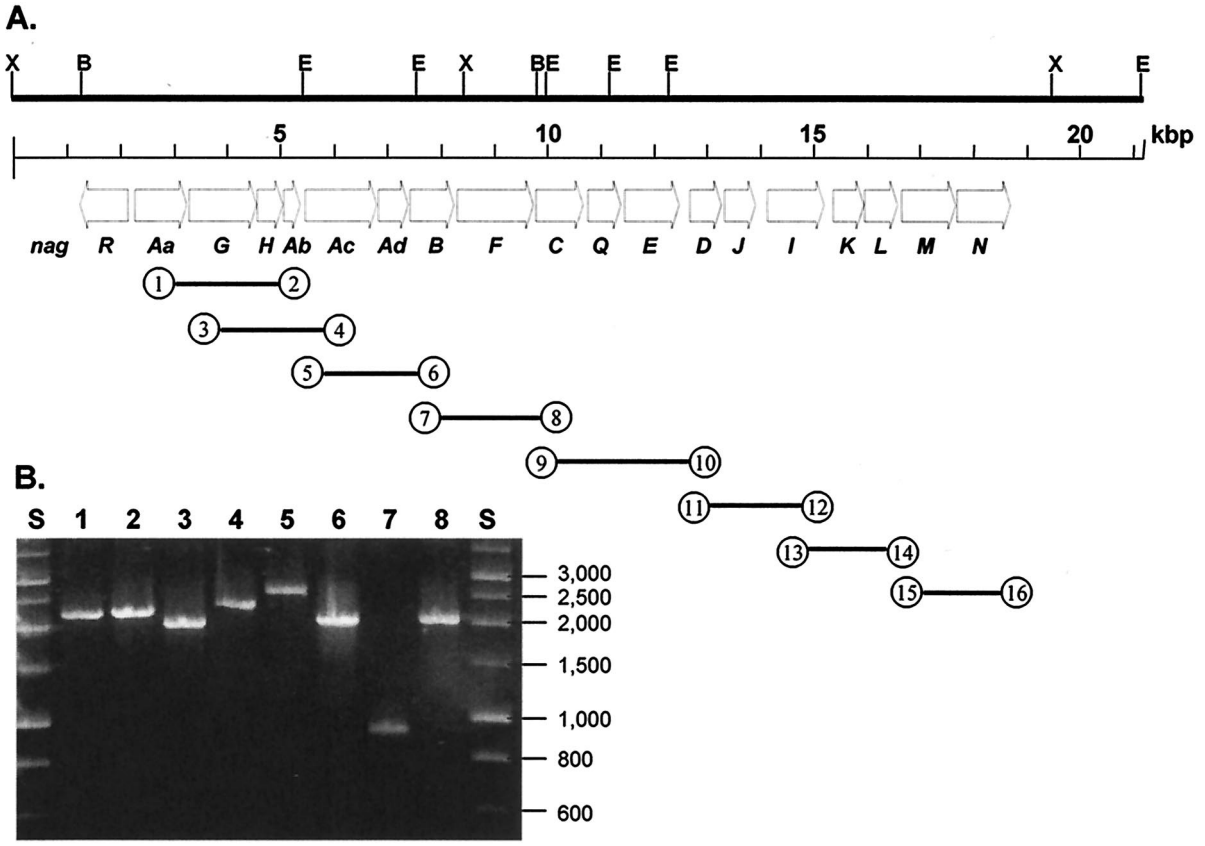

FIG. 1. (A) Physical map of the *nag* genes and the locations of RT-PCR amplicons, obtained by using mRNA purified from salicylate-grown *Ralstonia* sp. strain U2 as the template. The numbers in circles indicate the primers used for each amplification and correspond to the nagRT primers described in Table 2. Important restriction sites are indicated as follows: X, *Xho*I; B, *Bam*HI; E, *Eco*RI. (B) Agarose gel electrophoresis of RT-PCR products amplified from *Ralstonia* sp. strain U2 grown on salicylate. The sizes of molecular size markers in lanes S (HyperLadder I; Bioline, London, United Kingdom) are indicated on the right (in base pairs). Lane 1, nagRT-1 plus nagRT-2 (expected size, 2.168 kb); lane 2, nagRT-3 plus nagRT-4 (2.190 kb); lane 3, nagRT-5 plus nagRT-6 (1.999 kb); lane 4, nagRT-7 plus nagRT-8 (2.323 kb); lane 5, nagRT-9 plus nagRT-10 (2.582 kb); lane 6, nagRT-11 plus nagRT-12 (2.030 kb); lane 7, nagRT-13 plus nagRT-14 (0.920 kb); lane 8, nagRT-15 plus nagRT-16 (2.051 kb). No detectable products were obtained in control reactions with each pair of primers from which RT had been omitted or in reactions carried out with succinate-grown cells (data not shown).

at 30°C, the cells were washed off the filter into 2 ml of minimal medium, and appropriate dilutions were spread onto selective medium. Donor-only and recipient-only controls were treated in the same way.

β-Galactosidase assays. Cultures were grown overnight in 5 ml of minimal media containing 10 mM succinate with or without inducer. Cells were lysed with chloroform and sodium dodecyl sulfate, and β-galactosidase activities were determined as described by Miller (17).

DNA sequencing and sequence analysis methods. Nucleotide sequences of both DNA strands were determined by MWG-Biotech Ltd. PCR primers were designed with the aid of the Lasergene software package (DNAStar, Inc., Madison, Wis.).

RESULTS

Inactivation of NagR. To demonstrate the role of NagR as the regulatory protein, a 4-bp frameshift deletion in *nagR* was created by digesting pWWF24 (Table 1) at the unique *Nsi*I site within *nagR* and filling in the overhanging ends with T4 DNA polymerase, creating pWWF116. pWWF24 harbors *nagR* and all of the *nagAaGHAb* genes coding for the salicylate 5-hydroxylase gene products which are responsible for catalytic conversion of salicylate to gentisate. *E. coli*(pWWF24) accumulates gentisate when it is cultured in LB media containing salicylate, which can be detected by the characteristic UV spectrum and by accumulation of a brown color due to autoxidation. No such accumulation of gentisate occurred in *E. coli*(pWWF116) under the same growth conditions. Moreover, spectrophotometric assays of cell extracts of *E. coli* DH5α(pWWF24) showed that there was NADH-linked conversion of salicylate to gentisate, whereas no conversion was detected when extracts of *E. coli* DH5α(pWWF116) were used (data not shown).

Operon structure of *nag* genes. RT-PCR of RNA purified from salicylate-grown *Ralstonia* sp. strain U2 cells amplified products that extended across the boundaries between the *nag* genes (Fig. 1A). The amplified products were analyzed by agarose gel electrophoresis (Fig. 1B). The presence of amplified DNA fragments obtained with each primer pair suggests that all of the *nag* genes are transcribed on the same message. No amplification product was obtained when RT was omitted from the reaction mixture.

Mapping the transcriptional start site of *nagAa*. In order to map the site of initiation of transcription of *nagAa*, 5′ RACE PCR experiments were carried out with RNA extracted from

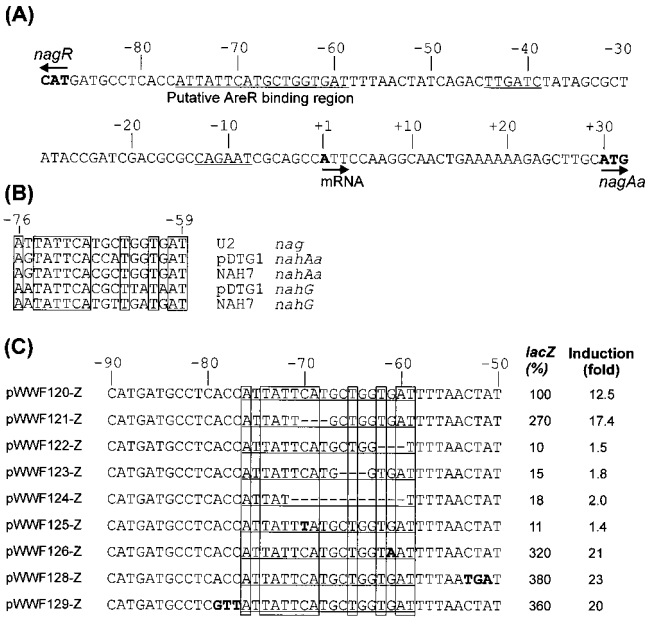

FIG. 2. (A) Schematic representation of the organization of the *nag* promoter region. The arrows indicate the starts of transcription and translation. The transcriptional initiation nucleotide (+1) and the putative −35 and −10 motifs are underlined, as is the putative NagR-binding motif. (B) Alignment of the conserved regions of the upstream regions of operons controlled by NagR and NahR regulators. Conserved residues are enclosed in boxes. The sequence of pDTG1 was obtained from GenBank (accession number AF491307). (C) Plasmids and relevant sequences of the mutations in and around the putative binding domain for NagR. Deletions are indicated by dashes, and substitutions are indicated by boldface type. The wild-type sequence is the sequence of pWWF120-Z. The *lacZ* activity is expressed as a percentage of the salicylate-induced activity of the wild type (pWWF120-Z), and the induction value is the ratio of salicylate-induced activity to uninduced activity.

Ralstonia sp. strain U2 grown on salicylate. The nucleotide sequence of the 5′ RACE PCR product showed that transcription starts at the A located 28 bases upstream of the *nagAa* translational start site (data not shown). Upstream of this transcriptional start site is a putative −10 sequence, CAGAAT, which is separated by 25 bp from a putative −35 sequence, TTGATC (Fig. 2A); these sequences are the best candidates for sequences that resemble the −10 and −35 promoter consensus sequences, TATAAT and TTGACA, respectively (6).

Upstream of this *nagAa* promoter, between bases −59 and −76, is the sequence ATTATTCATGCTGGTGA (Fig. 2A and B). This sequence contains the symmetrical dyad motif $TTCAN_6TGAT$, identified by footprinting as the core of the NahR interaction with both of the naphthalene promoters on plasmid NAH7 (10, 25). Alignment of the 18-bp sequence with the similar sequences upstream of both the naphthalene catabolic operons on plasmids NAH7 and pDTG1 showed that there are 11 conserved bases in all five sequences. This conservation is in the context of very little other homology between the upstream regions of the *nag* and *nah* operons (8).

Determination of coinducers by using a *nagR-nagAa*::*lacZ* fusion. We compared the levels of expression of the *nagR-nagAa::lacZ* fusion constructed in pWWF120F-Z. Attempts to select transconjugants of pWW120F-Z in *Ralstonia* sp. strain U2 were unsuccessful due to the high levels of intrinsic antibiotic resistance, and therefore, the construct was mated into *Pseudomonas putida* PaW340 (Table 1). PaW340(pWWF120F-Z) was grown in the presence of a range of aromatic compounds and assayed for β-galactosidase expression (Table 3). Only salicylate induced expression, and neither naphthalene nor gentisate resulted in detectable induction of *nagAa*. Of the other substituted aromatic compounds tested as potential gratuitous inducers, only 2-nitrobenzoate was marginally able to induce *nagAa* expression. 2-Aminobenzoate, a gratuitous inducer of the classical *nah* operons (1), did not function for the *nag* system.

TABLE 3. β-Galactosidase activities expressed from *nagR-nagAa*::*lacZ* fusions with different aromatic compounds

Inducer[a]	β-Galactosidase activity of PaW340(pWWF120F-Z) (*nagR-nagAa*::*lacZ*)	
	Miller units[b]	% of salicylate-induced culture
None	61 (6.5)	3.6
Salicylate	1,700 (43.2)	100
4-Methylsalicylate	65 (4.8)	3.8
5-Methylsalicylate	61 (3.2)	3.8
4-Chlorosalicylate	64 (1.6)	3.5
5-Chlorosalicylate	59 (1.6)	4.1
Benzoate	70 (6.5)	3.9
Gentisate	67 (9.7)	3.1
Naphthalene	53 (3.2)	3.9
2-Aminobenzoate	66 (4.8)	4.0
5-Aminobenzoate	68 (6.5)	3.8
2-Chlorobenzoate	65 (3.2)	3.7
2-Nitrobenzoate	135 (8.1)	7.9
3-Nitrobenzoate	58 (2.4)	3.5
4-Nitrobenzoate	60 (6.5)	3.2
2-Nitrotoluene	54 (3.2)	3.2
3-Nitrotoluene	56 (2.4)	3.3
4-Nitrotoluene	58 (6.5)	3.4
2,4-Dinitrotoluene	62 (9.7)	3.6
2,6-Dinitrotoluene	57 (5.7)	3.4
Nitrobenzene	58 (6.5)	3.4
Ethyl salicylate	65 (3.2)	3.8

[a] Inducers were present at a concentration of 1 mM.
[b] The values are averages for three independent trials, each conducted in duplicate. The values in parentheses are standard deviations.

Analysis of expression of *nagR-nagAa*::*lacZ* fusions containing mutations in the activator-binding motif. To test whether the region from bp −78 to −59 upstream of the *nag* promoter (Fig. 2A and B) is required for activation of transcription, we compared the salicylate-induced and uninduced β-galactosidase activities in eight strains carrying the *nagR-nagAa*::*lacZ* fusion and designed mutations in or around the putative regulator binding site (Table 4; Fig. 2C). Four mutations, incorporated into plasmids pWWF122-Z, pWWF123-Z, pWWF124-Z, and pWWF125-Z, resulted in failure of the system to express β-galactosidase. In contrast, four other mutations, incorporated into pWWF121-Z, pWWF126-Z, pWWF128-Z, and pWWF129-Z, resulted in a threefold increase in activity compared to the wild-type activity, but none of these mutations was constitutively expressed and all of them remained salicylate inducible.

DISCUSSION

As previously proposed based on the nucleotide sequence (28), the complete *nag* genes in *Ralstonia* sp. strain U2 have

TABLE 4. β-Galactosidase activities expressed from wild-type and mutated *nagR-nagAa*::*lacZ* fusions

Plasmid harbored in PaW340[a]	β-Galactosidase activity (Miller units)[b]	
	Salicylate induced	Uninduced
pRK415-Z	61 (4)	59 (2.4)
pWWF120R-Z	72 (14)	63 (6)
pWWF120F-Z	1,700 (50)	136 (5)
pWWF121-Z	4,530 (230)	260 (10)
pWWF122-Z	180 (13)	120 (7)
pWWF123-Z	250 (20)	140 (5)
pWWF124-Z	306 (24)	150 (4.0)
pWWF125-Z	192 (10.6)	135 (6)
pWWF126-Z	5,400 (170)	260 (14)
pWWF128-Z	6,500 (160)	280 (18)
pWWF129-Z	6,000 (400)	300 (25)

[a] The plasmids contained mutations in the *lysR*-type activator-binding motif as shown in Table 1 and Fig. 2.
[b] The values are averages for three independent trials conducted in duplicate. The values in parentheses are standard deviations.

now been shown to be expressed as a single operon. Particular care was taken in RT-PCR for the *nagD-nagJ-nagI* intergenic regions since it is in one of these regions that a separate promoter for the gentisate pathway might be expected; they are the regions where the similarity to the classical *nah* upper-pathway operon (the 3′ gene of which is *nahD*) ends and the gentisate pathway genes start, but both RT-PCR of the *nagDJI* intergenic regions and the failure to obtain a RACE amplification product with a *nagI*-specific primer indicate that all of the *nag* genes are cotranscribed as an operon. This includes the two downstream genes, *nagMN*, which are homologous to each other but do not appear to determine any enzyme activity in the catabolism of gentisate and for which no function has been described yet (28).

Elevated levels of expression of the pathway genes were measured only when the inducer was salicylate, as observed for other *nah*-like genes (24). The only other gratuitous inducer which we found was 2-nitrobenzoate, which is weakly able to induce the *nag* genes by a factor of two, although the level is 10-fold less than the level at which salicylate induces the operon. Much of the interest in the *nag* operon lies in its similarities and differences with the two other catabolic routes, (i) the classical naphthalene degradative pathway (*nah*) found on plasmids pDTG1 (also known as pWW60-1 [5]) and NAH7 and (ii) the nitroaromatic pathways involving an initial dioxygenase attack (see below).

In the well-established *nah* pathways naphthalene is converted to salicylate by the enzymes encoded by the upper-pathway operon, and the salicylate is converted via catechol and meta (extradiol) cleavage to central metabolites by the enzymes encoded by the lower- or meta-pathway operon (genes *nahG* to *nahM*). On both plasmid pDTG1 and plasmid NAH7 the two operons are separated, but the relative orientations are different, and both operons are regulated by a LysR-type regulator, NahR; the gene that encodes NahR is divergently transcribed from *nahG*, the upstream gene of the meta-pathway operon.

The organization of the *nag* genes has similarities but also significant differences. The *nag* genes that encode enzymes which convert naphthalene to salicylate are homologous to and in the same order as the genes of the *nah* upper-pathway operon, demonstrating that the genes have a common ancestral origin (Fig. 1). The genes differ (i) by the insertion between *nagAa* and *nagAb* of *nagGH* encoding the catalytic subunits of salicylate 5-hydroxylase, which converts salicylate to gentisate, and (ii) by their fusion to create a single operon with the genes for the gentisate pathway. In this study we found that both *nag* and *nah* genes are regulated in the same way, by homologous LysR-type regulatory proteins, but that the genes are located in entirely different relative positions. Whereas the entire intergenic region between *nahR* and *nahG* is relatively conserved for classical *nah*-type genes (19), the corresponding region in the *nag* system exhibits sequence similarity with the classical system only in the region of the putative LysR-type activator-binding motif (8). A few systematic mutations in this motif in the *nag* region resulted in varied responses when induction was with salicylate. Four mutants with mutations in the activator-binding motif, namely, pWWF122-Z and pWWF123-Z (both 3-bp deletions), pWWF124-Z (12-bp deletion), and pWWF125-Z (a single substitution), exhibited major decreases in gene expression, reducing the level of induction to less than twofold, compared with about 12-fold reduction for the wild-type (Fig. 2C; Table 4); all of the mutations involve bases which are conserved in the *nah* and *nag* systems (Fig. 2B). The likely effect of these changes is either that NagR is unable to bind to the mutant motif or that binding still occurs but the mutations render NagR unable to relieve the DNA bending which results in activation of transcription. In a corresponding study with the *nah* genes of plasmid NAH7, performed with only substitution mutants, Schell and Poser (24) found that there were only two bases in the putative NahR-binding site which, when changed, produced an equivalent uninducible phenotype, corresponding to bases −72 and −73 (Fig. 2B).

Surprisingly four of our mutations significantly increased the specific activity of induced cells without resulting in a major increase in the activity of uninduced cells, thus making salicylate a more effective inducer. The mutation in pWWF121-Z deleted three bases of the proposed NagR-binding motif (bases −68 to −70) and changed the dyad motif (10) from $TTCAN_6TGAT$ to $TTATN_6TGAT$. This removed a guanine, thought to be an obligatory element of the dyad (10), from the 5′ end of the motif, and yet it made salicylate a more potent inducer. Similarly, a G-to-A substitution mutation in pWWF126-Z at position −61 also increased the effectiveness of salicylate as an inducer, and it removed the guanine from the 3′ end of the dyad motif. However, this base is not an absolutely conserved base since in the pDTG1 *nahG* promoter-proximal region it is an A, not a G (Fig. 2B), so it is perhaps not surprising that the change from one base to the other merely modulated activity rather than destroying it. More surprising were the results obtained with mutants pWWF128-Z and pWWF129-Z, which were included as controls and were expected to behave like the wild type, pWWF121-Z. In these mutants the substitutions were outside the conserved binding motif, but they caused increased levels of gene expression in induced cultures and, to a lesser extent, in uninduced cultures. The expression was not constitutive, and binding of and transcription initiation by the regulator protein NagR still had to occur, but these processes occurred more effectively than they occurred with the wild type. This suggests that the base changes which we introduced

at these positions produced additional or alternative promoter or binding sites or that the composition of the DNA on either side of the NagR binding site had an important modulating effect on the initiation of transcription.

So far, we have had little success in trying to purify NagR and have been unable to carry out either footprinting assays or, on a less discriminating level, gel shift assays. Further productive examination of this region requires obtaining purified protein.

We investigated the inducing role of nitro-substituted aromatic compounds because of the clear similarity between the upstream end of the *nag* operon and the genes encoding the oxygenases involved in the first attack upon nitrobenzene (*nbz*) (16), 2-nitrotoluene (*ntd*) (18), and 2,4-dinitrotoluene (*dnt*) (12). In all of these cases, the four genes (*AaAbAcAd*) encoding the nitroaromatic dioxygenase are homologs of naphthalene dioxygenase genes, but the sequences are more similar to the sequences of the *nag* genes than to the sequences of the *nah* genes (8, 12, 29). Additionally, between the *Aa* and *Ab* genes they all contain residual sequences homologous to *nagGH*, the genes encoding salicylate 5-hydroxylase (8, 29). This fact has been interpreted as showing that the nitroaromatic dioxygenases evolved by recruitment of *nag*-like naphthalene dioxygenase genes carrying the *nagGH* insert but subsequently acquired inactivating mutations in the *nagGH* DNA but the *nagGH* DNA was not completely deleted (12, 18, 29). Further evidence of the close relationship of the nitroaromatic dioxygenase genes with the corresponding *nag* genes rather than the *nah* genes, which has particular relevance to this study, includes (i) the presence of a *nagR* homolog (>98% amino acid identity) upstream of and divergently transcribed from the dioxygenase genes in all the nitroaromatic pathways (12, 16, 18), (ii) the 99% identity of the nucleotide sequences of the intergenic regions between the *Aa* genes and the divergently transcribed *R* genes, and (iii) the fact that salicylate still is able to induce the *nbz* genes, although various nitroaromatic compounds can also induce their expression (R. E. Parales, personal communication). It is possible that the evolution of the nitroaromatic pathways from *nag*-like ancestral genes occurred because of a combination of the broad specificity of the dioxygenase and the ability of the induction mechanisms of the *nag*-like systems to adjust to nitroaromatic compounds as inducers. Our data give some minimal credence to this hypothesis because of the induction by 2-nitrobenzoate, but none of the nitro-substituted hydrocarbons tested had any effect as an inducer (Table 3); additional stronger evidence is required to explain why a *nag*-like system is the apparent ancestor of the nitroaromatic pathways.

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REFERENCES

1. **Barnsley, E. A.** 1975. Induction of enzymes of naphthalene metabolism in *Pseudomonas* by salicylate and 2-aminobenzoate. J. Gen. Microbiol. **88:**193–196.
2. **Bauchop, T., and S. R. Elsden.** 1960. The growth of microorganisms in relation to energy supply. J. Gen. Microbiol. **23:**457–469.
3. **Bosch, R., E. Garcia-Valdes, and E. R. Moore.** 1999. Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. Gene **236:**149–157.
4. **Bosch, R., E. Garcia-Valdes, and E. R. Moore.** 2000. Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. Gene **245:**65–74.
5. **Cane, P. A., and P. A. Williams.** 1986. A restriction map of the catabolic plasmid pWW60–1 and the location of some of its catabolic genes. J. Gen. Microbiol. **132:**2919–2929.
6. **deHaseth, P. L., M. L. Zupancic, and M. T. Record, Jr.** 1998. RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. J. Bacteriol. **180:**3019–3025.
7. **Fuenmayor, S. L., and V. Rodriguez-Lemoine.** 1992. Characterization of polycyclic aromatic hydrocarbons degradative soil *Pseudomonas.* Acta Cient. Venez. **43:**349–354.
8. **Fuenmayor, S. L., M. Wild, A. L. Boyes, and P. A. Williams.** 1998. A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J. Bacteriol. **180:**2522–2530.
9. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
10. **Huang, J. Z., and M. A. Schell.** 1991. In vivo interactions of the NahR transcriptional activator with its target sequences. Inducer-mediated changes resulting in transcription activation. J. Biol. Chem. **266:**10830–10838.
11. **Jeenes, D. J., and P. A. Williams.** 1982. Excision and integration of degradative pathway genes from TOL plasmid pWW0. J. Bacteriol. **150:**188–194.
12. **Johnson, G. R., R. K. Jain, and J. C. Spain.** 2002. Origins of the 2,4-dinitrotoluene pathway J. Bacteriol. **184:**4219–4232.
13. **Jones, R. M., V. Pagmantidis, and P. A. Williams.** 2000. *sal* genes determining the catabolism of salicylate esters are part of a supraoperonic cluster of catabolic genes in *Acinetobacter* sp. strain ADP1. J. Bacteriol. **182:**2018–2025.
14. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
15. **Kokotek, W., and W. Lotz.** 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene **84:**467–471.
16. **Lessner, D. J., G. R. Johnson, R. E. Parales, J. C. Spain, and D. T. Gibson.** 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765 Appl. Environ. Microbiol. **68:**634–641.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Parales, J. V., A. Kumar, R. E. Parales, and D. T. Gibson.** 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. Gene **181:**57–61.
19. **Park, W., P. Padmanabhana, S. Padmanabhan, G. J. Zylstra, and E. L. Madsen.** 2002. *nahR*, encoding a LysR-type transcriptional regulator, is highly conserved among naphthalene-degrading bacteria isolated from a coal tar waste-contaminated site and in extracted community DNA. Microbiology **148:**2319–2329.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Schell, M. A.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. Gene **36:**301–309.
22. **Schell, M. A.** 1986. Homology between nucleotide sequences of promoter regions of *nah* and *sal* operons of NAH7 plasmid of *Pseudomonas putida*. Proc. Natl. Acad. Sci. USA **83:**369–373.
23. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
24. **Schell, M. A., and E. F. Poser.** 1989. Demonstration, characterization, and mutational analysis of NahR protein binding to *nah* and *sal* promoters. J. Bacteriol. **171:**837–846.
25. **Schell, M. A., and P. E. Wender.** 1986. Identification of the *nahR* gene product and nucleotide sequences required for its activation of the *sal* operon. J. Bacteriol. **166:**9–14.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains—nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
27. **Yen, K.-M., and I. C. Gunsalus.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA **79:**874–878.
28. **Zhou, N.-Y., S. L. Fuenmayor, and P. A. Williams.** 2001. *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. J. Bacteriol. **183:**700–708.
29. **Zhou, N. Y., J. Al-Dulayymi, M. S. Baird, and P. A. Williams.** 2002. Salicylate 5-hydroxylase from *Ralstonia* sp. strain U2: a monooxygenase with close relationships to and shared electron transport proteins with naphthalene dioxygenase. J. Bacteriol. **184:**1547–1555.