Effector Specificity Mutants of the Transcriptional Activator NahR of Naphthalene Degrading *Pseudomonas* Define Protein Sites Involved in Binding of Aromatic Inducers*

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This work reports a genetic analysis of the interactions between NahR, the LysR-type regulator of the NAH operons for biodegradation of naphthalene in *Pseudomonas*, and its aromatic effectors. Six mutants encoding NahR variants responsive to salicylate analogs such as benzoate, which is not an inducer for the wild type regulator, were isolated with a polymerase chain reaction-based saturation mutagenesis protocol. Most mutants displaying a specific change of effector profile bore single amino acid substitutions within a short protein segment of 60 residues located at the central portion of the NahR sequence. Some of the protein variants exhibited an increased affinity for salicylate and also for otherwise suboptimal effectors, with apparent K'_s values 5–100-fold lower than those of the wild type NahR protein. In addition, all mutants were activated by inducers bearing novel substituents at positions 1 or 2 of the aromatic ring and displayed also an enhanced tolerance to changes at positions 3 and 4. Correlation between mutations in NahR and the structures of the new effectors suggested that protein sites Met[116], Arg[132], Asn[169], and Arg[248] are involved in effector recognition and binding during the earlier steps of the process leading to transcriptional activation of cognate NAH promoters.

Transcriptional regulators belonging to the LysR-type family (hereafter referred to as LTTRs)[1] have protein sizes ranging from 276 to 324 amino acids, and they are widely distributed among prokaryotes (1). Individual members of the family share their being responsive to chemical effectors, their being transcribed divergently from the genes that they regulate (frequently accompanying an autorepression mechanism), and their mode of transcriptional activation of target promoters. All LTTRs studied so far in this respect remain bound to equivalent promoter-proximal target DNA sequences regardless of the presence or absence of inducer. Effector binding and sub-

sequent transcriptional activation seem to result in the relief of the DNA bending caused by LTTR binding under noninduced conditions (reviewed in Ref. 2). In fact, the most conserved portion of LTTRs is precisely the N-terminal segment that contains the helix-turn-helix DNA-binding motif that accounts for binding to DNA (3).

Apart from changes in DNA structure, the precise mechanism by which inducer binding is ultimately translated in transcriptional activation remains largely unknown. LTTRs contain a poorly conserved central domain (see Fig. 1) believed to interact with the chemical inducers. The divergence of the central portions of LTTRs seems to reflect the variety of inducers to which these proteins respond (1). This notion is supported by comparisons between various NodD proteins of *Rhizobium* species. These LTTRs are similar and bind the same target DNA sequences, but minor differences in their central domain account for their responsiveness to distinct flavonoid derivatives (4, 5). Furthermore, constitutive, inducer-independent mutants of various LTTRs have been found to bear changes in their central domain (6–9). However, evidence of direct interactions between specific residues of LTTRs and their cognate inducers is generally scarce. A particularly useful LTTR to address this issue is the NahR protein of *Pseudomonas*, the salicylate-responsive transcriptional regulator of two operons for biodegradation of naphthalene by strains carrying the catabolic plasmid NAH7 (Refs. 1, 3, and 10; see Fig. 1). Because the native effector of NahR is a simple aromatic compound, an entire variety of salicylate analogs is commercially available to search for altered effector specificities. In this work, we show that mutations at the central domain of NahR expand the range of aromatic compounds that can activate the protein into a transcriptionally competent form. On the basis of these results, we have identified a region of the NahR protein that may interact with the aromatic inducers and tentatively pinpoint those amino acid residues involved in effector specificity.

MATERIALS AND METHODS

Strains, Plasmids, Media, and General Procedures—The bacterial strains and plasmids used in this work are listed in Table I. Plasmids bearing an R6K origin of replication (*i.e.* mini-Tn*5* delivery vectors) were always propagated in *Escherichia coli* CC118λ*pir*. Bacteria were grown in rich LB medium (11) supplemented where required with 150 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Km), 25 μg/ml streptomycin (Sm), and 40 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Recombinant DNA techniques were made following published protocols (12). Insertion of hybrid mini-transposons into the chromosome of target bacteria was made as described in detail elsewhere (13) using *E. coli* S17–1λ*pir* as the donor strain of suicide delivery plasmids. DNA sequencing of NahR variants was carried out on double-stranded templates with an automatic Applied Biosystems instrument and a dye terminator cycle protocol with *Taq* polymerase.

Construction and Analysis of Reporter Strains—Activity of NahR was monitored in all cases by following its ability to cause accumulation of β-galactosidase in an *E. coli* strain bearing a transcriptional *Psal::lacZ*

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[1] The abbreviations used are: LTTR, LysR-type transcriptional regulator; Ap, ampicillin; Km, kanamycin; Sm, streptomycin; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; kb, kilobase pair(s); PCR, polymerase chain reaction.

fusion. To this end, a hybrid between *nahG* (the first gene transcribed from *Psal*; see Fig. 1) and *lacZ* was made by cloning a 0.5-kb *Sal*I-*Pst*I fragment of pMS15 (spanning the *Psal* promoter; Ref. 3) in the corresponding sites of *lacZ* vector pNM482 plasmid (14). This fused the first 70 codons of *nahG* to the eighth codon of *lacZ*. The same fusion was recloned in pUJ9 (13) by exchange of *Eco*RI-*Sac*I segments between the two plasmids. The entire *Psal::nahG'-'lacZ* fusion (simplified, *Psal::lacZ*) was then excised as a *Not*I fragment from the resulting plasmid and placed into the *Not*I site of pUT/mini-Tn*5*Sm (15). This gave rise to pTSAL1, a delivery vector for a hybrid mini-transposon bearing the *Psal::lacZ* fusion, which was placed into the chromosome of *E. coli* CC118 as explained in de Lorenzo and Timmis (13). Such an arrangement places strong transcriptional terminators upstream and downstream of the reporter cassette (see Fig. 1) to prevent readthrough transcription from nearby chromosomal promoters after insertion. The resulting strain (*E. coli* SAL1) was used as the recipient of plasmids bearing the *nahR* variants as described below. Induction of the *Psal* promoter in *E. coli* cells bearing *Psal::lacZ* fusions was made by growing the corresponding cultures in LB medium at 37 °C up to an A_{600} of 0.4, after which inducers were added at the concentrations indicated in each case. Incubation was then continued for 5 h, and the accumulation of β-galactosidase was measured in cells permeabilized with chloroform and sodium dodecyl sulfate as described by Miller (11). β-Galactosidase activity values given throughout this paper represent the average of at least three independent experiments, each of which was conducted in duplicate samples, with deviations being less than 15–20%.

Mutagenesis of the nahR Gene—The plasmid pNR, used as the DNA template for the protocol described below, was produced by transferring an 1.2-kb *Nco*I-*Hin*dIII fragment from pMS15 (3) bearing the entire *nahR* sequence to the same sites of vector pFH2 (16). For mutagenesis, the DNA regions of interest within pNR were amplified by the polymerase chain reaction (PCR) using the oligos NR*Bgl* (5′-ACCGACAT-TGGCGAGATCT-3′) and NR*Sph* (5′-CTTGTGGTACTTCGCATGC-3′) under conditions favoring errors of the *Taq* DNA polymerase. 100-μl PCR reactions were set up in a buffer containing 0.1 mM MgCl₂, 0.5 mM MnCl₂, 1 mM dGTP, 1 mM dTTP, 1 mM dCTP, 0.25 mM dATP, 0.5 μg of each of the primers, and 5 units of *Taq* DNA polymerase. 1–50 ng of pNR template was added to the reactions, the amount depending on the rate of mutations desired. The resulting 0.6-kb amplification products were digested with *Bgl*II and *Sph*I, purified, and used to replace the equivalent segment of pNR. A second DNA template, namely plasmid pUC/NR, was alternatively employed for mutagenesis of a different *nahR* portion (see Fig. 2). pUC/NR bears also the entire *nahR* gene assembled as the result of a tripartite ligation between *Not*I-digested pUC18*Not* (17), a 0.6-kb *Not*I-*Bgl*II fragment of pUC/NRC (see Table I) and a 0.4-kb *Bgl*II-*Not*I fragment of pNR. pUC/NR has an unique *Hin*dIII site that simplifies insertion when required of the 0.6-kb *Hin*dIII-*Sph*I segment of *nahR* sequence resulting from error-prone PCR with primers NR*H5* (5′-ATCTGGCCGAGCCCGTC-3′) and NR*Sph*. As before, the mutated *Hin*dIII-*Sph*I DNA segments were purified and cloned in the same sites of the larger fragment of pUC/NR. After mutagenesis, screening for NahR variants with altered effector specificity included the individual culture of each of the *E. coli* SAL1 transformants with the reconstructed *nahR*⁺ plasmids in microtiter plates, followed by their replication on separate LB plates amended with Ap, X-Gal, and 1 mM of each of the aromatic compounds under scrutiny. Differences in the intensity of the color developed in equivalent spots in each plate after incubation was considered an indication of altered NahR activity.

Construction of nahR/Psal-lacZ Insertions—Mini-Tn*5* derivatives bearing not only a *Psal-lacZ* fusion but also the mutant *nahR* variants named *nahR3* and *nahR4* were made as follows. pNR derivatives encoding the mutated genes (pNR3 and pNR4, respectively) were partially digested with *Hin*dIII and ligated to a 2.1-kb *Hin*dIII Ω-Km fragment (18). The location and orientation selected was the same than that present in the reference wild type *nahR/Psal-lacZ* mini-transposon bore by pCNB4*lacZ* plasmid (Ref. 19; see Fig. 1). The resulting plasmids (pNR3*Km* and pNR4*Km*, respectively) were then digested with *Nco*I, which produced 2.2-kb DNA fragments spanning the entire mutant *nahR* sequence and part of the Ω-Km fragment. The equivalent *Nco*I segment pCNB4*lacZ* was then replaced by each of these *Nco*I segments, thus replacing the wild type *nahR* sequence by the mutant variants and reconstructing Ω-Km interposon. These manipulations originated transposon delivery plasmids pCNB43*lacZ* and pCNB44*lacZ* that carry hybrid mini-transposons containing *nahR3/Psal-lacZ* and *nahR4/Psal-lacZ* reporter elements, respectively. Once inserted into the chromosome of *E. coli* CC118 (Table I), the accumulation of β-galactosidase by cells exposed to aromatic inducers was carried out as described above.
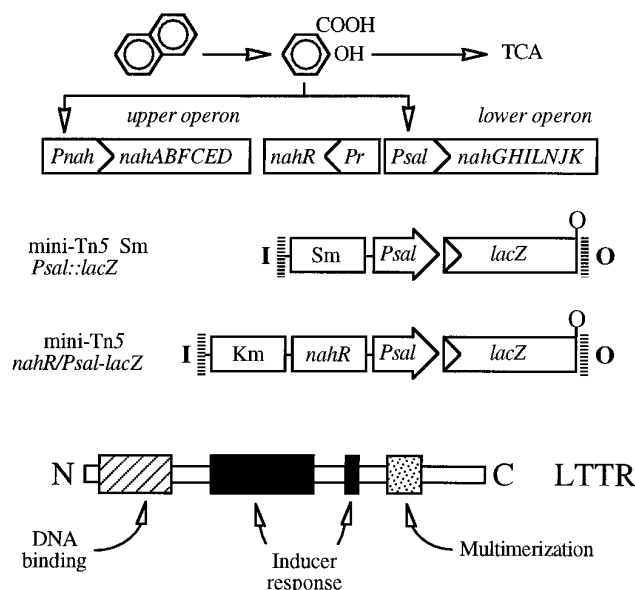
FIG. 1. **Biodegradation of naphthalene and organization of LysR-type transcriptional regulators.** Naphthalene catabolism by *Pseudomonas* strains carrying plasmid NAH7 is determined by two operons. The *upper operon* gives rise to the various activities required for the oxidative transformation of naphthalene into salicylate, whereas the *lower operon* determines the subsequent metabolism of this compound onto Krebs cycle intermediates. Transcription of these two operons requires NahR, a member of the LysR family of regulators (1). The salicylate produced from naphthalene as the result of the low constitutive expression of the *upper operon* allows NahR to activate the promoters of both the upper (*Pnah*) and the lower (*Psal*) operons through a positive feedback mechanism (3, 10). Some of the regulatory elements of the pathway are included in the mini-Tn*5* *Psal::lacZ* and mini-Tn*5* *nahR/Psal-lacZ* transposons shown *below* the pathway. Their scheme shows (not to scale) the arrangement of other segments, *i.e.* Sm or Km resistance genes, the transcriptional terminator at the end of the *lacZ* reporter, and the I and O terminal 19-base pair sequences of the wild type transposon Tn*5*. The figure at the *bottom* summarizes the location of potential functional domains in LTTRs, which are distributed along protein sizes of 276–324 amino acids (1, 6), NahR being 300 amino acids (3). As discussed in the text, the multimerization domain may also participate in effector recognition.

RESULTS

Structural Requirements of NahR Effectors—In order to determine which specific portions of the salicylate molecule were engaged in activation of NahR, we examined the degree of structural permissiveness of the regulator toward structural variants of the natural inducer. To this end, we assembled the entire segment of the NAH7 plasmid that spans the wild type *nahR* gene and adjacent regions in a mini-Tn*5* transposon vector (Fig. 1), thereby faithfully reconstructing in *E. coli* all the regulatory elements that participate in the activation of the *Psal* promoter in *Pseudomonas*. In this construct, *nahR* is expressed under its own promoter and transcribed divergently from the *Psal* promoter, followed by a promoterless *lacZ* reporter gene (Fig. 1). This element was then placed into the chromosome of *E. coli*, and the resulting strain (*E. coli* RSL9) was challenged with a variety of aromatic compounds as described under "Materials and Methods." Reproduction of the system in *E. coli* prevented any metabolism of the aromatic compounds leading to other intermediates than those added to the culture and ensured their transport to the cell cytoplasm through a broad specificity facilitated diffusion system (20).

The results of measuring accumulation of β-galactosidase under the different inducers is summarized in Table II. One important conclusion of this experiment is that the presence of a carboxyl group in the effector is an absolute requirement for NahR activation. As shown in Table II, salicylamide (compound

TABLE I
Bacteria and plasmids

E. coli K12 strain	Relevant genotype/characteristics	Reference
CC118	Δ(*ara-leu*), *araD*, Δ*lacX74*, *galE*, *galK*, *phoA*	17
CC118λ*pir*	CC118 lysogenized with λ*pir* phage	17
S17–1λ*pir*	Tp^r Sm^r *recA*, *thi*, *hsdRM*$^+$, RP4∷2-Tc∷Mu∷Km Tn7, λ*pir* phage lysogen	31
SAL1	Sm^r, CC118 bearing a chromosomal insertion of mini-Tn5 Sm/*Psal*∷*lacZ*	This work
RSL9	Km^r, CC118 bearing a chromosomal insertion of mini-Tn5 *nahR*/*Psal-lacZ*	This work
R3SL1	Km^r, same as RSL9, but mini-Tn5 *nahR*/*Psal-lacZ* insertion bearing mutant *nahR3* variant	This work
R4SL1	Km^r, same as RSL9, but mini-Tn5 *nahR*/*Psal-lacZ* insertion bearing mutant *nahR4* variant	This work

Plasmids	Genotype/phenotype/characteristics	Reference
pMS15	Ap^r, *nahR*/*Psal* sequence from NAH7 plasmid of *Pseudomonas* cloned as a 1.6-kb *Hin*dIII-*Pst*I insert in pUC8	3
pNM482	Ap^r, '*lacZ* promoter probe vector	14
pUJ9	Ap^r, '*lacZ* promoter probe vector, entire fusion excisable as a *Not*I restriction fragment	15
pUT/mini-Tn5 Sm	Ap^r, Sm^r, R6K*oriV*, RP4*oriT*, mini-Tn5 Sm transposon vector delivery plasmid	15
pTSAL1	Ap^r, Sm^r, *Psal-nahG'-'lacZ* (*Psal*∷*lacZ*) fusion inserted as a *Not*I fragment in pUT/mini-Tn5 Sm	This work
pFH2	Ap^r, identical to pBKT7–0 but with *Not*I sites flanking the multiple cloning site	16
pNR	Ap^r *nahR* gene cloned as a *Nco*I-*Hin*dIII fragment in pFH2. pNR series (pNR3, pNR4 . . .) encode *nahR* variants *nahR3*, *nahR4*, etc.	This work
pNR3*Km*	Ap^r, Km^r, pNR3 inserted with an Ω-Km interposon at the *Hin*dIII site downstream of the *nahR3* sequence of pNR	This work
pNR4*Km*	Ap^r, Km^r, same as pNR3, but bearing *nahR4*	This work
pUC18*Not*	Ap^r, same as pUC18 (32) but with *Not*I sites flanking the multiple cloning site	17
pUC/NRC	Ap^r, pUC18Not inserted with the *Hin*dIII/*Eco*RI segment of pCNB4 (19) bearing the wild type *nahR* sequence	This work
pUC/NR	Ap^r, pUC18*Not* inserted with the wild type *nahR* gene. pUC/NR series encode numbered *nahR* variants	This work
pCNB4	Ap^r, Km^r, *ori*R6K, *mob*RP4, delivery vector for mini-Tn5 *nahR*/*Psal*	19
pCNB4*lacZ*	Same as pCNB4, but inserted with a promoterless *trp'-'lacZ* reporter downstream of the *Psal* promoter	19
pCNB43*lacZ*	Same as pCNB4*lacZ*, but bearing the *nahR3* mutant	This work
pCNB44*lacZ*	Same than pCNB4*lacZ*, but bearing the *nahR4* mutant	This work

B), 2-hydroxy-acetophenone (compound C), and salicyl alcohol (compound D) caused no accumulation of the reporter product. On the contrary, substitutions of the 2-hydroxyl group of salicylate were more tolerated, as indicated by the β-galactosidase activity produced after the addition of anthranilic acid (compound F), *o*-acetyl-salicylic acid (compound G), or to a lesser extent *o*-methoxy-benzoic acid (compound H). However, benzoate (compound E) or 2-chloro-benzoate (compound I) did not give rise to any significant induction of the reporter system. Chloro and methyl substituents in positions C-3, C-4, and C-5 of the aromatic ring of salicylate appeared also to be tolerated (Table II). Introduction of hydroxyl or methoxyl groups in sites C-4 or C-5 of salicylate decreased the induction ability of the compounds that were, however, still able to activate NahR to significant levels. Finally, although with reduced activity, di- or tri-chloro salicylate derivatives (compounds M and N) could induce also the reporter system.

In summary, it seems that specific contacts between substituents of the aromatic ring and the protein engage exclusively positions C-1 and C-2 of the salicylate, the minimal apparent requirements being the presence of a carboxyl group in C-1 and the presence of a small functional group with available electrons in C-2. NahR appeared to accept a wide variety of salicylate structural analogs as effectors, thus reflecting the permissiveness of the protein domain(s) that interacts with the aromatic molecules. The decrease and even lack of activity observed when bulkier groups are entered in other positions may reflect a steric hindrance related to the size of the "effector pocket" in the regulator and not to the disruption of specific protein-inducer interactions.

Isolation of NahR Mutants with Altered Effector Specificity—
Once the structural requirements of NahR effectors were determined, we set out to probe the location of the protein segments involved in interactions with the aromatic compounds. We assumed that changes in the amino acid residues involved in effector recognition may result in NahR variants that can be

activated by otherwise nonproductive inducers. On this basis, we designed a genetic strategy to identify NahR mutants that could respond to compounds found to be unable to act as effectors of wild type NahR (Fig. 2). In particular, we pursued the isolation of mutants responsive to benzoate, because we reasoned that NahR variants lacking the interactions with the quite permissive yet important position C-2 should appear at a reasonable frequency. To this end, we constructed an *E. coli* strain (named *E. coli* SAL1) bearing a chromosomal *Psal::lacZ* fusion, which was used as the recipient of plasmids bearing mutagenized *nahR* sequences. With the protocol described under "Materials and Methods," we first targeted the portion of NahR spanning amino acids 110–277, corresponding to a DNA segment of 0.49 kb (Fig. 2). This region was amplified through error-prone PCR from plasmid pNR. The ligation of the resulting *Bgl*II-*Sph*I fragment with pNR digested with the same enzymes was then electroporated in *E. coli* SAL1 and plated out on LB/Ap/benzoate/X-Gal plates. After incubation, about 1% of the Ap-resistant colonies were intense blue. In order to discard constitutive mutants, these blue colonies bearing plasmids encoding putative NahR mutants were replicated in LB/Ap/X-Gal plates with or without benzoate, and only those clearly regulated by the aromatic compound were kept for further analysis. This was the case with two-thirds of all colonies tested, *i.e.* only about one-third of all mutants gave a constitutive, effector-independent phenotype, whereas the rest were genuinely responsive to benzoate. In addition, none of the benzoate-responsive mutants lacked their responsiveness to salicylate.

Characterization of Benzoate-responsive NahR Variants—
Twenty of the *nahR*$^+$ pNR derivatives present in *E. coli* colonies giving a benzoate-responsive color in X-Gal plates were subjected to DNA sequencing. This revealed the existence of at least six independent mutations (in all cases single-base transitions) in the *nahR* sequence (Fig. 2) accountable for the distinct phenotype observed. Mutants NahR3 (R248C) and NahR4

TABLE II
Activation of NahR by structural analoges of salicylate

Measured as accumulation of β-galactosidase by *E. coli* RSL9 (*nahR/Psal-lacZ*). Figures correspond to Miller units (11) assayed in permeabilized cells from LB cultures induced for 5 h with 2 mM of each of the compounds indicated (excepting compound M, 3,5-chloro-salicylate, that was added at 1 mM). The addition of the aromatics to the cultures had no significant effect on growth rate at the concentrations employed. The numbers shown are the average of three independent assays run with duplicate samples. Positions R1–R6 correspond to the substituents in the aromatic ring.
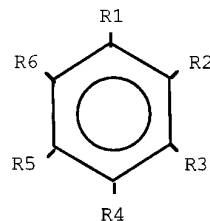
Compound	R1	R2	R3	R4	R5	R6	Induction[a]
A	-COOH	-OH	-H	-H	-H	-H	23
B	-CONH$_2$	-OH	-H	-H	-H	-H	1
C	-COCH$_3$	-OH	-H	-H	-H	-H	1
D	-CH$_2$OH	-OH	-H	-H	-H	-H	1
E	-COOH	-H	-H	-H	-H	-H	1
F	-COOH	-NH$_2$	-H	-H	-H	-H	20
G	-COOH	-OCOCH$_3$	-H	-H	-H	-H	36
H	-COOH	-OCH$_3$	-H	-H	-H	-H	3
I	-COOH	-Cl	-H	-H	-H	-H	2
J	-COOH	-OH	-Cl	-H	-H	-H	16
K	-COOH	-OH	-H	-Cl	-H	-H	30
L	-COOH	-OH	-H	-H	-Cl	-H	28
M	-COOH	-OH	-Cl	-H	-Cl	-H	9
N	-COOH	-OH	-Cl	-H	-Cl	-Cl	9
O	-COOH	-OH	-CH$_3$	-H	-H	-H	17
P	-COOH	-OH	-H	-CH$_3$	-H	-H	19
Q	-COOH	-OH	-H	-H	-CH$_3$	-H	27
S	-COOH	-OH	-H	-H	-OCH$_3$	-H	9
T	-COOH	-OH	-OH	-H	-H	-H	9
U	-COOH	-OH	-H	-OH	-H	-H	5
V	-COOH	-OH	-H	-H	-H	-OH	1

[a] Ratio between maximum accumulation of β-galactosidase and basal enzymatic activity of *E. coli* RSL9 in the absence of induction (approximately 50 Miller units).
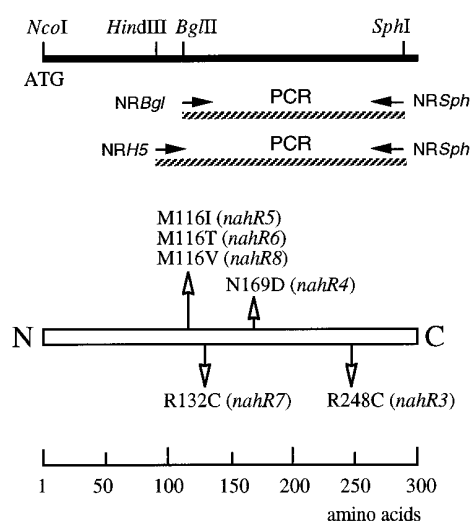

FIG. 2. **Strategy used for generation of benzoate-responsive NahR variants.** The amino acid sequence of NahR can be matched to a 0.9-kb DNA sequence that is nearly completely included in an *Nco*I-*Sph*I restriction fragment of the NAH7 plasmid (3). Oligonucleotides flanking the *nahR* sequence, (excluding the region corresponding to the DNA-binding protein domain) were employed to prime the region for a polymerase chain reaction under error-prone conditions. The mutagenized DNA segments were then cloned back to selected plasmids vectors in order to reconstruct the *nahR* sequence and transformed in *E. coli* SAL1 (*Psal::lacZ*) to reveal the phenotype of the resulting mutants (see "Results" for explanation). The *lower part* of the figure shows the position within the NahR sequence and the nature of the six benzoate-responsive mutants described in this work.

(N169D) bore a single-amino acid change that made the resulting proteins to respond to benzoate to the same extent as the wild type responds to salicylate (Table III). In contrast, mutants NahR5 (M116I), NahR6 (M116T), and NahR8 (M116V) displayed a remarkably high basal level of activity in the absence of any inducer, whereas they reached a similar degree of β-gal accumulation with either benzoate or salicylate (Table III). This semiconstitutive phenotype was caused in all three cases by the exchange of the Met[116] residue for neutral amino acids. Mutants NahR3 and NahR4 will be termed hereafter as variants type I, whereas mutants NahR5, NahR6, and NahR8 will be named variants type II. Interestingly, the same amino acid change bore by NahR5 was found by Huang and Schell (21) in their quest for salicylate-independent NahR proteins.

Mutant NahR7 (R132C), albeit similar to type I mutants, showed an intermediate phenotype because its basal activity was about twice that of the wild type and its induction by benzoate was significantly less than its responsiveness to salicylate. However, it gained a remarkable inducibility by other effectors. In fact, further analysis of the ability of various compounds to activate mutant NahR variants revealed that mutants type I and NahR7 (which bear single amino acid changes) had generally broadened their effector profile while maintaining a tight regulation. For instance, as indicated in Table III, NahR3 and NahR7 acquired the ability of being activated by salicylamide, and they displayed also an enhanced induction by chloro-derivatives, whereas NahR4 seemed to be more specific in gain of responsiveness to benzoate. Similarly, mutants type II generally broadened their effector profile.

TABLE III
Transcriptional activity of NahR variants induced with various salicylate analoges

Measured as accumulation of β-galactosidase by E. coli SAL1 (Psal-lacZ) transformed with each of the plasmids indicated, encoding the *nahR* variants specified in each case.

Plasmid	Gene	Codon exchanged	Amino acid substitution	*Psal*::*lacZ* activity[a]							
				None	B	2HB	SA	2HBA	2ClB	3ClB	4ClB
							Miller units				
pNR	*nahR*	None	None	58	60	1100	106	31	169	483	48
pNR3	*nahR3*	CGT → TGT	Arg[248] → Cys	64	923	1499	552	44	837	1008	146
pNR4	*nahR4*	AAT → GAT	Asn[169] → Asp	51	1158	1067	71	33	1104	840	40
pNR7	*nahR7*	CGC → TGC	Arg[132] → Cys	104	400	1061	1258	61	682	848	186
pNR5	*nahR5*	ATG → ATA	Met[116] → Ile	375	1050	1765	1109	330	1526	1303	983
pNR6	*nahR6*	ATG → ACG	Met[116] → Thr	359	1101	1247	288	268	1416	1326	949
pNR8	*nahR8*	ATG → GTG	Met[116] → Val	432	1049	1419	1330	344	1416	1387	1201

[a] Units of β-galactosidase (11) assayed in permeabilized cells from LB cultures induced for 5 h with 2 mM of the following inducers: B, benzoate; 2HB, salicylate; SA, salicylamide; 2HBA, 2-OH benzylalcohol; 2ClB, 2-chloro-benzoate; 3ClB, 3-chloro-benzoate; 4ClB, 4-chloro-benzoate.

However, because these mutants display an up phenotype, it was difficult to distinguish which compounds became authentic inducers because of a gain of function in NahR and which behaved as such because of an unspecific amplification of an otherwise residual responsiveness. At least in the case of salicylamide and 4 chloro-benzoate, it seems that two of the type II mutants acquired a genuine ability to respond to these aromatic compounds that was totally missing in the wild type regulator.

Affinity of Group I Mutants for Inducer Compounds—To gain some insight in the mechanism by which *group I* mutants broadened their effector profile, they were subjected to a series of experiments to compare the apparent affinities of NahR for four representative effectors, namely salicylate, benzoate, 3-chloro-benzoate and 3-methyl salicylate. To this end, we employed the procedure developed by Ramos *et al.* (20) to estimate *in vivo* the apparent $K_s^{'}$ values of effectors for prokaryotic transcriptional regulators. The procedure depends on the assumptions that (i) benzoate analogs are transported into *E. coli* cell through nonlimiting facilitated diffusion and (ii) the output of the reporter *lacZ* fusion reflects faithfully the activity of the regulator. Because these two conditions can be safely assumed in our system, we set out to examine the induction of a reporter *Psal-lacZ* fusion by varying effector concentrations. In order to decrease the number of variables in the procedure, we engineered the mutant *nahR3* and *nahR4* genes along with the reporter *Psal-lacZ* fusion within a mini-Tn5 vector (Fig. 1). These elements, along with that bearing wild type *nahR* (19), were introduced into *E. coli* CC118 by transposition, giving rise to strains *E. coli* RSL9 (wild type *nahR*), *E. coli* R3SL1 (*nahR3*), and *E. coli* R4SL1 (*nahR4*). As shown in Fig. 3, each of the strains was subjected to induction experiments with concentrations of the aromatic compounds ranging from 10^{-5} to 1 mM. It is interesting to note that the fully induced cells displayed a responsiveness to each of the aromatic compounds virtually identical to that observed when the reporter system carries the *nahR* variants in a multicopy plasmid (Table III). This ruled out that the phenotypes raised by the *nahR* mutants were simply due to different expression levels. On the other hand, the shape of the dose-response curves in Fig. 3 clearly indicated that the mutations did increase the affinity of NahR for specific aromatic compounds. This is particularly true for benzoate: as shown in Fig. 3*C*, the affinity increased from virtually nil to an apparent $K_s^{'}$ value in the range of 0.5 mM. The operative affinities of NahR3 for 3-chloro-benzoate and 3-methyl salicylate increased >100-fold and >10-fold, respectively, as compared with wild type NahR. Interestingly, NahR4 also seemed to remarkably increase its affinity toward salicylate. These results suggested that the mutations carried by NahR variants type I affected amino acid residues involved in inducer binding.
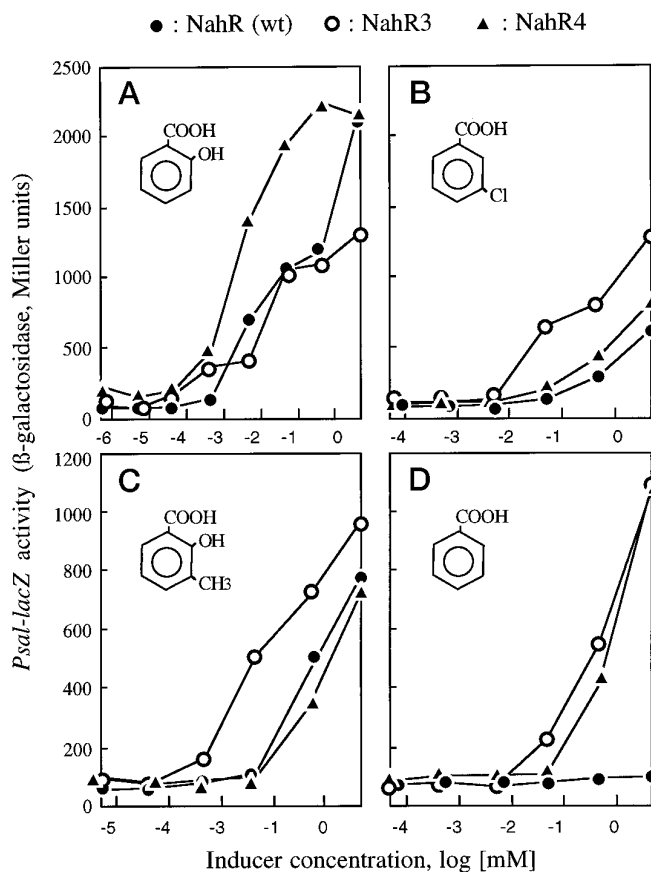

● : NahR (wt)   ○ : NahR3   ▲ : NahR4

FIG. 3. **Effect of the concentration of aromatic inducers on *Psal* activity promoted by NahR and its mutant derivatives.** Wild type *nahR* and its variants *nahR3* and *nahR4* were assembled in mini-Tn5 vectors along with a reporter *Psal-lacZ* fusion, as explained under "Materials and Methods." Strains *E. coli* RSL9 (wild type *nahR*[+]), *E. coli* R3SL1 (*nahR3*[+]), and *E. coli* R4SL1 (*nahR4*[+]), bearing each of the mobile elements in the chromosome (Table I) were then subjected to induction experiments with varying concentrations of the aromatic effector indicated in each case: salicylate (*A*), 3-chloro-benzoate (*B*), 3-methylsalicylate (*C*), and benzoate (*D*). The β-galactosidase levels determined in permeabilized cells after 5 h of induction are shown.

Benzoate-responsive Multiple Mutants Share the Same Key Changes in the NahR Sequence—In an attempt to identify additional residues that could simultaneously be involved in interactions with the aromatic effectors, we repeated the mutagenesis protocol under conditions in which error-prone PCR was expected to enter more than one base change per molecule of amplified DNA (see "Materials and Methods"). Furthermore, we employed primers NR*H5* and NR*Sph*, which amplified a longer portion of the NahR sequence (Fig. 2) along with plas-

mid pUC/NR (Table I). This facilitated the recovery of the mutagenized DNA segment. The procedure yielded 47 benzoate-responsive clones. On the basis of their responsiveness to selected inducers (salicylate, *o*-acetyl-salicylate, 3-methyl-benzoate, 2,3-chloro-benzoate, 2,6-chloro-benzoate, anthranylate, 5-chloro-salicylate, salicylalcohol, phenol, and salicylamide), they fell equally (23 and 24 clones) within the two types of mutants generated with the single-mutation protocol. As before also, none of the mutants lost its responsiveness to salicylate. Out of the entire collection, we selected 12 clones displaying a phenotype of low basal activity and broadening of their effector profile. All turned out to largely behave as NahR3, NahR4, or NahR7. Six of these were sequenced. The variant *nahR138* was altered in five bases, three of them producing no amino acid changes, and the other two causing mutations N125S and N169D. Two other mutants were found to bear changes in two bases, both resulting in amino acid exchanges: *nahR105* (R248C, A272T) and *nahR141* (M139T,N169D). Finally, two mutants bore three changed residues: *nahR135* (Q158R,N169D,E208G) and *nahR130* (N156S,V226M,R248C), whereas the remaining one (renamed *nahR123*) was again, N169D. Therefore, four of the new six variants had the change N169D, already studied in *nahR4*, whereas the other two, alike *nahR3*, bore the change R248C. Therefore, the benzoate-responsive effect of the multiple mutants was attributed to the presence of mutations already identified and not because of the other amino acid changes.

DISCUSSION

A number of NahR mutants had been isolated in the past that could not activate the *Psal/Pnah* promoters because of their inability to bind DNA (1, 9). Not surprisingly, some of these were found to cluster within the the N-terminal portion of the protein bearing an helix-turn-helix motif (residues 23–45). However, other nondominant mutants unable to bind DNA were mapped closer to the C terminus (residues 239–291), thus suggesting that DNA binding required some multimerization through a different protein domain (1). In addition, a variety of noninducible NahR mutants were found within the region 126–206 that were still competent for DNA binding (9). This central region overlaps also with mutations that led to a constitutive, effector-independent behavior (mapping between residues 99–253; Ref. 21). These data, however, said little on whether salicylate interacts directly with the protein and, if so, which parts of the regulator are involved in such an interaction. In this work, we show that mutations that clearly alter the effector profile of NahR and affect its affinity for aromatic inducers map recurrently at distict sites of the regulator (Fig. 2). This provides an strong genetic evidence that these sites are engaged in effector binding during the earlier steps of the process leading to transcriptional activation of the *Psal* and *Pnah* promoters.

NahR mutants type I and the very related variant NahR7 appear to be genuinely affected in residues that participate in inducer recognition, because they show an altered effector specificity without any other detectable phenotypic change. Mutants NahR4 and NahR7 are particularly meaningful in this respect. As shown in Table III and Fig. 3, NahR4 appeared to gain the ability to respond to benzoate in a fashion that seemed to affect only the OH substituent at position C-2 of salicylate. On the contrary, NahR7 gained a remarkable ability of induction by salicylamide, suggesting that the protein residue(s) interacting with the otherwise essential carboxyl group of salicylate have also undergone some change. Mutant NahR3 seems, on the other hand, to increase its general reponsiveness to virtually all salicylate analogs examined, which may reflect a flexibilization of the effector pocket in NahR but not so much

the change of a residue directly involved in effector recognition. Finally, mutants type II pinpoint the pivotal role of amino acid 116 in both effector recognition and NahR activation. That the mutations in this residue lock the protein in an intermediate activation step is indicated by the degree of inducer-independent activity observed. But that the 116 residue may also participate in interactions with the carboxyl group of salicylate is suggested by the observation (Table III) that the changes M116I and M116V make the proteins respond to salicylamide, whereas M116T does not. A number of amino acid residues critical for effector binding are therefore placed in a protein segment not larger than 60 amino acids, spanning positions 116 and 169. However, the contribution of more distant locations (*i.e.* 248) cannot be ruled out. These conclusions generally fit those found in the OccR protein of *Agrobacterium tumefaciens*, another archetypical LTTR, in which mutations within the central portion of OccR (F113L and G148D) increased the affinity of the protein for its natural effector, octopine (8). Ours is, however, the first case reported of single mutations within an LTTR that alter the effector profile of a protein of this type.

It would be highly speculative to draw structural or mechanistic conclusions from the data presented in this work. However, the remarkable similarity (approximately 50%) between two segments present in NahR and NodD proteins (residues 140–200 and 207–266; Ref. 3) allows some considerations. These two regions, which include NahR variants N169D and R248C, span a potential β-turn-β motif in NodD that might be a ligand-binding crevice (1, 22). In fact, segments 109–171 and 189–216 of NodD resemble a consensus steroid-binding domain, thus suggesting an involvement in effector binding (22). Furthermore, residue 248 of NahR, which is mutated in variant *nahR3*, is located within a region conserved in many LTTRs that could be involved in multimerization (1). Perhaps the protein segment around 248 links effector binding to a multimer conformation shift that leads to activation, as has been suggested for CysB (23).

The ease for obtaining variants with novel effector specificities makes NahR a system of choice for the generation of proteins responsive to predetermined aromatic molecules, a task of considerable interest for biotechnological applications (19, 24–27). In fact, the proneness of NahR to mutate to forms with a broader effector specificity might not be alien to the fact that LTTRs are frequently found as regulators of bacterial operons for biodegradation of xenobiotic compounds (28), the assembly of which requires a very fast protein evolution (29). This is true also for the NodD proteins of rhizobia, another well known case of LTTR, which are necessary for specific interactions with plants (5). NodD proteins must interact with similar DNA targets (the so-called *nod* boxes) in response to an array of flavonoid derivatives present in the plant exudates. The sequences available from various NodD proteins indicate that the ability to recognize novel flavonoids resides in the divergence of the amino acid sequences of the central protein domains (4, 30). This substantiates the notion that changes within a few amino acid residues in LTTRs give rise to repertoires of regulators responsive to chemical signals that become instrumental for transcriptional control of many different genes or operons along evolution (29).

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Effector Specificity Mutants of the Transcriptional Activator NahR of Naphthalene Degrading *Pseudomonas* Define Protein Sites Involved in Binding of Aromatic Inducers

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