Effect of Specific 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 analogues 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 *Kₐ* 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 Met¹¹⁶, Arg¹³², Asn¹⁶⁹, and Arg¹⁸⁸ 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) 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 subsequent 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 inducer. 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 analogues 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-Tn5 delivery vectors) were always propagated in *Escherichia coli* CC118pir. 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* S17–1pir 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* reporter.
fusion. To this end, a hybrid between nahG (the first gene transcribed from PsaI; see Fig. 1) and lacZ was made by cloning a 0.5-kb Sall-PstI fragment of pMS15 (spanning the PsaI 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 reacted in pUC/NR (19) by exchange of EcoRI-SalI segments between two plasmids. The entire Psal::nahG′-lacZ fusion (simplified, Psal::lacZ) was then excised as a NotI fragment from the resulting plasmid and placed into the NotI site of pUT/mni-Tn5Sm (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 SAL1 as explained by de Lorenzo and Timmis (13). Attachment 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 A600 of 0.4, after which inducers were added to 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 transfecting an 1.2-kb NcoI-HindIII fragment from pMS15 (3) bearing the entire nahR sequence to the same sites of vector pFF2 (16). For mutagenesis, the DNA regions of interest within pNR were amplified with the polynucleotide chain reaction (PCR) using the oligos NB9 (5′-ACCGCATG-TGGCGGAGATCCT-3′) and NRSp (5′-CTTGTGGAATTCCTGCGATC-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 dATP, 1 mM dCTP, 0.25 mM dTTP, 0.5 μM 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 BglII and SpI1, 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). pUCNR bears also the entire nahR gene as assembled in the result of a tripartite ligation between NcoI-digested pUC18(NorI) (17), a 0.6-kb NorI-BglII fragment of pUC1NR (see Table I) and a 0.4-kb BglII-NcoI fragment of pNR. pUCNR has an unique HindIII site that simplifies insertion when required of the 0.6-kb HindIII-Sphl segment of nahR sequence resulting from error-prone PCR with primers NRH5 (5′-ATCTGGCCGAAGCCCGTC-3′) and NRSp. As before, the mutated HindIII-Sphl 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 efficiencies 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. Loadings 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-Tn5 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 HindIII and ligated to a 2.1-kb HindIII-Ω-Km fragment (18). The location and orientation selected was the same as that present in the reference wild type nahR′/Psal-lacZ mini-transposon bore by pCNB4lacZ plasmid (Ref. 19; see Fig. 1). The resulting plasmids (pNR3Sm and pNR4Km, respectively) were then digested with NcoI, which produced 2.2-kb DNA fragments spanning the entire mutant nahR sequence and part of the Ω-Km fragment. The equivalent NcoI segment pCNB4lacZ was then replaced by each of these NcoI segments, thus producing the type nahR′′ mini-transposon sequence by the mutant sequenced by the Ω-Km interposon. These manipulations originated transposon delivery plasmids pCNB4lacZ and pCNB44lacZ 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.

**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-Tn5 transposon vector (Fig. 1), thereby faultlessly 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, ensuring their transport to the cell cytoplasm and into 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
On the contrary, substitutions of the 2-hydroxyl group of salicylate structural analogs as effectors, thus reflecting the apparent requirements being the presence of a carboxyl group (compound F), or 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 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.

| E. coli K12 strain | Relevant genotype/characteristics | Reference |
|-------------------|----------------------------------|-----------|
| CC118             | Δ(ara-leu), araD, ΔlacX74, galE, galK, phoA | 17        |
| CC118Aprir        | CC118 lysogenized with Aprir phage | 17        |
| S17-Lpir          | Ty Sm’ recA, thi, hsdRM’, RP4 2-Tc: Mu: Km Tn7, lpir phage lysogen  | 31        |
| SAL1              | Sm’, CC118 bearing a chromosomal insertion of mini-Tn5 Sm/Psal::lacZ | This work |
| RSL9              | Km’, CC118 bearing a chromosomal insertion of mini-Tn5 nahR/Psal-lacZ | This work |
| RSL9              | Km’, same as RSL9, but mini-Tn5 nahR/Psal-lacZ insertion bearing mutant nahR3 variant | This work |
| R41L              | Km’, same as RSL9, but mini-Tn5 nahR/Psal-lacZ insertion bearing mutant nahR4 variant | This work |

### Bacteria and plasmids

| Plasmids | Genotype/phenotype/characteristics | Reference |
|----------|-----------------------------------|-----------|
| pMS15    | Ap’, nahR/Psal sequence from NAH7 plasmid of Pseudomonas cloned as a 1.6-kb HindIII-PsalI insert in pUC8 | 3         |
| pNM482   | Ap’, lacZ promoter probe vector | 14        |
| pU9J     | Ap’, lacZ promoter probe vector, entire fusion excisable as a NotI restriction fragment | 15        |
| pUT/mini-Tn5 Sm | Ap’, Sm’, 86KorY, RP4oriT, mini-Tn5 Sm transposon vector delivery plasmid | 15        |
| pTSAL1   | Ap’, Sm’, Psal-nahG’-lacZ (Psal::lacZ) fusion inserted as a NotI fragment in PUT/mini-Tn5 Sm | This work |
| pFH2     | Ap’, identical to pBK7T-0 but with NotI sites flanking the multiple cloning site | 16        |
| pNR      | Ap’ nahR gene cloned as a NotI-HindIII fragment in pHF2, pNR series (pNR3, pNR4 ...) encode nahR variants nahR3, nahR4, etc. | This work |
| pNR3Km   | Ap’, Km’, pBR3 inserted with an 8-kb interposon at the HindIII site downstream of the nahR3 sequence of pNR | This work |
| pNR4Km   | Ap’, Km’, same as pNR3, but bearing nahR4 | This work |
| pUC18Not | Ap’, same as pUC18 (32) but with NotI sites flanking the multiple cloning site | 17        |
| pUC/NRC  | Ap’, pUC18Not inserted with the HindIII/EcoRI segment of pCNB4 (19) bearing the wild type nahR sequence | This work |
| pUCNR    | Ap’, pUC18Not inserted with the wild type nahR gene, pUCNR series encode numbered nahR variants | The work |
| pCN4B    | Ap’, Km’, oriR6K, mobRP4, delivery vector for mini-Tn5 nahR/Psal | 19        |
| pCN4lacZ | Same as pCN4B, but inserted with a promoterless trp’-lacZ reporter downstream of the Psal promoter | 19        |
| pCN4lacz | Same as pCN4lacZ, but bearing the nahR3 mutant | This work |
| pCN4lacz2 | Same than pCN4lacZ, but bearing the nahR4 mutant | This work |

| Twenty of the nahR+ pNR derivatives present in E. coli colonies giving a benzoate-responsive color in X-Gal plates were picked from each of the above colonies. 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/ ApX-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...
Boreasingle-aminoacidchangethatmadetheresulting 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 Met116 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 II

| Compound | R1   | R2   | R3 | R4 | R5 | R6 | Induction* |
|----------|------|------|----|----|----|----|------------|
| A        | -COOH| -OH  | -H | -H | -H | -H | 23         |
| B        | -CONH₂| -OH  | -H | -H | -H | -H | 1          |
| C        | -COCH₃| -OH  | -H | -H | -H | -H | 1          |
| D        | -CH₂OH| -OH  | -H | -H | -H | -H | 1          |
| E        | -COOH| -H   | -H | -H | -H | -H | 1          |
| F        | -COOH| -NH₄| -H | -H | -H | -H | 20         |
| G        | -COOH| -OC(O)CH₃| -H | -H | -H | -H | 36         |
| H        | -COOH| -OCH₃| -H | -H | -H | -H | 3          |
| I        | -COOH| -Cl  | -Cl| -H | -H | -H | 2          |
| J        | -COOH| -OH  | -H | -Cl| -H | -H | 16         |
| K        | -COOH| -H   | -Cl| -H | -H | -H | 30         |
| L        | -COOH| -OH  | -H | -H | -H | -H | 28         |
| M        | -COOH| -OH  | -Cl| -H | -Cl| -Cl| 9          |
| N        | -COOH| -OH  | -Cl| -H | -Cl| -Cl| 9          |
| O        | -COOH| -OH  | -CH₃| -H | -H | -H | 17         |
| P        | -COOH| -OH  | -H | -CH₃| -H | -H | 19         |
| Q        | -COOH| -OH  | -H | -H | -H | -OH| 1          |
| S        | -COOH| -OH  | -H | -H | -H | -OH| 9          |
| T        | -COOH| -OH  | -H | -H | -H | -OH| 9          |
| U        | -COOH| -OH  | -H | -H | -H | -OH| 5          |
| V        | -COOH| -OH  | -H | -H | -H | -OH| 1          |

* 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 NcoI-SphI 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 transform into 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 Met116 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.
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 salicylate 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 $K_a$ values for effectors of 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 multicity 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. 3C, the affinity increased from virtually nil to an apparent $K_a$ 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.

### Table III

| Plasmid | Gene | Codon exchanged | Amino acid substitution | Psal-lacZ activity$^a$ (Miller units) |
|---------|------|----------------|------------------------|-------------------------------------|
| pNR3    | nahR | None           | None                   | None                                |
| pNR4    | nahR4| CGT $\rightarrow$ TGT | Arg$^{G38}$ $\rightarrow$ Cys | 58 | 60 | 1100 | 106 | 31 | 169 | 146 |
| pNR7    | nahR7| CGC $\rightarrow$ TGC | Arg$^{G12}$ $\rightarrow$ Cys | 51 | 1158 | 1067 | 71 | 33 | 1104 | 840 |
| pNR5    | nahR5| ATG $\rightarrow$ ATA | Met$^{116}$ $\rightarrow$ Ile | 375 | 1950 | 1765 | 1109 | 330 | 1526 | 1303 |
| pNR6    | nahR6| ATG $\rightarrow$ ACG | Met$^{116}$ $\rightarrow$ Thr | 359 | 1101 | 1247 | 288 | 268 | 1416 | 1326 |
| pNR8    | nahR8| ATG $\rightarrow$ GTG | Met$^{116}$ $\rightarrow$ Val | 432 | 1049 | 1419 | 1330 | 344 | 1416 | 1387 |

$^a$ Units of $\beta$-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.

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**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 $\beta$-galactosidase levels determined in permeabilized cells after 5 h of induction are shown.

Benzozoate-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 NRH5 and NRSpH, 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 mutated gene segment. The procedure yielded 47 benzoate-responsive clones. On the basis of their responsiveness to selected inducers (salicylate, α-acetyl-salicylate, 3-methyl-benzoate, 2,3-chloro-benzoate, 2,6-chloro-benzoate, anthranilate, 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 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 N-terminal portion of the protein bearing an helix-turn-helix motif (residues 23–45). However, other nonadominate 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 distinct 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 responsiveness 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 carbonyl 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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