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Construction and Evaluation of *nagR-nagAa::lux* Fusion Strains in Biosensing for Salicylic Acid Derivatives

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Abstract

The NagR protein is a response regulatory protein found in the bacterium *Ralstonia* sp. U2 that is involved in sensing for salicylic acid and the subsequent induction of the operon just upstream of its gene. The genes encoded for in this operon are involved in the degradation of salicylic acid. *Escherichia coli* strain RFM443 carrying a fusion of the *Photorhabdus luminescens luxCDABE* operon with the *nagR* gene and upstream region of the *nagAa* gene was constructed and characterized with respect to its optimum temperature, its response time and kinetics, and its ability to detect numerous benzoic acid derivatives. Although capable of detecting 0.5 m*M* salicylic acid at any temperature between 28 and 40°C, this *E. coli* strain, labeled DNT5, showed its greatest relative activity at 30°C, i.e., the temperature at which the largest induction was seen. Furthermore, experiments done with numerous benzoic acid derivatives found the NagR protein to be responsive to only a few of the compounds tested, including salicylic acid and 3-methyl salicylic acid, and acetyl salicylic acid was the strongest inducer. The lower limits of detection for these compounds with *E. coli* strain DNT5 were also established, with the native inducer, salicylic acid, giving the most sensitive response and detectable down to a concentration of about 2 µ*M*. A second *lux* fusion plasmid was also constructed and transformed into an NahR background, *Pseudomonas putida* KCTC1768. Within this strain, NAGK-1768, the supplemental activity of the NahR protein on the *nagAa* promoter, was shown to extend both the range of chemicals detected and the sensitivity.

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Index Entries: Bacterial biosensor; bioluminescence; NahR; naphthalene; salicylic acid.

Introduction

The degradation of polyaromatic hydrocarbons (PAHs) is a research area that has received much attention because PAHs are recalcitrant and persist within the environment for many years. Numerous bacterial species have been isolated and characterized based on their ability to degrade naphthalene *(1–4)*, phenanthrene *(5,6)*, fluorene *(6)*, anthracene *(7)*, and pyrene *(8,9)*, with some pure cultures capable of degrading several PAHs *(7,10)*. The genes responsible for degradation have typically been found on large plasmids *(11–14)*, and the genes responsible for naphthalene degradation have been cloned and its enzymatic pathway mapped out *(2)*.

It was found that two distinct operons, the *nah* operon and the *sal* operon, were required for the complete mineralization of naphthalene in most strains. The *nah* operon gene products are responsible for the formation of salicylic acid, which acts as the inducer of both the *nah* and *sal* operons, from naphthalene, and the *sal* operon gene products further degrade salicylic acid to catechol and finally to pyruvate and acetaldehyde *(15)*. Both of these operons are upregulated by the same protein, NahR, which detects salicylic acid produced during the degradation of naphthalene *(16–19)*. However, one study showed that the presence of different compounds that are structurally similar to salicylic acid leads to higher transcriptional activities from the *nah* and *sal* promoters compared with the activities seen from salicylic acid *(20)*. Furthermore, the gene encoding NahR has been cloned and sequenced *(21,22)*, allowing several groups to study the effects of different amino acid substitutions on its effector recognition *(20,23)*.

Recently, in *Ralstonia* sp. U2 a different naphthalene degradation pathway encoded by the *nag* operon was discovered *(14,24)*. The degradation of salicylic acid in this pathway occurs via gentisic acid, maleylpyruvate, pyruvate, and fumarate *(14)*. Expression of the *nag* operon appears to be regulated by a single promoter located upstream from the operon. The positive regulator of the system is salicylic acid. In the present study, the gene coding for the NagR protein from *Ralstonia* sp. U2, along with the upstream *nag*-operon promoter, were fused with the *lux* genes from *Photorhabdus luminescens*, present in plasmid pDEW201 *(25)*. The *Escherichia coli* strain DNT5 carrying this construct allowed us to characterize the NagR protein with respect to its temperature dependence, effector specificity, and response kinetics to different derivatives of salicylic acid. A second strain, *Pseudomonas putida* strain KCTC1768, was also transformed with a broad-host range plasmid, pNAGK, which harbors a fusion of the *nag*-operon promoter region with the *lux* operon from *Vibrio fischeri* present within plasmid pUCD615 *(26)*. Several other studies have previously examined the use of *nahR-lux* fusions, using the same plasmid backbone, to study the degradation of naphthalene and salicylic acid *(27–29)*. In the

Table 1
Chemical Names and Structures of Compounds Tested

Chemical	Position on benzene ring					
	C1	C2	C3	C4	C5	C6
1. Benzoic acid	-COOH	-H	-H	-H	-H	-H
2. Salicylic acid	-COOH	-OH	-H	-H	-H	-H
3. Gentisic acid	-COOH	-OH	-H	-H	-OH	-H
4. 3,4-Dihydroxy benzoic acid	-COOH	-H	-OH	-OH	-H	-H
5. 3,5-Dihydroxy benzoic acid	-COOH	-H	-OH	-H	-OH	-H
6. 2-Chloro benzoic acid	-COOH	-Cl	-H	-H	-H	-H
7. 3-Chloro benzoic acid	-COOH	-H	-Cl	-H	-H	-H
8. 4-Chloro benzoic acid	-COOH	-H	-H	-Cl	-H	-H
9. 3-Methyl salicylic acid	-COOH	-OH	$-CH_3$	-H	-H	-H
10. 4-Methyl salicylic acid	-COOH	-OH	-H	$-CH_3$	-H	-H
11. 5-Methyl salicylic acid	-COOH	-OH	-H	-H	$-CH_3$	-H
12. 4-Chloro salicylic acid	-COOH	-OH	-H	-Cl	-H	-H
13. 5-Chloro salicylic acid	-COOH	-OH	-H	-H	-Cl	-H
14. Acetyl salicylic acid	-COOH	$-OC(O)CH_3$	-H	-H	-H	-H
15. *m*-Anisic acid	-COOH	-H	$-OCH_3$	-H	-H	-H
16. *p*-Anisic acid	-COOH	-H	-H	$-OCH_3$	-H	-H
17. Salicylamide	$-CONH_2$	-OH	-H	-H	-H	-H
18. Salicylhydroxamic acid	-C(O)NHOH	-OH	-H	-H	-H	-H
19. Anisole	$-OCH_3$	-H	-H	-H	-H	-H
20. Veratrole	$-OCH_3$	$-OCH_3$	-H	-H	-H	-H
21. Benzyl alcohol	$-CH_2OH$	-H	-H	-H	-H	-H
22. 3,4-Dimethoxybenzyl alcohol	$-CH_2OH$	-H	$-OCH_3$	$-OCH_3$	-H	-H
23. Benzylamine	$-CH_2NH_2$	-H	-H	-H	-H	-H
24. Salicylaldoxime	-CNOH	-OH	-H	-H	-H	-H
25. Benzonitrile	-CN	-H	-H	-H	-H	-H

present study, however, the activity of the NahR protein on the *nagAa* promoter was investigated. This is possible because the binding sequences for the NagR and NahR proteins are nearly identical *(19,30)*.

Materials and Methods

Chemicals

All chemicals are listed in Table 1 and were purchased from Sigma-Aldrich. A stock solution of each chemical was prepared using absolute alcohol (Merck). The stock was diluted to ensure that the alcohol concentration in the samples was <0.25%, a level that did not show any significant response from *E. coli* strain DNT5 (data not shown).

Molecular Cloning Techniques

Ralstonia sp. U2 *(14)* was grown overnight in 3 mL of Luria-Bertani (LB) broth in a 15-mL tube at 30°C and 250 rpm. Using a Soil DNA Extraction Kit from Mo Bio Research, a total of 1.5 mL from the culture was used to purify the chromosomal DNA and to conduct polymerase chain reaction

(PCR) of the regions of interest. All DNA-modifying enzymes were purchased from New England Biolabs except those for PCR, which was performed using a Qiagen HotStart® Kit. The primers used for PCR of *nagR*, the divergent promoter, and the upstream *nagAa* region were NTI (5'-CTTGTCTTCAGCATCAACCTGCTG-3') and NT2 (5'-GGTTCTGGTACC AGTTCCATGCAA-3'). The underlined region corresponds to the *Kpn*I site used later to clone the PCR product into pDEW201 *(25)*. The 2.2-kb PCR product was gel purified (Qiagen Gel Extraction Kit) and ligated into pGEM-T Easy vector from Promega (Madison, WI). After transformation, two colonies were isolated because they had both possible orientations of the insert. One plasmid was selected and named pNTL3 (P_{lac}::*nagR*). The 2-kb *Eco*RI-*Kpn*I fragment was then digested out of pNTL3 using *Eco*RI and *Kpn*I and ligated into pDEW201, which was digested using the same two enzymes. After transformation, bioluminescent colonies were selected and characterized with salicylic acid. A positive clone was grown and its plasmid DNA, pDNT5, purified and confirmed (Fig. 1).

Plasmid pNAGK1 was constructed through several steps. The first was the amplification of the *nagR-nagAa'* region with the primers NL1 (5'-CCGCGTCTAGAATGCTA ATTGAGGGG-3') and NR1 (5'-GTCACCAAT ATGGACCAGGCAACGC-3'), which correspond to the region between nucleotides 973 and 2303 from NCBI accession no. AF036940. The underlined region corresponds to the *Xba*I site used later to clone the PCR product. After removing the *luc* gene from plasmid pSP-Luc+ (Promega) with an *Hind*III and *Xba*I digest, the plasmid backbone was ligated with the NL1-NR1 PCR product digested with the same enzymes, giving plasmid pNAG9 (Fig. 1C). This plasmid was then digested with *Kpn*I and *Eco*RI, and the 1.3-kb fragment was ligated with the 11-kb *Kpn*I and *Eco*RI fragment digested out of pUCD615, which carries the *lux* genes from *V. fischeri (26)*, giving pNAG1. This plasmid conferred resistance only to ampicillin because the kanamycin gene was disrupted. After digesting another prep of pUCD615 with *Kpn*I and purifying the 7-kb fragment, this fragment was ligated into pNAG1, which was also digested with *Kpn*I, and colonies that grew on kanamycin were selected for further tests. The presence of the *nagR* gene and upstream promoter region was confirmed through digestion and PCR (data not shown).

Bacterial Strains and Growth Conditions

The host *E. coli* bacterium used for the characterization experiments was RFM443 (rpsL$^-$ [StrR], galK2, lacΔ74). Plasmid pDNT5 was constructed as described in the previous section and then transformed into RFM443, giving *E. coli* strain DNT5, and colonies were grown on LB agar (Difco, Detroit, MI) with 75 µg/mL of ampicillin added to maintain the plasmid. Single colonies were transferred to 3 mL of LB broth, with ampicillin, and grown overnight in a 15-mL test tube at 30°C. For the flask experiments, 300 µL of the overnight DNT5 cultures was transferred to 50 mL of sterile LB, with ampicillin, in a 250-mL flask and grown under the same condi-

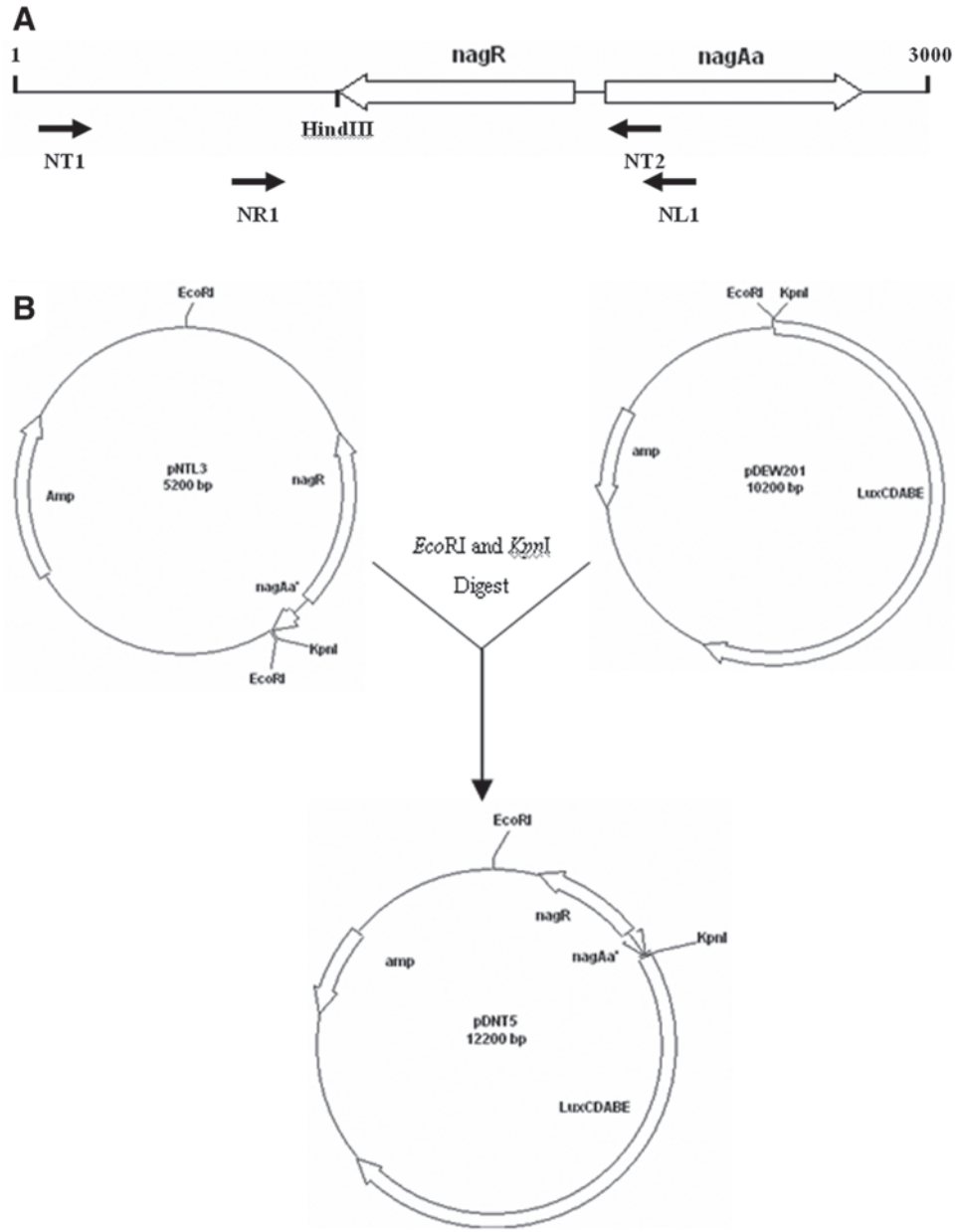

Fig. 1. Construction of plasmids used: **(A)** Direction of *nagR-nagAa* region **(B)** plasmid pDNT5; **(C)** broad-host plasmid pNAGK1. Sizes are approximate *(Fig. 1C continued on next page)*.

tions. When the optical density (OD) reached 0.08 at 600 nm, the chemicals to be tested were added to achieve the final concentration desired. The OD and bioluminescence (BL) of the cultures were then measured using a spectrophotometer and a Turner 20-e Luminometer, respectively, every 20 min for 3 h. The BL was measured using a 100-µL sample of the culture. For the

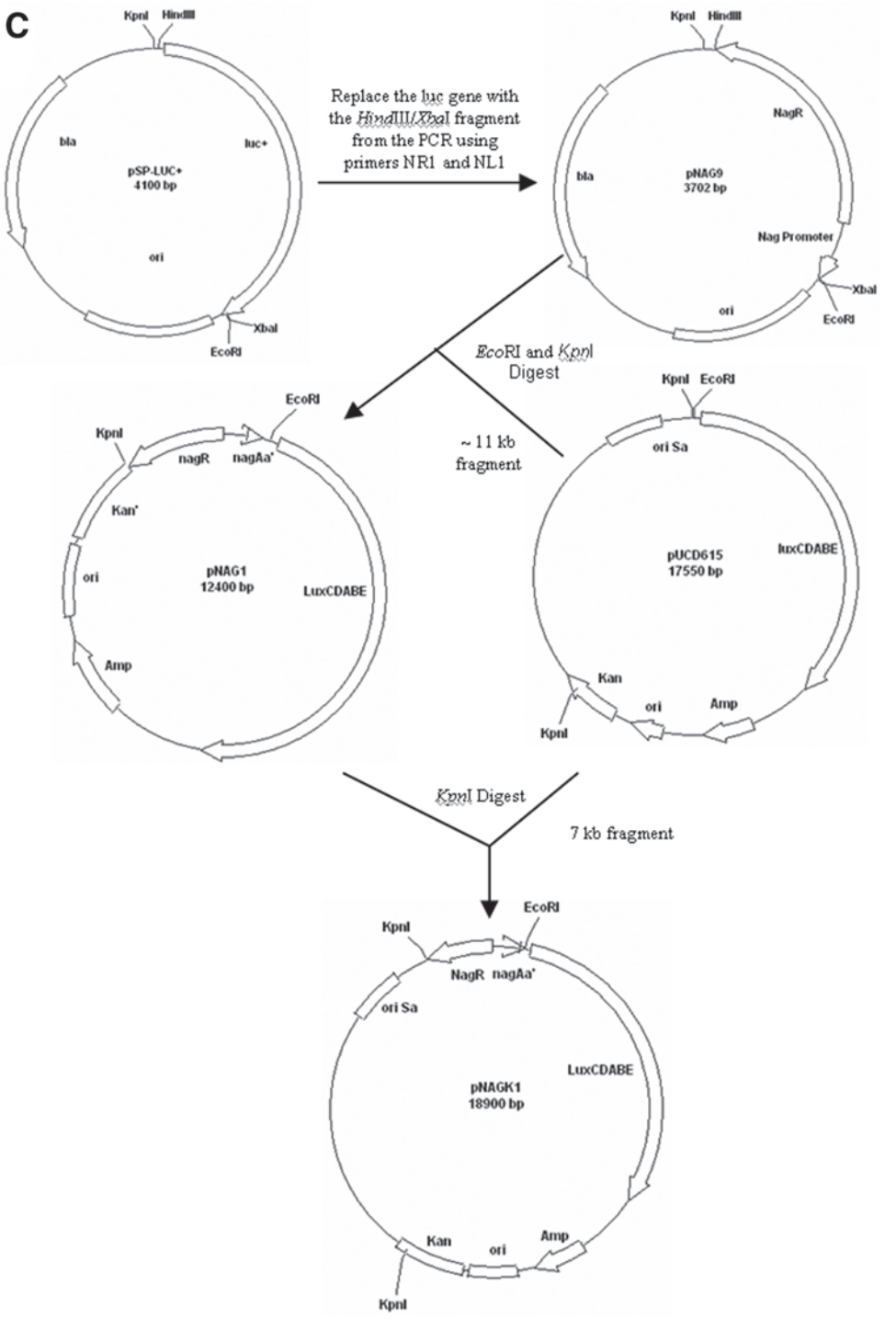

Fig. 1. *(continued)*.

temperature studies, the relative specific bioluminescence (RSBL), defined as the $(BL_{Sample}/OD_{Sample})/(BL_{Control}/OD_{Control})$, was determined when the cultures were at an OD of 0.3 at a wavelength of 600 nm.

Likewise, for the 96-well experiments, after the culture reached an OD of 0.08, 100 µL was transferred to the wells of an opaque 96-well plate (Microplate 1; Dynex) into which the test chemicals had already been serially diluted using LB medium with a final volume within each well (before addition of the culture) of 100 µL *(31)*. The control sample was the same culture mixed with 100 µL of LB medium, i.e., no chemical. A plate luminometer (Microtitre Plate Reader; MLX) was set at a temperature of 30°C, and the speed of the luminometer shaker was set to high for a duration of 1 s. The plate was shaken just before each reading, which was done automatically every 10 min. The results were transferred to Microsoft Excel™ and analyzed and are presented as either the BL or relative bioluminescence (RBL), which is defined as the ratio of the sample's BL to that of the control at the same time point. Responses were considered significant when the RBL exceeded a value of 2.

To develop a biosensor capable of detecting salicylic acid while also degrading it, *P. putida* KCTC1768, which was purchased from the Korean Collection for Type Cultures, was transformed with pNAGK1, giving strain NAGK-1768, and was grown under the same conditions as RFM443 but with kanamycin as the antibiotic resistance marker. This strain is capable of degrading naphthalene because it carries the functional *nah* and *sal* operons.

Data Analysis

All samples were performed in triplicate for error analysis and are the average values from three flasks or samples grown under the same conditions. Although the experiments were repeated, only representative data points are shown for clarity. The standard deviations for the results are shown as error bars within the figures. The minimum detectable concentration (MDC) is defined as the lowest concentration that led to a twofold induction in BL.

Results

Effects of Temperature on Response of E. coli *Strain DNT5 to Salicylic Acid*

In *Ralstonia*, the NagR protein is used to detect the production of salicylic acid. Within *E. coli* strain DNT5, therefore, the presence of salicylic acid will activate the NagR protein and consequently lead to a higher BL. To determine the optimum temperature to detect the presence of 0.5 m*M* salicylic acid with this construct, experiments were performed using flask cultures at various temperatures between 25 and 40°C. Figure 2 shows the RSBL, defined as $(BL_{Sample}/OD_{Sample})/(BL_{Control}/OD_{Control})$, seen at each of the temperatures tested when the DNT5 (RFM443/p*NagR-lux*) cultures were at an OD of 0.3. This value was used to compare the responses because

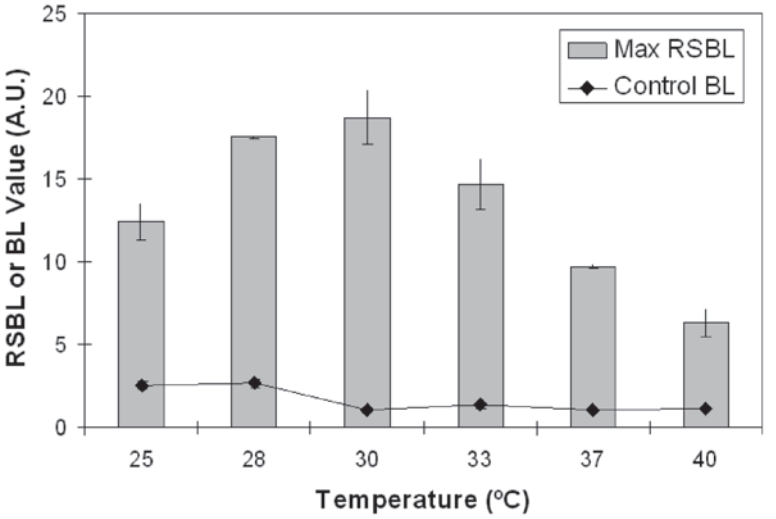

Fig. 2. RSBL responses seen from DNT5 when 0.5 m*M* salicylic acid was added to cultures growing at different temperatures. The RSBL was determined when the cultures were at an OD of 0.3 at 600 nm and is defined in Materials and Methods. The BL from the control flasks at the same time point is also shown. Tests were performed in flasks and were done independently on separate days. A.U., arbitrary units.

it takes into consideration both the bioluminescent (BL) induction and the OD and presents the data as the RBL per cell. The results show the greatest response from *E. coli* strain DNT5 when tested at 30°C, whereas above this the RSBL values decreased linearly with further increases in temperature, with approx 30% activity at 40°C. This loss cannot be owing to the stability of the luciferase proteins because they have a maximum activity at 37°C *(25)*, and, thus, this loss in response is believed to be attributable to the activity of the NagR protein. Therefore, all further experiments were performed at 30°C.

Response and Sensitivity of DNT5 to Salicylic Acid

The response from *E. coli* strain DNT5 for a wide range of salicylic acid concentrations was then measured using a 96-well plate format and is shown in Fig. 3. In Fig. 3A, during the first 2 h of exposure the BL increased linearly with time ($R^2 > 0.95$ for all salicylic acid concentrations tested up to 2.5 m*M*). As well, *E. coli* strain DNT5's response time was reasonably quick, requiring only about 20 min after exposure for a significant induction to be seen for some of the higher concentrations of salicylic acid, with the maximum RBL values seen in about 1 h. Furthermore, as shown in Fig. 3B, DNT5 is capable of responding to salicylic acid over a wide range of concentrations. The minimum detectable salicylic acid concentration with *E. coli* strain DNT5 was about 2.4 µ*M*, which led to an approximately twofold induction in the bioluminescent signal. However, when the concentration exceeded 2.5 m*M*, *E. coli* strain DNT5 experienced a severe toxicity owing to the

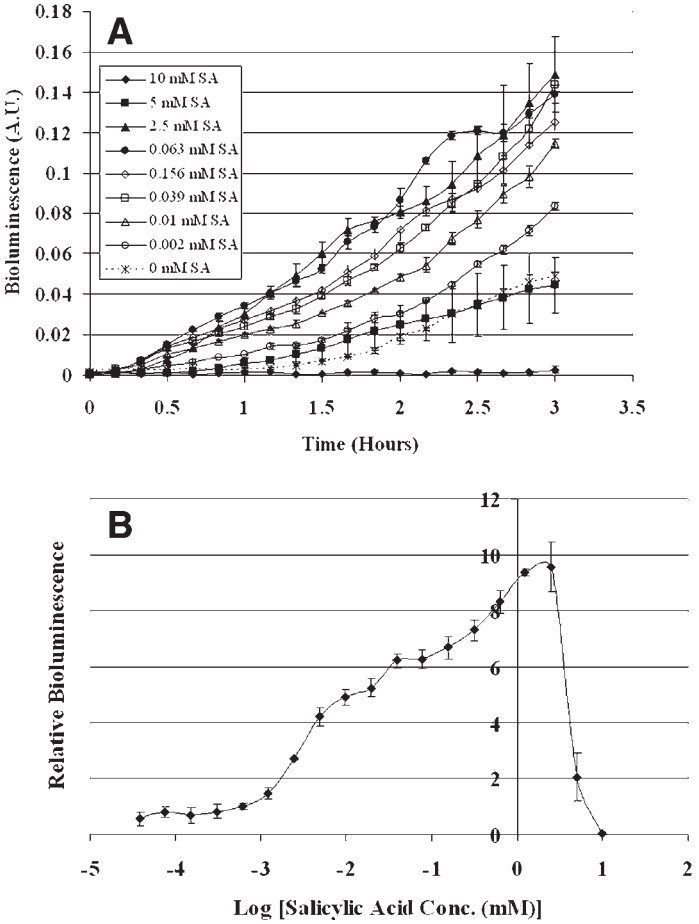

Fig. 3. **(A)** Time-course bioluminescent responses from *E. coli* strain DNT5 with addition of salicylic acid (SA). **(B)** RBL results for each salicylic acid concentration tested. The RBL values were determined using the BL values in (A) at a time of 1.5 h. The RBL is defined as the $BL_{Sample}/BL_{Control}$ for the same time point. Tests were performed in a 96-well luminometer. A.U., arbitrary units.

uncoupling action of salicylic acid on bacteria and, consequently, this strain's ability to respond was greatly hindered (Fig. 3).

Response of DNT5 to Various Salicylic Acid Derivatives

Because it was previously reported that the NahR protein can detect numerous salicylic acid derivatives *(20)*, in this study the chemical promiscuity of the NagR protein was tested using the 25 compounds listed in Table 1. Figure 4 presents the maximum RBL values seen from *E. coli* strain DNT5 when exposed to each of these compounds. It is obvious that the NagR protein is not very wanton in its recognition and responsiveness to the different effectors, because of the 25 compounds tested, only 5 showed a significant induction, i.e., an RBL value of at least 2. Of these, two were

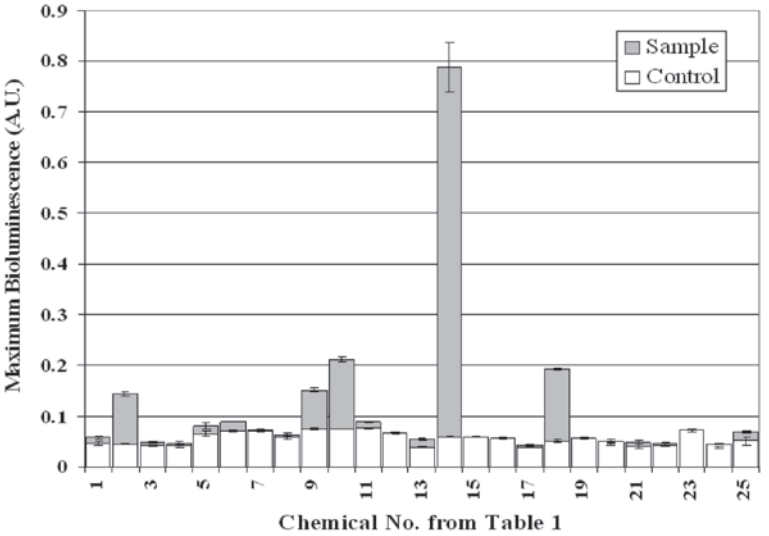

Fig. 4. Maximum bioluminescent responses from *E. coli* strain DNT5 when exposed to various benzoic acid derivatives. The BL values were taken at a time of 3 h. The maximum BL from the control wells is shown as well for comparison. Tests were performed in a 96-well luminometer. A.U., arbitrary units.

Table 2
Concentrations of Chemicals Leading to Maximum Response and an RBL of 2 From *E. coli* Strain DNT5

Chemical	MDC (μ*M*)[a]	MRC (μ*M*)[b]
Salicylic acid	2.4	2500
3-Methyl salicylic acid	3	780
4-Methyl salicylic acid	50	780
Acetyl salicylic acid	24	1560
Salicylhydroxamic acid	11.4	102.9

[a]MDC, minimum detectable concentration—the lowest concentration showing an RBL value of at least 2.

[b]MRC, maximum responsive concentration—the concentration showing the highest RBL value.

found to be more potent inducers than the native inducer, salicylic acid, with the strongest being acetyl salicylic acid. The concentrations of these five compounds that gave the highest response, as well as their MDCs, are all listed in Table 2. The MDC values for these compounds show that salicylic acid was detected more effectively than the other compounds, with an MDC of approx 2 μ*M* (Table 2 and Fig. 3), and that acetyl salicylic acid, the strongest inducer of *E. coli* strain DNT5, also had the highest MDC.

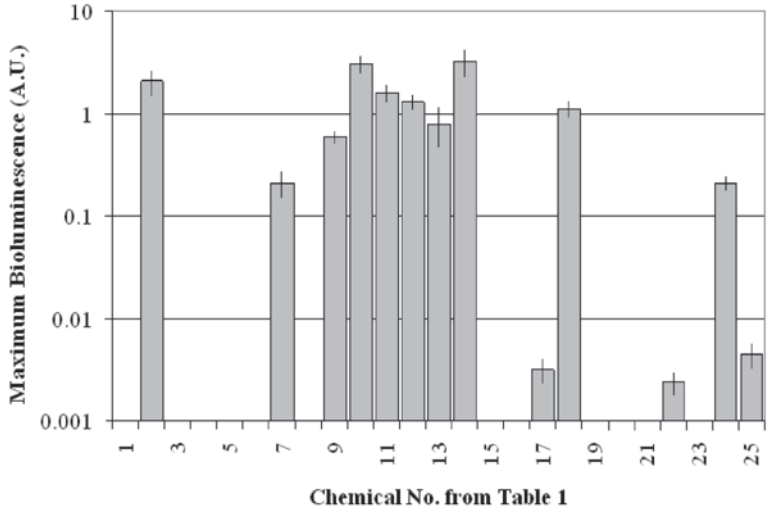

Fig. 5. Maximum BL values seen from strain NAGK-1768 when exposed to chemicals listed in Table 1. The BL values are from a time of 3 h. The maximum BL from the control wells cannot be shown because they were always below the lower limit of detection (approx 0.001 A.U.). Tests were performed in a 96-well luminometer. A.U., arbitrary units.

Construction of Plasmid pNAGK1 and Its Use Within Strain Pseudomonas putida *KCTC1768*

To use the responses of the NagR protein in the detection of salicylic derivatives within other bacterial strains, plasmid pNAGK was constructed using the broad-host range plasmid pUCD615 *(26)* because the origin of replication in plasmid pDEW201 does not permit this plasmid to replicate in *Pseudomonads*. Although attempts to transform *Ralstonia* sp. U2 were not successful, the recognition sequences for the NagR and NahR proteins are nearly identical *(19,30)*, suggesting that the NahR protein should bind to and activate the *nagAa* promoter within plasmid pNAGK. Therefore, an NahR strain, *P. putida* KCTC1768, was transformed with this plasmid, and its bioluminescent responses to the chemicals listed in Table 1 were characterized (Fig. 5). The values are listed as the maximum BL, not the RBL, because the control's BL was consistently below the lower limit of the plate luminometer, making the determination of a reliable RBL value almost impossible. A comparison between the responses of *E. coli* strain DNT5 and this strain, NAGK-1768, clearly shows that NAGK-1768 is more promiscuous and responds to the presence of 13 compounds, as opposed to the 5 recognized by *E. coli* strain DNT5. This broader effector recognition may be attributed to the activity of the NahR protein, which was shown previously to recognize several of these salicylic acid derivatives *(20)*, on the *nagAa* promoter. Another difference that may be attributed to the NahR activity is the more sensitive response of this strain to salicylic acid, with significantly higher BLs seen when strain NAGK-1768 was exposed to only

about 0.03 μ*M* salicylic acid (data not shown). Moreover, tests with colonies grown on LB agar for 2 d showed them to be highly responsive to naphthalene vapors ($BL_{Exposed}$ 20/$BL_{Unexposed}$ 0.01), making this strain useful for the detection of both naphthalene and various derivatives of salicylic acid.

Discussion

In nature, numerous bacterial species are capable of degrading the recalcitrant PAH naphthalene. Although the regulatory protein is typically NahR, a novel LysR-type regulatory protein, NagR, was recently discovered in *Ralstonia* sp. U2. In the present study, the NagR protein was initially characterized with respect to its temperature dependence, response kinetics, sensitivity, and recognition of different derivatives of benzoic and salicylic acid using a fusion with the *lux* operon from *P. luminescens*. The optimum temperature for detecting salicylic acid was found to be 30°C, which is slightly higher than the optimum growth temperature for *Ralstonia* sp. U2, 28°C. However, the NagR protein was still responsive at a temperature of 40°C, with about 30% its maximum activity, based on the RSBL values. Furthermore, in tests with different concentrations of salicylic acid, which is the native inducer within *Ralstonia* sp. U2, the bioluminescent signal increased consistently over time, with significant BL values seen only 20 min after its addition. These responses were also dose dependent, with a maximum response at a concentration of 2.5 m*M* and the MDC for salicylic acid of approx 2.4 μ*M*, which gave an RBL value of just more than 2.0. When the salicylic acid concentration exceeded 2.5 m*M*, the responses from *E. coli* strain DNT5 were stunted owing to the inherent toxicity of this compound. Therefore, tests with high concentrations of salicylic acid and its derivatives should be performed using serial dilutions to ensure a proper exposure and response.

Tests with different benzoic acid and salicylic acid derivatives found only a small handful of chemicals that led to increased promoter activation. Of the five that led to an enhanced P_{nagAa} activity, 4-methylsalicylic acid and acetylsalicylic acid were more potent inducers than salicylic acid. However, both also had significantly higher MDC values than salicylic acid, which was detected the most sensitively. A comparison with one previous study of the NahR protein and its effector recognition reveals that the NagR protein is much more specific in its affinity for and activation by different compounds; eight of the chemicals tested in the present study (no. 2, 6, and 9–14 in Table 1) were previously tested with an *nahR-lacZ* fusion strain *(20)*, and all eight of these compounds led to a significant induction of β-galactosidase activity in the previous study but only four led to an enhanced BL from *E. coli* strain DNT5 in this study. However, this is likely attributed to differences in the NagR and NahR amino acid sequences, which are only 57% identical and 73% similar. Another recent study also examined effector recognition and the NagR protein *(24)*. In that study, a fusion of the *nagR*

gene and upstream divergent promoter region to the *lacZ* gene was used and also eight of the compounds used here were tested. All except 4 methylsalicylate showed similar responses. However, note that the concentration used was 1 m*M*, which was slightly higher than the maximum inducible concentration found in our study. Therefore, the lack of induction in the previous study may be due owing in part to the inherent toxicity of the sample, which would lead to cell death and an overall lower response ratio.

The results with strain NAGK-1768 indirectly show how poorly strain DNT5 responds to each chemical: the range of chemicals, relative induction, and maximum BL were all higher with strain NAGK-1768. Although a different host was used, including a different set of *lux* genes, and thus a direct comparison between the activities of the NagR and NahR proteins is not possible, a study previously done in our laboratory showed the *V. fischeri* fusion strains to be generally less bioluminescent than their *P. luminescens* counterparts *(32)*. When comparing the maximum values in Fig. 4 with those in Fig. 5, the fact that the maximum BL from *E. coli* strain DNT5 was only about 0.15 A.U. whereas that of strain NAGK-1768 was 2.1 A.U. when both strains were exposed to salicylic acid strongly suggests that the NahR protein within strain NAGK-1768 is not only more promiscuous, but it is far more responsive. This greater activity is also seen in the MDC of strain NAGK-1768, which is capable of detecting salicylic acid at a concentration 100 times lower than the MDC of *E. coli* strain DNT5.

Several other studies have also examined the use of *nahR-lux* fusions to study the degradation of naphthalene and salicylic acid *(27–29)*. In each of these studies, the backbone of the plasmid was pUCD615, the same plasmid used to construct pNAGK. One major difference, however, is the presence of the *nagAa* promoter and its being acted on *in trans* by the NahR protein. This is possible because the binding sequences for the NagR and NahR proteins are nearly identical *(19,30)*. As with the constructs in the other studies, strain NAGK-1768 responded to salicylic acid and naphthalene vapors, as well as numerous salicylic acid derivatives.

Conclusion

A novel strain carrying a fusion of the *nagR* gene from *Ralstonia* sp. U2 fused to the *lux* genes present within plasmid pDEW201 was constructed and characterized. This study focused on the temperature dependence and response to different chemical effectors. It was shown that *E. coli* strain DNT5 is responsive to several derivatives of salicylic acid but, for the most part, was very specific in its recognition. Salicylic acid, as the native inducer within *Ralstonia* sp. U2, was the most sensitive inducer, with significant bioluminescent responses seen at concentrations down to about 2 µ*M*. However, this was not the most potent inducer. Acetyl salicylic acid led to a higher BL level from *E. coli* strain DNT5 when compared with salicylic acid, as did 4-methyl salicylic acid. However, both also had significantly higher MDC levels. Finally, a new plasmid, pNAGK1, was successfully

transformed into *P. putida* strain KCTC1768, giving a recombinant strain that can detect naphthalene vapors as well as a wider range of salicylic acid derivatives owing to the more promiscuous nature of the NahR protein.

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References

1. Garcia-Valdes, E., Cozar, E., Rotger, R., Lalucat, J., and Ursing, J. (1988), *Appl. Environ. Microbiol*. **54,** 2478–2485.
2. Grund, A. D. and Gunsalus, I. C. (1983), *J. Bacteriol*. **156,** 89–94.
3. Kuhm, A. E., Stolz, A., and Knackmuss, H. J. (1991), *Biodegradation* **2,** 115–120.
4. Mihelcic, J. R. and Luthy, R. G. (1988), *Appl. Environ. Microbiol*. **54,** 1188–1198.
5. Kiyohara, H., Nagao, K., Kouno, K., and Yano K. (1982), *Appl. Environ. Microbiol*. **43,** 458–461.
6. Weissenfels, W. D., Beyer, M., and Klein, J. (1990), *Appl. Microbiol. Biotechnol*. **32,** 479–484.
7. Grifoll, M., Selifonov, S. A., Gatlin, C. V., and Chapman, P. J. (1995), *Appl. Environ. Microbiol*. **61,** 3711–3723.
8. Heitkamp, M. A., Franklin, W., and Cerniglia, C. E. (1988), *Appl. Environ. Microbiol*. **54,** 2549–2555.
9. Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W., and Warshawsky, D. (1996), *Appl. Environ. Microbiol*. **62,** 13–19.
10. Stringfellow, W. T. and Aitken, M. D. (1995), *Appl. Environ. Microbiol*. 61**,** 357–362.
11. Sanseverino, J., Applegate, B. M., King, J. M., and Sayler, G. S. (1993), *Appl. Environ. Microbiol*. **59,** 1931–1937.
12. Tsuda, M. and Iino, T. (1990), *Mol. Gen. Genet*. **223,** 33–39.
13. Yen, K. M. and Gunsalus, I. C. (1985), *J. Bacteriol*. **162,** 1008–1013.
14. Zhou, N. Y., Fuenmayor, S. L., and Williams, P. A. (2001), *J. Bacteriol*. **183,** 700–708.
15. Yen, K. M. and Serdar, C. M. (1988), *Crit. Rev. Microbiol*. **15,** 247–268.
16. Huang, J. Z. and Schell, M. A. (1991), *J. Biol. Chem*. **266,** 10830–8.
17. Schell, M. A. (1985), *Gene* **36,** 301–309.
18. Schell, M. A. (1986), *Proc. Natl. Acad. Sci. USA* **83,** 369–373.
19. Schell, M. A. and Poser, E. F. (1989), *J. Bacteriol*. **171,** 837–846.
20. Cebolla, A., Sousa, C., and de Lorenzo, V. (1997), *J. Biol. Chem*. **272,** 3986–3892.
21. Schell, M. A. and Wender, P. E. (1986), *J Bacteriol*. **166,** 9–14.
22. You, I. S., Ghosal, D., and Gunsalus, I. C. (1988), *J. Bacteriol*. **170,** 5409–5415.
23. Schell, M. A., Brown, P. H., and Raju, S. (1990), *J. Biol. Chem*. **265,** 3844–3850.
24. Jones, R. M., Britt-Compton, B., and Williams, P. A. (2003), *J. Bacteriol*. **185,** 5847–5853.
25. Van Dyk, T. K. and Rosson, R. A. (1998), in *Methods in Molecular Biology*: *Bioluminescence Methods and Protocols*, vol. 102, LaRossa, R. A., ed., Humana, Totowa, NJ, pp. 85–95.
26. Rogowsky, P. M., Close, T. J., Chimera, J. A., Shaw, J. J., and Kado, C. I. (1987), *J. Bacteriol*. **169,** 5101–5112.
27. Burlage, R. S., Sayler G. S., and Larimer F. (1990), *J. Bacteriol.* **172,** 4749–4757.
28. King, J. M. H., DiGrazia, P. M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F., and Sayler, G. S. (1991), *Science* **249,** 778**–**781.

29. Heitzer, A., Webb, O. F., Thonnard, J. E., and Sayler, G. S. (1992), *Appl. Environ. Microbiol*. **58,** 1839–1846.
30. Fuenmayor, S. L., Wild, M., Boyes, A. L., and Williams, P. A. (1998), *J. Bacteriol*. **180,** 2522–2530.
31. Lee, H. Y., Choi, S. H., and Gu, M. B. (2003), *Biotechnol. Bioprocess. Eng.* **8,** 101–105.
32. Mitchell, R. J., Ahn, J. M., and Gu, M. B. (2005), *J. Microbiol. Biotech.*, in press.