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# Bioluminescent Reporters for Catabolic Gene Expression and Pollutant Bioavailability



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Running head: Pollutant Bioavailability, *lux*-gene fusion

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## INTRODUCTION

The biodegradation of polyaromatic hydrocarbon pollutants is frequently limited due to their poor bioavailability which is caused by low aqueous solubilities and high adsorption to soil particles. For an efficient process optimization to biologically decontaminate polluted soils, it is therefore important to develop biological methods to monitor pollutant bioavailability and degradation. For this purpose, naphthalene has been chosen as a model compound because of the well established biochemical and genetic determinants of its degradation (8). Recently, two bioluminescent catabolic reporter bacteria have been constructed to monitor catabolism of naphthalene and its degradation intermediate salicylate, which acts as an inducer of the biodegradation pathway (1,5). One of these reporter strains, *Pseudomonas fluorescens* HK44, contains a transcriptional gene fusion between the *nahG* gene and a *luxCDABE* gene cassette from *Vibrio fischeri* (5) located in a reporter plasmid. Since this strain contains the *luxAB* genes encoding luciferase as well as the *luxCDE* genes necessary for aldehyde synthesis, no exogenous addition of the substrate aldehyde is necessary (6). Exposure of this strain to either naphthalene, salicylate or anthranilate results in an increase in *nahG* gene expression which can be measured in a growing culture *in situ*, on-line, using a photomultiplier in conjunction with a fiber-optic cable. Since such increased bioluminescence reflects both, the transport of the pollutant into the bacterial cell and the activation of catabolic gene expression, it can be used as a measure for the presence, bioavailability and biodegradation of naphthalene and salicylate.

## RESULTS AND DISCUSSION

Experiments to investigate the application of *P. fluorescens* HK44 for the quantitative and specific detection of naphthalene and salicylate bioavailability have been conducted and are

summarized in Table 1. The reporter strain was used in different physiological states, namely: as starved culture, as immobilized culture and as exponentially growing culture.

Starved cultures were obtained by harvesting exponentially growing cultures at a predefined optical density and resuspending the washed culture in an equal volume of buffered mineral salts medium without any carbon source for 3 hours. Such cultures responded rapidly and quantitatively within 0.1 and 1 h upon exposure to either naphthalene or salicylate. The experiments were conducted in 25 ml mineralization vials containing 2ml test solution and 2ml bacterial culture with an optical density of 0.35 at 546nm. However, exposure of these cultures to either 0.55mM acetate, 0.55mM succinate or 0.55mM glucose resulted in significant *nahG* gene expression, measured as bioluminescence increase, compared to the control without any carbon substrate. This observation can be explained by a low, but significant basal expression level of both naphthalene operons in growing bacterial cultures (7). The presence of readily utilizable carbon substrates enables the reporter bacteria to grow depending on the amount and type of carbon substrate. Therefore, this approach may not be convenient for further use in bioavailabilty assays, because a response to a low target substrate concentration could not be distinguished from a bioluminescence increase caused by readily utilizable carbon substrates without the additional measurement of biomass increase, which can be difficult and time consuming in complex environmental samples.

In contrast, such effects were found to be less problematic when cultures, immobilized in alginate were used. The immobilization procedure was done as outlined in reference 4. A good linear relationship between naphthalene or salicylate concentration and the maximum light response was obtained with suspended alginate beads. Figure 1 shows such a calibration curve for different salicylate concentrations. While the exposure of alginate entrapped cells to 1 mM concentrations

of either acetate, succinate or glucose as sole carbon substrates resulted only in a minor increase in culture bioluminescence, dual substrate systems, such as 1mM glucose + 0.05mM salicylate etc., resulted in a carbon substrate dependent increase in the total light response to a given salicylate concentration. The response to the mixture containing acetate was indistinguishable from the response to salicylate alone while addition of succinate resulted in about a doubling and glucose in an inrease by a factor of 3 as compared to the salicylate control. The addition of a complex medium containing 12.5 mg/l yeast extract and 125 mg/l peptone resulted in a similar maximal response as exposure to glucose. The physiological basis of these observations remains to be elucidated. The results suggest that the presence of a readily utilizable carbon substrate might have an effect on the biochemical reactions involved in the bioluminescence response. In addition, it is also possible that slow bacterial growth in the alginate matrix ocurred. It was therefore concluded, that the addition of a rich carbon substrate would be an advantage to avoid unspecific responses due to readily utilizable carbon substrates. Further, maintaining the reporter culture under conditions where the *nah* operon is expressed at the constitutive low basal level should be of an additional advantage.

Therefore, further experiments were conducted using exponentially growing cultures of *P. fluorescens* HK44 in a rich yeast extract-, peptone-, glucose-medium. Cultures were grown to a defined optical density in the exponential growth phase and subjected to a variety of carbon substrates which did not result in any bioluminescence changes as compared to a control. A calibration curve for naphthalene is shown in Figure 2. Light readings were taken after 1 hour exposure to naphthalene at the concentrations indicated. A linear relationship could be established for naphthalene concentrations between 0.045 mg/l and 5.6 mg/l, reflecting about two orders of magnitude. With this method a series of soil aqueous extracts from polluted sites were

investigated. A significant bioluminescence response could be observed, reflecting the bioavailability of naphthalene. The presence of naphthalene in the samples was analytically confirmed. These results are very promising with respect to further environmental applications of this assay method. However, questions concerning possible other compounds which might induce a *nah*-gene expression because they are degraded through the same catabolic routes (2), have to be investigated. In addition, the possible effects of other pollutants such as heavy metals on the bioluminescence reaction have to be addressed.

## SUMMARY AND OUTLOOK

The application of visualized catabolic *nah*-gene expression using a *luxCDABE* gene fusion provides a valuable method to measure quantitatively and specifically naphthalene and salicylate bioavailability. It has been demonstrated that the physiological state of the test culture together with the intrinsic regulation mechanisms of the naphthalene degradation pathway as well as the physiological aspects of the *lux* gene fusion have to be taken into account.

The method presented provides a high potential for *in situ* bioprocess monitoring. In addition, the results obtained with immobilized cells provide a basis for the development of biosensors for environmental applications in specific pollutant monitoring in waste streams and soil slurry systems but, as a general method, also for more conventional biotechnological process control.

## ACKNOWLEDGEMENTS

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## DISCLAIMER

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FIGURE LEGENDS

Figure 1. Relationship between salicylate concentration and *nahG* gene expression, measured as bioluminescence in alginate immobilized *Pseudomonas fluorescens* HK44. For the assay 0.5 g beads, containing ca. $4x10^9$ cells, suspended in a total liquid volume of 4 ml were used. Assay temperature and pH were 27°C and 7, respectively. a) Time course of the culture bioluminescence after exposure to different salicylate concentrations. b) Bioluminescence peak levels plotted against salicylate concentration.

Figure 2. Relationship between naphthalene concentration and *nahG* gene expression, measured as bioluminescence in exponentially growing *Pseudomonas fluorescens* HK44. Measurements were taken 1 hour after incubation. The biomass concentration, measured as optical density at 546nm was for all samples 0.355 at the bioluminescence reading time. Assay temperature and pH were 27°C and 7, respectively.

**TABLE 1.** Comparison[a] of different culture preparation methods for the application of the bioluminescent naphthalene catabolism reporter *Pseudomonas fluorescens* HK44 to quantitatively and specifically assess naphthalene and salicylate bioavailability.

| **Physiological culture state** | **Measurement method** | **Linear range tested so far** | | **Specificity in complex substrate mixtures** [a] |
|---|---|---|---|---|
| | | **Salicylate** | **Naphthalene** | |
| **Starved cells** | reading after defined incubation period | n.t. | 0.5 - 50%[b] | Readily utilizable carbon substrates other than naphthalene or salicylate can cause a light response and cellular growth which reduces the specificity and makes a quantitative interpretation of the data difficult |
| **Immobilized cells** (suspended alginate beads) | maximum peak level | 1.7- 27.6ppm | 0.8 - 25%[b] | The quantitative light response to a given salicylate concentration can be affected by the presence of readily utilizable carbon substrates. The presence of such carbon sources alone results in minor light responses as compared to starved cells |
| **Exponentially growing cells** | reading after defined incubation period | 0.4- 13.8ppm | 0.045-5.6ppm | No unspecific responses with other carbon substrates have been observed |

[a] For more details see text.
[b] Data are given as percent of a saturated naphthalene solution at 25°C which contains 31.7 mg/l (3).
n.t. not tested

Figure 1

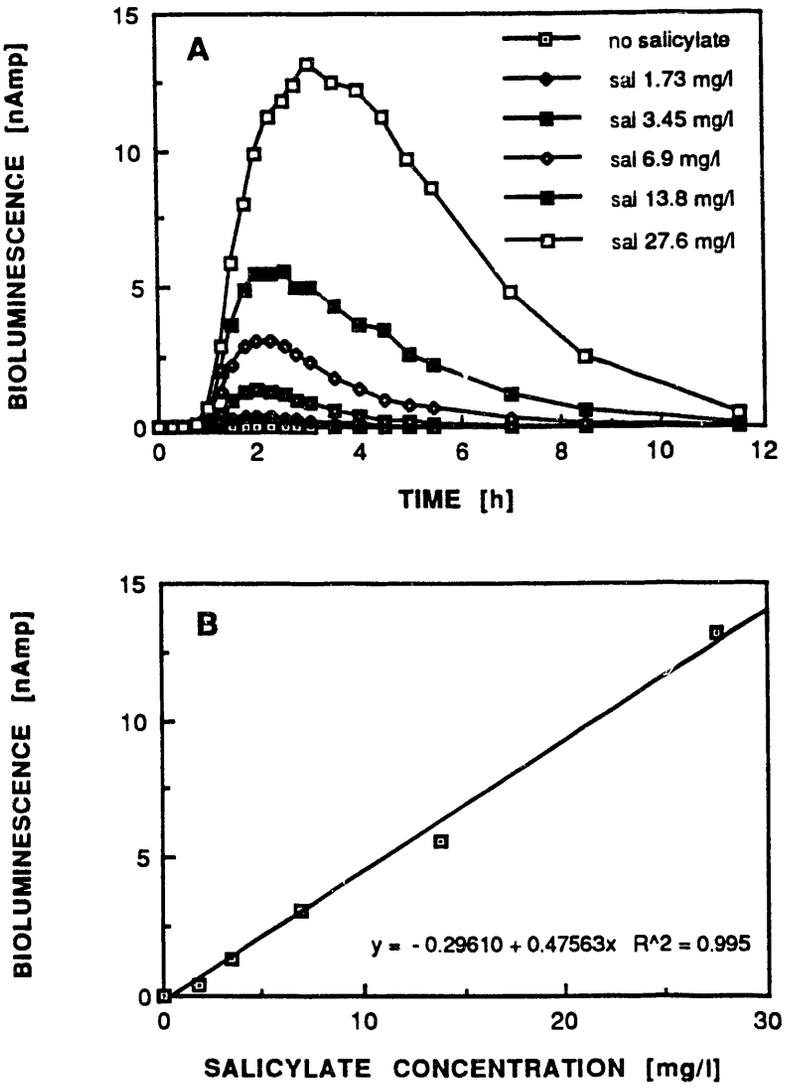
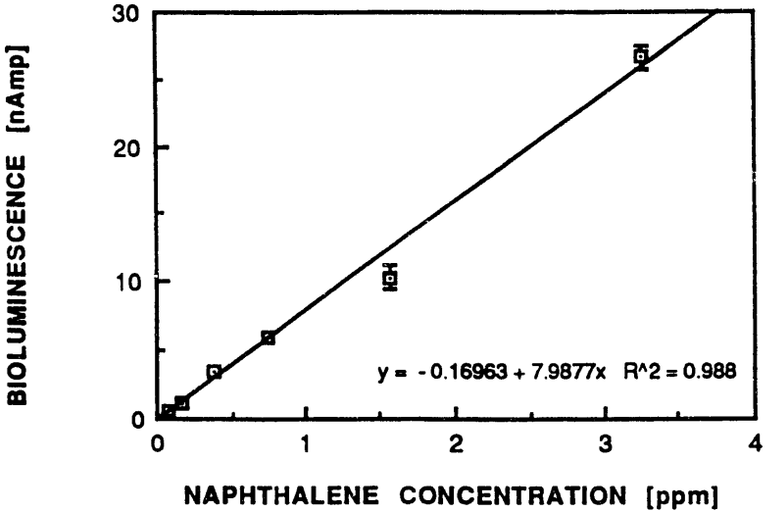

A
no salicylate
sal 1.73 mg/l
sal 3.45 mg/l
sal 6.9 mg/l
sal 13.8 mg/l
sal 27.6 mg/l
BIOLUMINESCENCE [nAmp]
TIME [h]
0
5
10
15
0
2
4
6
8
10
12
B
y = - 0.29610 + 0.47563x R^2 = 0.995
BIOLUMINESCENCE [nAmp]
SALICYLATE CONCENTRATION [mg/l]
0
5
10
15
0
10
20
30

Figure 2


BIOLUMINESCENCE [nAmp]
30
20
10
0
0
1
2
3
4
y = -0.16963 + 7.9877x R^2 = 0.988
NAPHTHALENE CONCENTRATION [ppm]

# END

# DATE FILMED

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