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Sphingomonas paucimobilis is capable of modifying polycyclic aromatic compounds present at sub-nanomolar concentrations in a complex medium

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Abstract: *Sphingomonas paucimobilis was incubated in a complex medium amended with 95 pM to 950 nM fluorescein or resorufin. The samples were centrifuged and the supernatants analyzed by capillary electrophoresis utilizing laser-induced fluorescence detection. The growing cells failed to affect the fluorescein concentration but decreased the resorufin concentration by approximately 90% at all concentrations tested. The decrease in resorufin was due to conversion into a non-fluorescent product rather than adsorption to the cells.*

Key words: *Biodegradation, Capillary electrophoresis, Nanomolar, Polycyclic aromatic, Sphingomonas paucimobilis.*

Introduction

Contamination of soils with persistent organic pollutants is of considerable environmental concern and their degradation by bacteria has been studied as a cost-effective means of remediation (Perei *et al.*, 2001; Kharoune *et al.*, 2002; Bosma *et al.*, 2002). Studies have used polycyclic aromatic hydrocarbons (Baldwin *et al.*, 2000; Johnsen *et al.*, 2002), individual pollutants as the sole carbon source, mixtures of different pollutants (Baraniecki *et al.*, 2002), pollutants in the presence of other carbon sources (Johri *et al.*, 2000), and the presence of surfactants (Luning Prak and Pritchard., 2002). In these studies the pollutants were present at relatively high concentrations. Since soils containing even low amounts of many pollutants are still considered contaminated, an interesting question is whether bacteria can chemically modify such compounds when present at trace levels. Such a condition may be further complicated by several alternative carbon sources being present at much higher levels. This study was undertaken to determine if a test bacteria, *Sphingomonas paucimobilis*, is capable of modifying test polycyclic aromatic compounds, resorufin and fluorescein, when present at nanomolar concentrations in a complex media.

Materials and Methods

Organism: *Sphingomonas paucimobilis* was isolated from the pipe biofilm of a drinking water fountain.

Chemicals: Fluorescein and resorufin were obtained from Molecular probes (Eugene, OR). Yeast extract and hydrolyzed casein were from Becton Dickinson (Cockeysville, MD) and Difco (Detroit, MI) respectively. All other reagents were from Sigma (St. Louis, MO).

Instrumentation: Samples were analyzed using an in-laboratory constructed capillary electrophoresis (CE) instrument equipped with laser-induced fluorescence detection. Details of the instrument have been published (Zhao *et al*, 1992; Chen and Dovichi, 1994). Sheath and running buffers were 40 mM sodium borate (pH 9.3). A 30 cm long, 10 µm internal diameter, 145 µm external diameter fused silica capillary was used. Five second, 167 Vcm^{-1} electrokinetic injections were performed followed by electrophoretic separation at 600 Vcm^{-1}. Excitation was by the 15 mW output at 488 nm of an Ar^{+} laser (fluorescein) or the 1 mW ouput at 543.5 nm of a HeNe laser (resorufin). Emission was passed through a 535df45 (fluorescein) or 580df40 (resorufin) optical filter and detected using a photomultiplier tube.

Assay: *S. paucimobilis* was grown aerobically in 9.1 g/l M9 salts, 9.1 g/l glucose, 0.91 g/l hydrolyzed casein and 0.91 g/l yeast extract at 30 ºC with constant shaking at 50 rpm for 2 days. Prior to use, the 2-day culture was mixed with an equal volume of fresh medium and further incubated 2 hr. This stock culture was used to inoculate the samples.

The stock culture was diluted 20-fold with fresh medium containing 95 pM, 950 pM, 9.5 nM, 95 nM and 950 nM fluorescein. Sterile controls were run. Samples were made in triplicate and incubated with 50 rpm shaking at 30 ºC for 4 days. At the end of the incubation period the optical density (550 nm) of the culture was approximately 2. A similar series of experiments was performed using resorufin rather than fluorescein and a 67 hr incubation.

Samples were also prepared without dye and incubated at 30ºC for 67 hr. These samples were heated to 95ºC for 15 min and cooled. Resorufin was then added to 1.0 µM, 10 nM and 100 pM and the samples incubated at 30ºC for 22 hr. Controls contained no *S. paucimobilis*.

An aliquot of each sample was centrifuged at 13,000xg and the supernatant analyzed by CE-LIF. A second aliquot of each sample was mixed with an equal volume of DMSO, incubated at 30 ºC for 1 hr and centrifuged prior to CE analysis of the supernatant.

In order to avoid saturation of the detector, all samples 95 and 950 nM dye were diluted 10 and 100 fold in running buffer prior to injection.

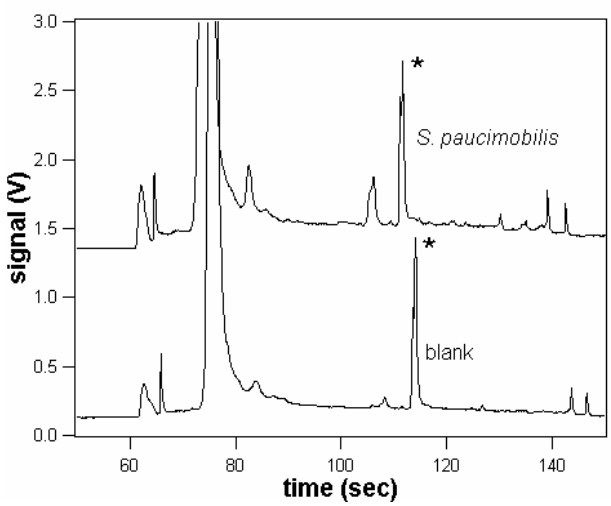

Fig. 1: Electropherogram of the incubation of a medium containing fluorescein with *S. paucimobilis.* Medium containing 95 pM fluorescein was incubated with S. paucimobilis for 4 days. The sample was centrifuged and the supernatent analyzed by CE-LIF. The resultant electropherogram is shown (top). The lower trace is that of a blank which contained no bacteria but was otherwise treated identically. The fluorescein peaks are denoted with an asterix.

Results and Discussion

Measurement of the change in concentration of a compound of interest present at very low concentration in the presence of a myriad of many other compounds present at concentrations that are an order of magnitude higher, which is also changing in concentration, is a challenging task. Such a task is greatly simplified if the compounds of interest exhibit strong molecular fluorescence and this property is not shared with the large majority of the other components of the sample. In this study resorufin and fluorescein were used as test compounds. Both are a polycyclic aromatic compound, which is a class of compounds that includes many persistent organic pollutants, and exhibit strong molecular fluorescence, although neither compound is typically considered itself to be a pollutant.

This assay can not measure if the complete degradation of the test compounds occurs. What can be observed is conversion into compounds of different electrophoretic mobilities or disappearance of signal due to conversion to a compound that does not fluoresce under the conditions used. It was not the objective of the current study to determine what products the dyes were converted into, nor the properties of these products. The objective was to determine if it is possible for bacteria to modify the test polycyclic aromatic compounds present at trace levels in a complex medium.

Fig. 1 shows the electropherogram of the medium containing 95 pM fluorescein incubated in with *S. paucimobilis* for 4 days at 30 ºC. There was no substantial difference in the amount of fluorescein detected relative to the control, which contained no *S. paucimobilis*. This was observed at all concentrations used. The splitting of the fluorescein peak was due to the large differences in the composition of the sample and running buffers, particularly with respect to conductive solutes, and is not uncommon with CE separations using electrokinetic injections. This was not observed when the samples were diluted with running buffer prior to injection, which made the sample and buffer compositions more similar. The shifting of the peak retention time in the sample containing the bacterium relative to the sterile control was because of differences in the sample compositions due to the metabolism of the medium. Again, this is not uncommon with CE separations. This shift in retention time was not observed when the samples were diluted prior to injection Fig. 2 shows the electropherogram of the medium containing 95 pM resorufin incubated in the presence of *S. paucimobilis* for 67 hr at 30 ºC. In the presence of the bacterium the resorufin was reduced to below the detection level. Samples were also prepared containing 950 pM, 9.5 nM, 95 nM and 950 nM resorufin. In these samples, the concentration of resorufin was reduced to 9.8 ± 0.2%, 11.1 ± 0.4%, 9.7 ± 0.6% and 10.8 ± 3.9% relative to that in the sterile control. At the lower 3 concentrations the sample was not diluted prior to analysis and the resorufin was detected as a split peak. In these samples the fraction of dye remaining reported was determined from the height of the first peak. Comparison of the second peak yielded similar results. The height of the set of three peaks seen at a migration time of 130 sec in the sample did not increase with increasing resorufin concentration. This indicates that this cluster was not due to a degradation product of resorufin but rather represents some chemical species that were produced by the growing bacteria.

The use of fluorescent dyes as test compounds in this study had two distinct advantages over the use of non-fluorescent compounds. Firstly, as the objective of this study was to study trace levels of test compounds, a method with high sensitivity, such as fluorescence spectroscopy, was required. Secondly, the measurement of a change in concentration of a particular chemical species in the presence of a very large number of different chemical species, which are also changing in concentration due to the bacterial metabolism, requires a high degree of selectivity. Such a study is greatly simplified if the test compounds provide a signal while the large majority of the remaining compounds do not. Since there are relatively few metabolites that fluoresce under the conditions used and the sensitivity for fluorescein and resorufin is sufficient to allow for sub-nanomolar quantification, these dyes where chosen as test polycyclic aromatic compounds.

A simpler means of performing this study would be to detect fluorescent signal directly from the mixture without a separation. Two major concerns arise with such a strategy. Firstly, fluorescence signal obtained from a sample can be substantially affected by the presence of other molecules which may interact with the fluorophore itself or the emitted light. Differences in the levels of such molecules in the samples and the blanks, due to the action of the bacteria, leads to inaccurate

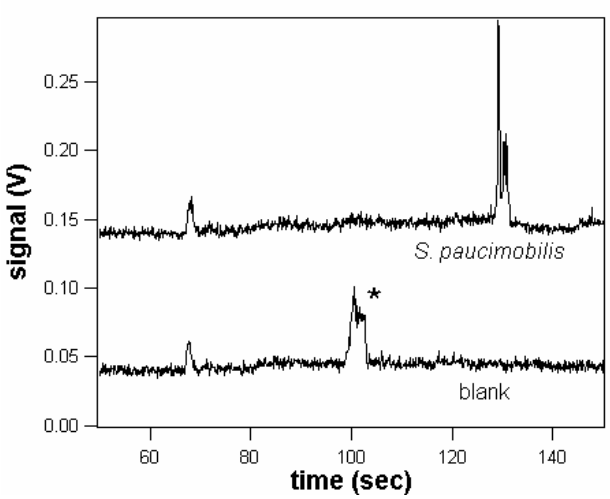

Fig. 2: Electropherogram of the incubation of media containing resorufin with *S. paucimobilis*. Media containing 95 pM resorufin was incubated with *S. paucimobilis* for 67 hours. The sample was centrifuged and the supernatent analyzed by CE-LIF. The resultant electropherogram is shown (top). The lower trace is that of a blank which contained no bacteria but was otherwise treated identically. The resorufin peak is denoted with an asterix.

measurements. A second concern is the production or degradation of other molecules in the medium by the bacterium that produce a detectable signal, which would not occur in the blank. Both concerns can be at least partially alleviated by the separation of the compound of interest from the interfering components.

An additional advantage is improved sensitivity. Detection limits increase with increasing background signals. In the absence of a separation, differences in the fluorescent signal of the molecule of interest must be detected in the presence of the background from the other signal producing molecules. A separation method allows for the detection of the dye in the absence of this background signal, providing a lower detection limit.

Fig. 1 and 2 demonstrate the usefulness of the separation. In figure 1, the peak with a migration time of 75 seconds was greater than 10 V. Of the entire fluorescent signal obtained, only a small fraction was due to fluorescein. In the absence the separation, sensitivity for the fluorescein would have been greatly diminished due to the high background caused by the other signal producing chemical species.

In Fig. 2 there are few species producing signal other than resorufin. In the absence of the bacteria, a resorufin split peak is observed that is not present in the run containing the bacteria. However in the latter there is an additional set of three peaks at a migration time of 130 sec, with a similar area to that of the resorufin peak. In the absence of a separation method these signals would not have been differentiated and would have caused the false conclusion that there was little change in the concentration of resorufin. Figure 1 also shows that the concentrations of the background signal producing compounds did not remain constant.

In order to determine if the decrease in resorufin in the incubation medium was due to adsorption to the bacterial cells rather than degradation, two series of experiments were performed. Samples without resorufin were prepared and incubated in parallel and in an identical manner to those containing resorufin. After the 67 hr incubation the samples were heated to 95 ºC for 15 min to kill the bacteria. Resorufin was then added and the samples re-incubated at 30 ºC for 22 hrs to allow for any adsorption to the dead cells to occur. Blanks contained no *S. paucimobilis*. The samples were centrifuged and the supernatents assayed. Resorufin signal from the samples incubated in 1.0 µM, 10 nM and 100 pM resorufin in the presence of the dead cells was indistinguishable from that of the controls, indicating no detectable adsorption occurred.

Additional aliquots of the samples containing resorufin and *S. paucimobilis* and incubated for 67 hr were mixed with an equal volume of DMSO and re-incubated for 1 hr. Resorufin has a relatively low solubility in water but is very soluble in DMSO. The addition of DMSO was performed to solubilize any adsorbed resorufin. Subsequent assay of the supernatents measured the total resorufin in the sample, both dissolved and adsorbed. Resorufin signal in the presence of the cells showed a similar decrease relative to that in the absence of the cells. This is consistent with the drop in resorufin having been due to degradation by *S. paucimobilis* rather than adsorption.

The bacterium transformed the resorufin into a non-fluorescent product at all of the concentrations used. The lower limit for the resorufin concentration used was set by the sensitivity of the instrument and not the ability of the bacteria to modify it. It may be possible for the bacteria to modify the resorufin at lower concentrations than those used. Fluorescein was not affected by incubation with *S. paucimobilis* at any of the concentrations studied. Presumably this was either due to an inability of this dye to enter the cell or an inability of the cells metabolic machinery to alter it.

In summary, this study was undertaken to determine if bacteria can chemically modify test polycyclic aromatic compounds present at trace levels in a complex medium. *S. paucimobilis* was used as the test bacteria and fluorescein and resorufin as the test compounds and were present at 95 pM to 950 nm. In the case of fluorescein, no alteration in concentration occurred. *S. paucimobilis* caused an approximately 90% decrease in the resorufin present after a 67 hr incubation at 30ºC. This decrease occurred at all concentrations studied. This decrease was not due to adsorption to the bacterium but rather due to the conversion of the dye into a non-fluorescent product.

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