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Detection of Buried Human Remains Using Bioreporter Fluorescence

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Summary

The search for buried human remains is a difficult, laborious and time-consuming task for law enforcement agencies. This study was conducted as a proof of principle demonstration to test the concept of using bioreporter microorganisms as a means to cover large areas in such a search. These bioreporter microorganisms are affected by a particular component of decaying organic matter that is distinct from decaying vegetation. The diamino compounds cadaverine and putrescine were selected as target compounds for the proof-of-principle investigation, and a search for microorganisms and genes that are responsive to either of these compounds was conducted. One recombinant clone was singled out for characterization based on its response to putrescine. The study results show that small concentrations of putrescine increased expression from this bioreporter construct. Although the level of increase was small (making it difficult to distinguish the signal from background), the results demonstrate the principle that bioreporters can be used to detect compounds resulting from decaying human remains and suggest that a wider search for target compounds should be conducted.

Introduction

The decomposition of human remains is a complex process that varies extensively with local environmental conditions. It is probably safe to assume that no two bodies degrade in exactly the same way, since the microorganisms that perform catabolic activity vary considerably from site to site, and environmental conditions such as rainfall, soil composition, and temperature all play a part in degradation. Nevertheless, there are probably some chemical intermediates of degradation that are common to most every instance. For this project we chose cadaverine and putrescine because they are closely associated with the decomposition process, as breakdown products of proteins. Although vegetation provides ample examples of carbohydrate breakdown products, there are relatively few proteins in leaf litter.

The presence of either of these compounds certainly suggests a burial site for a decomposing body, but detecting such chemicals in the field is not trivial. In high concentrations they are apparent to human smell. In lower concentrations, they are apparent only to cadaver-locating dogs and flies. As has been proven true for many other chemicals, the use of dogs is far from ideal. Dogs cannot be calibrated in the ordinary sense of the word, and, thus, performance varies from day to day. In addition, very few dogs nationwide have been trained for this purpose, their maintenance is costly, and they can only cover small areas before they grow tired of the task. All of these make them rather expensive to utilize.

What is needed is a means to cover a large area with a predictable, sensitive, yet inexpensive sensor. We have produced bacterial strains that recognize specific compounds or groups of structurally related compounds and respond to those compounds by activating specific genes. When a bioreporter gene is inserted in tandem with these genes, a bioreporter strain is created.

Whenever the gene of interest is expressed, the bioreporter gene will be expressed and will produce a signal that can be detected.

We have used two bioreporter genes extensively in our studies. The *lux* genes from *Vibrio fischeri* produce a bioluminescent (i.e. light-producing) response. This is easily detected and is especially valuable because present detection equipment is able to eliminate most background (ambient light) signal. In addition, it is possible to obtain relative comparisons of signal strength based on light quantitation, allowing the investigator to choose strong responders.

The other bioreporter gene in common use is the fluorescent GFP gene. This gene was isolated from a jellyfish and produces a protein that has exceptional fluorescence in the green region of the spectrum. This makes it a powerful tool for field detection, although the tremendous stability of the protein means that it accumulates in the cell, rendering futile most attempts at quantification of expression. For these reasons our usual procedure is to identify useful genes using the lux genes and, then, to isolate the genes of interest and clone the essential fragments to the GFP gene for field work. This report represents the first part of that work, in which we attempt to find genes that will express in the presence of diamino compounds.

Methods

Bacterial strains used in this project include a cadaverine-utilizing bacterium isolated in the soil underneath decomposing human remains. It is identified as a *Pseudomonas putida* and degrades phenol, another compound associated with early decompositional events. It will be referred to in this report as *P. putida*. Putrescine-degrading bacteria were also isolated from soil and were identified as *E. coli*. In addition, other bacteria were isolated from soil slurries that had been amended with either cadaverine or putrescine. Soil samples (1 g) were slurried in 20 mL of minimal salts medium containing 0.1 % glucose and one of the target substrates. These were grown for several days at room temperature (approximately 26°C) with vigorous shaking. At intervals the culture was reinoculated into fresh media in order to select for strains that could utilize the target compounds and, thus, have a growth advantage. Culture media containing only the target compound was also tried, although growth of the bacteria was poor and no culture selection was obtained.

Cadaverine (1,5 diaminopentane) and putrescine (1,4 diaminobutane) (Figure 1) were obtained from Sigma Chemical Co. and used as the target compounds for this project. These are well-known breakdown products of proteins and are known to be associated with decaying bodies. They were typically used at 1-mM final concentration, although lower concentrations were sometimes used.

Selected strains were mutagenized with transposon TN4431, a lux promoterless transposon containing a tetracycline resistance gene for selection of successful recombinants. This transposon was supplied in *E. coli* HB101, which could be easily selected against on the basis of *trp* deficiency. Therefore, recombinants were selected on minimal media containing tetracycline at 15 mg/L. To perform this procedure the donor and recipient strains were grown to mid-exponential phase of growth in a rich undefined medium. A 1-mL aliquot of each culture was added to a 0.45-micron sterile filter in a filter tower apparatus, and a vacuum was used to draw the mixed culture onto the filter. The filter was placed on nutrient agar plate overnight at room

temperature. After this incubation period the filter was suspended in 10 mL of sterile phosphate buffered saline (PBS) (Table 1) and vortexed to remove the cells from the filter. Aliquots of 0.1 mL were then spread plated on the selective media plates and were incubated for 48 hours at room temperature. With this procedure, a set of transposon insertion mutants was created.

Individual colonies that arose on the selective agar plates were screened for a clone that had the transposon inserted in the right orientation and position to report on a gene activated by the target chemical. Individual colonies were picked with sterile toothpicks onto selective agar. After growth at room temperature for 24 hours, the colonies were examined in a darkroom for constitutive bioluminescent expression, which is not unusual for this method. These colonies were typically marked with a red marker. Then the plates were sprayed with a 0.1% solution of the target compound using an atomizer. The plates were allowed to sit for several hours and were visually examined in a darkroom for bioluminescence at intervals during that time. Bioluminescent colonies were marked and isolated, typically with a green marker. Any colonies marked with green and not red were selected, streaked to obtain purified clones, and then retested.

Clones of interest were tested quantitatively using a Turner Designs ATP photometer. Briefly, the assay was performed as follows. The culture was grown in media until a mid-exponential rate of growth was attained, or sometimes until stationary phase was attained. The culture was then split into equal portions. One portion was left as a negative control while the other was amended with the target compound at 1-mM final concentration. The cultures were left stationary at room temperature, and at hourly intervals they were tested by examining 1-mL aliquots in the ATP photometer. Clones that demonstrated a clear response to the target compound were then tested under various conditions to obtain the optimum light output.

Results

Enrichment

Several bacterial strains were isolated after growth in enrichment cultures. These were typical gram negative aerobic bacilli. The *P. putida* strain was maintained on minimal salts medium supplemented with glucose and cadaverine, as described above. It was apparent that the strain became very mucoid, and this was considered a detriment to filter mating. Therefore, for some mating procedures, the strain was grown with succinate as the carbon source.

A series of filter matings was performed to obtain recombinant clones that would give a measurable response to one of the target compounds. The HB101 strain containing the Tn4431 plasmid was mated with the *Pseudomonas* strain and with several other isolated strains. Clones were selected on minimal salts media containing tetracycline in order to select against both parent strains. Although some clones were obtained for each strain in this manner, results were extremely variable. Working with the *Pseudomonas* strain was very difficult. The strain had some natural resistance to tetracycline, and colonies would eventually grow on the selective agar without the transposon. Higher concentrations of tetracycline were used (50 mg/L) but the efficiency was always very poor, typically about 10 colonies/mL of the mating mixture. After several attempts to obtain reasonable numbers of clones, this strain was abandoned.

Other strains, isolated from enrichment cultures, proved more pliable, and several hundred colonies were examined for activity. Of these, between 3% and 5 % were constitutive light producers and were of no use. One clone outperformed the others on the initial screening, and this clone, called RB1437, was used in subsequent characterization experiments. This strain is tentatively identified as a *Pseudomonas* species, based on its ability to grow on Pseudomonas Isolation Agar. It grows well at room temperature in both rich undefined and minimal media with glucose as carbon source.

Characterization

Since both cadaverine and putrescine are relatively poor sources of carbon and energy, it was initially believed that they would be utilized as a source of fixed nitrogen. In order to screen the samples, the target chemicals were diluted in distilled water, and it was assumed that the strains would be functional in an isotonic, nutrient-free mixture such as PBS. Therefore, the first test of this strain involved growth in LB medium followed by washing in PBS. The cells were resuspended in PBS before adding the target chemicals. Surprisingly, the light output from the strains under all conditions was greatly reduced. However, after overnight incubation the culture with added putrescine showed significant activity.

Subsequent experiments used M9 minimal salts medium (Table 1). An M9 recipe without added nitrogen was used to starve the cells for nitrogen, in the hopes that this would stimulate activity. While the light output in both the cadaverine and putrescine tests was increased (and were above the values seen with the PBS test), the values did not demonstrate a significant increase over the negative control. Only the time point at 18 hours (overnight) showed a clear increase. This suggested that the strain had significant reserves of fixed nitrogen and should be starved extensively before testing to obtain the fastest and clearest response.

To obtain a clear indication of the effect of added nitrogen on activity, a comparison was made between M9 with and without added nitrate (sodium nitrate, 20-mM final concentration). Two concentrations of the target compounds were used, 1.0 and 0.1 mM. These results are shown in Figures 2 and 3. Results are shown with a correction for initial output, so that the initial reading is zero. In cultures containing nitrate there is a significant response from putrescine only, which fades gradually over a period of hours. The effect is seen at both concentrations. Without added nitrate, the results are less clear, although putrescine still has the greatest and most sustained effect.

Since a starvation period seemed to be critical for gene expression, an approximation of the needed conditions was attempted. Cultures were starved for 8 hours and for 24 hours before adding the target chemicals. Once again, two concentrations were used. The results are shown in Figures 4 and 5. In both cases the response to putrescine is significant and sustained over the time course of the experiment. In the (8-hour) starved strain the response to putrescine is far greater than its response to cadaverine at 4.5 hours, showing a preference for one compound over the other in terms of gene expression. It should be emphasized, however, that we do not know what the fate of either compound is in these tests, only what the gene of interest is doing. In the extensive starvation test (24 hours), the response takes longer to achieve, presumably because the cells have entered a long-term starvation rate of growth, but the effect from putrescine is maintained longer.

These results suggest that the bioreporter strain must be grown to high density in a nutrient-rich medium and, then, starved for many hours in a defined medium before it can be used. This starvation period has the effect of suppressing background while permitting gene expression in the presence of putrescine. While overall light output is low, the proof-of-principle for the bioreporter concept is established.

Discussion

Useful clones from the several strains of this project were very rare, and only a single one was deemed suitable for in-depth work. This strain appears to be a pseudomonad and has been mutagenized with the lux transposon to create a bioluminescent bioreporter of cadaverine.

While it is apparent that some gene expression is favored in the presence of cadaverine, it is also clear that the activity is still very low and that background will continue to be a problem. It is unlikely that this genetic construction will be useful enough for fieldwork. However, the proof-of-principle has been established with these results, indicating that a bioreporter strain can be developed. Development of an acceptable tool for field use would require additional research to screen for other target chemicals and organisms and to develop sensitive detector.

Part of the problem lies with the choice of target chemical. Both cadaverine and putrescine are relatively small, chemically uncomplicated compounds. They may represent a source of fixed nitrogen but probably make poor growth substrates. They are inhibitory to growth only at relatively high concentrations. Therefore, in retrospect, it appears that the targets are not of sufficient importance to the bacterial cell to merit high-level expression of genes or tightly controlled regulation. That is, they are neither toxic nor an attractive source of nutrients.

If researchers are successful at identifying other, more complex products of decomposition, then these compounds should be used in subsequent searches for a bioreporter strain. For instance, aromatic compounds are recognized and degraded by a variety of operons that have evolved to be specific for structurally related compounds. However, even long-chain aliphatics like octane have specific genes that have evolved to degrade them. Such a search should then focus on abundance of relatively complex aromatic compounds to present the best chances for success.

Table 1. Formulae used in this study

Phosphate-buffered saline

NaCl, 8 g; KCl, 0.2 g ; Na_2HPO_4, 1.44 g; KH_2PO_4, 0.24 g per liter of distilled water

M9 medium

Na_2HPO_4, 12.8 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1.0 g

$MgSO_4$, 2 mM (final concentration)

$CaCl_2$, 0.1 mM (final concentration)

glucose 20 mM (final concentration)

per liter of distilled water

Cadaverine (1,5 diaminopentane)

$H_2NCH_2CH_2CH_2CH_2CNH_2$

Putrescine (1,4 diaminobutane)

$H_2NCH_2CH_2CH_2CNH_2$

Figure 1. Structures of the target compounds.

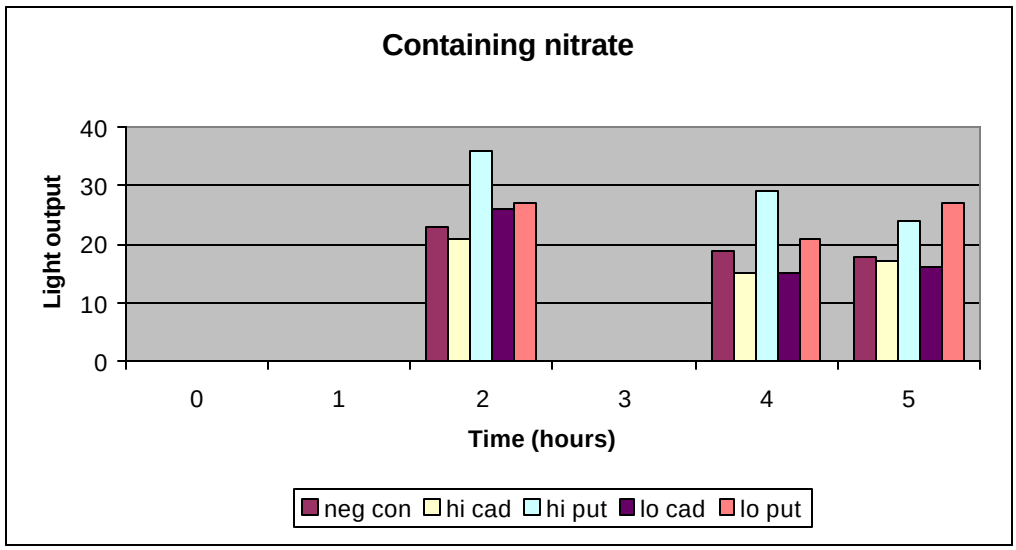

Figure 2. Cultures with added nitrate.

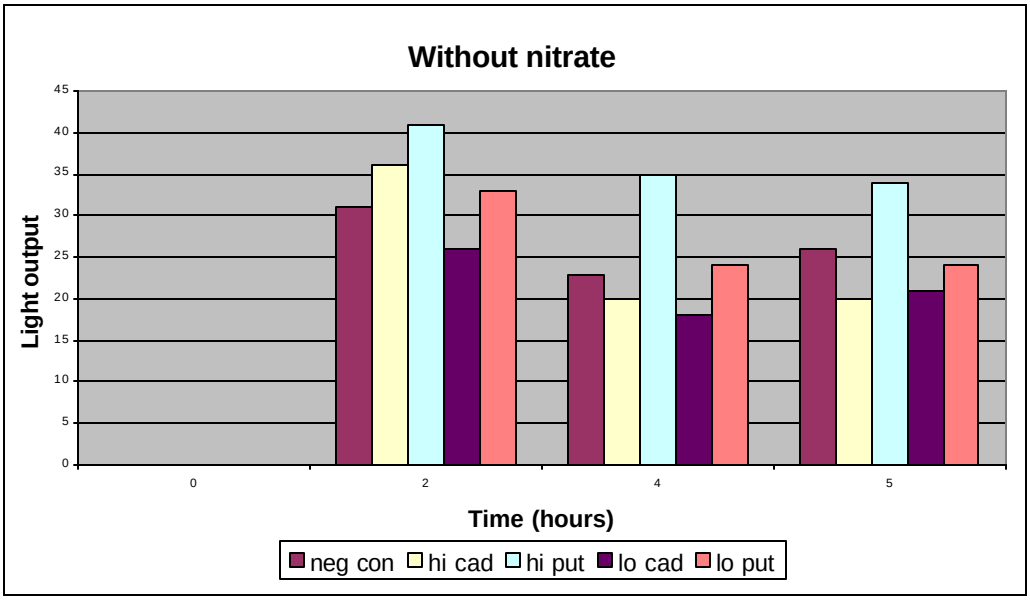

Figure 3. Cultures without added nitrate.

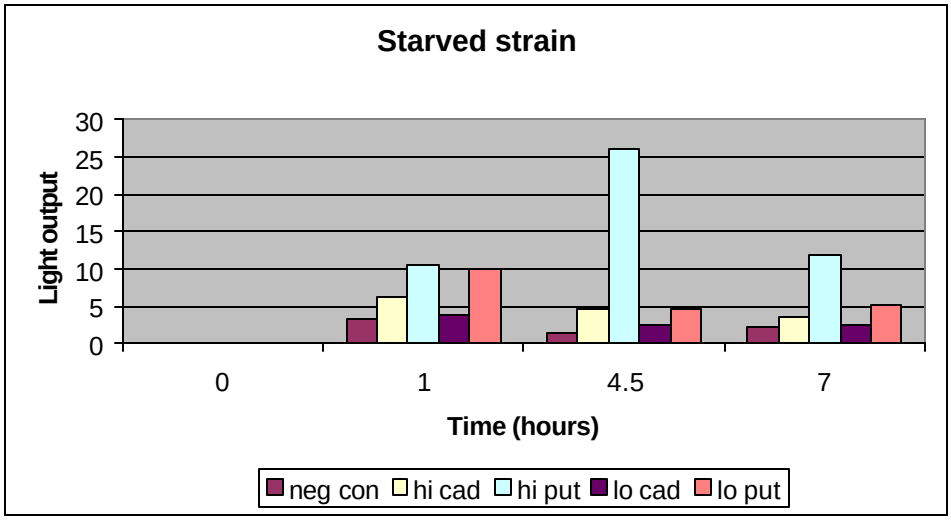

Figure 4. Starved cultures.

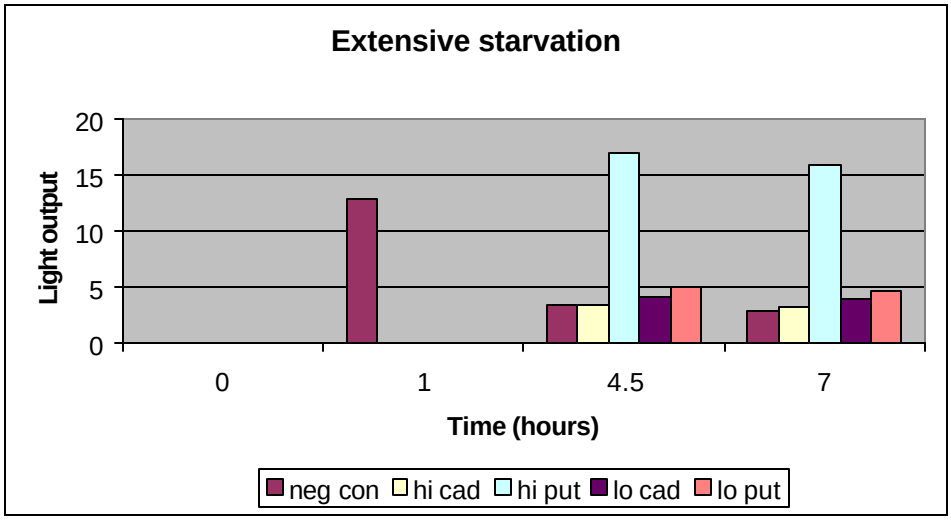

Figure 5. Extensive starved cultures.