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Silver nanotoxicity using a light-emitting biosensor *Pseudomonas putida* isolated from a wastewater treatment plant

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ABSTRACT

The effect of silver ions, nano- and micro-particles on a luminescent biosensor bacterium *Pseudomonas putida* originally isolated from activated sludge was assessed. The bacterium carrying a stable chromosomal copy of the lux operon (luxCDABE) was able to detect toxicity of ionic and particulate silver over short term incubations ranging from 30 to 240 min. The IC_{50} values obtained at different time intervals showed that highest toxicity (lowest IC_{50}) was obtained after 90 min incubation for all toxicants and this is considered the optimum incubation for testing. The data show that ionic silver is the most toxic followed by nanosilver particles with microsilver particles being least toxic. Release of nanomaterials is likely to have an effect on the activated sludge process as indicated by the study using a common sludge bacterium involved in biodegradation of organic wastes.

1. Introduction

Engineered nanomaterials, that have seen significant increases in use recently, are mainly composed of metallic nanoparticles. The knowledge of the ecotoxicology of nanoparticles (NP) to bacteria and other microbes is still limited, even though some manufactured nanoparticles (NP; materials with three dimensions between 1 and 100 nm) [1] such as silver and titanium, which are constantly released into the environment, are known as antibacterial agents. As such it is essential that technology that fully assesses their effects on natural microbial organisms, and on biogeochemical cycling in the environment, is available. Clearly there is a concern that these novel materials could be released into the environment.

Whole microbial cell biosensors are now widely used as research tools in the testing of substances likely to elicit cytotoxic and genotoxic events, and, in the determination of bioavailability of chemicals [2]. They embrace genetically engineered bacteria that have a toxicant detecting gene that is coupled with a reporter gene (e.g. luminescence gene such as lux or luc) capable of producing a detectable response on activation. Wiles et al. [3] argue that autochthonous microorganisms would be appropriate in toxicity testing with the potential for *in situ* relevance.

Silver, a metal used extensively in various consumer products because of its effective antimicrobial properties, is subject to release to sewer the sewerage system. Therefore it is important to determine whether has an effect on activated sludge microorganisms. Silver nanoparticles are well known for their antibacterial activity [4–7] and have become one of the nanomaterials mostly used in consumer product [8]. Being considered as the most prevalent of engineered materials [9] is likely to enter the wastewater treatment plants as nanowaste. Blaser et al. [10] pointed out that silver released to wastewater is incorporated into sewage sludge and may spread further on agricultural fields where will mainly stay in the top layer of soils [11].

This work aims to assess the effect of silver nanoparticles on the activated sludge process by examining the response of a common bacterial member of the sludge consortium involved in sewage organic degradation. In this study ecotoxicological was performed using a genetically-modified *Pseudomonas* isolated from a polluted, phenolic-rich, wastewater treatment system by transposon mutagenesis [3]. The *Pseudomonas* carries a stable chromosomal copy of the lux operon (luxCDABE) derived from *Photorhabdus luminescens* with continuous output of light. The bioluminescence bioassay performed in this study has the advantage of allowing monitoring the presence of these nanoparticles, specially silver nanoparticles, the object of this study.

2. Materials and methods

2.1. Silver chemicals

All of the chemicals used were analytical grade. Silver powder nanoparticles (average size: 35 nm, 99.5% metal basis, spherical morphology and cubic crystallographic structure) and

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microparticles (average size: 0.6–1.6 μm) were obtained from Nanoamor (Nanostructured and Amorphous Materials Inc., TX, USA). Stock suspensions of silver ion ($AgNO_3$), silver nanoparticles (Ag-NP) and silver microparticles (Ag-MP) were freshly made in 20 ml universal flasks and placed into an ultrasonic bath (XB6 Grant Instruments Cambridge Ltd., UK) at 25 kHz, 25 °C for 30 min. Two fold dilutions were externally prepared of these suspensions and added to the 96 well black microtitre plates (Sterling, Caerphilly, UK) to give final toxicant concentration in the range to be tested.

Two different stabilisers were added to the nanoparticles and microparticles working solutions: 0.1% citric acid, 0.1% BSA (bovine serum albumin) and comparisons were also made with preparations without stabilisers. Previous studies have used BSA for stabilisation of ZnO nanoparticles [12] and of carbon black nanoparticles [13]. Thus improving the stability of particle suspension reducing particle agglomeration and settling over time [13]. Citric acid is known to act as a chelating agent and may thus be able to also act to quench toxicity of dissolved metal toxicants. All working solutions were light protected and used within 30 min of preparation. In order to assess any shading effects of the particles on the bacterial cells, the light output was measured before and after addition of particles. No shading effects were determined.

2.2. Media and growth conditions

The bacterial strain used in this study, *Pseudomonas putida* BS566::luxCDABE was constructed based upon chromosomal expression of the luxCDABE operon derived from an entomopathogenic nematode symbiont, *P. luminescens* [14]. Originally isolated from the treatment system [15], this reporter organism encompassing a dynamic xenobiotic sensing range, is suitable for placement around an industrial processing system to monitor remediation in multiple compartments. Cultures were grown in Luria–Bertani (LB) broth (10.0 g L^{-1} of Tryptone; 5.0 g L^{-1} of yeast extract; 5.0 g L^{-1} of sodium chloride), containing 100 mg L^{-1} Kanamycin, and overnight at 26 °C in shaking conditions (200 rpm) to late exponential stage. Prior to the assay, cultures were diluted to approximately 10^7 cells/ml and regrown under the same conditions for two to three generations without Kanamycin. When OD (optical density) reached 0.2 (approximately 10^8 cells/ml) toxicity tests were carried out.

2.3. Biosensor assay

Luminescence measurements were undertaken using a 96-well plate luminometer (FLUOstar Optima, BMG Labtech, UK) in 96 well black microtitre plates (Sterling, Caerphilly, UK) whereby each well contained bacterial inoculum and toxicant at the required concentration in 100 μL volumes, using an integration time of 1 s at a temperature of 28 °C. Readings were taken every 30 min for 240 min. Control wells containing LB broth with *P. putida* BS566::luxCDABE were run and changes in toxicity for the test systems expressed as percentages of the control. Luminescence values were expressed in the instrument's arbitrary relative light units (RLU). The maximal response ratios were the highest ratios of luminescence in the sample-containing wells to luminescence in wells containing untreated cells determined during a specified period: 30, 90, 180 and 240 min [16].

Inhibitory concentration which represents 50% inhibition of light output (IC_{50}) in relation to the control was assessed for all toxicants tested at each time point. All experiments were run at least 3 times (most were ran 4 times) at different dates with different batches.

2.4. Calculation of IC_{50} (IC, inhibitory concentration) values

The IC values were calculated using a statistical program developed in-house. The program fits a three parameter logistic model to the logarithm of the concentration by weighted least squares. The parameters are the initial response, the slope and the intercept. It is assumed that the response would decline to zero at sufficiently high concentrations. The initial response effectively uses the information from both the controls (if present) and low concentrations. The weights used are taken to be proportional to the fitted response but with adjustments for high and low responses; this is to protect against bias due to "hormesis" effects (stimulatory effects causing increased light output when challenged with low toxicant concentrations) and the effective omission of data respectively.

2.5. Data analysis

For error analysis, all of the experiments were conducted 3 times on different plates. Data from eight wells were used for one concentration and coefficients of variation (CV) between independent assays were calculated using Microsoft Excel 97. Differences among treatments were tested using a two-way analysis of variance (ANOVA) to determine which treatments were statistically different ($P < 0.05$).

3. Results and discussion

IC_{50} values following challenge with $AgNO_3$, Ag-NP and Ag-MP (with and without dispersant) are shown in Table 1. The experimental uncertainty of these bioluminescence bioassays is within the coefficients of variation. Calculated coefficients of variation (CV) between independent assays were found to be between 1 and 15%.

Fig. 1 shows the light output reduction by *P. putida* BS566::luxCDABE when challenged with different concentrations up to 2500 μg L^{-1} silver ion. Among the silver species tested, $AgNO_3$ is by far the most toxic to *P. putida* highlighting the action of ionic Ag^+ ($P < 0.05$). It is well known that silver ion and silver based compounds are highly toxic to micro organisms and have strong biocide effects to many bacteria species [17–20].

Figs. 2 and 3 show the light output reduction by *P. putida* BS566::luxCDABE when challenged with Ag nanoparticles and Ag microparticles. In the presence of BSA as stabilizer, Ag nanoparticles showed to be statistically more toxic than Ag microparticles ($P < 0.05$). The same was observed when using citric acid as stabilizer. The highest toxicity (Table 1) was observed after 90 min incubation and indeed this was the case for all silver species tested with or without stabilisation. The order in toxicity was Ag^+ > Ag-NP (35 nm) > Ag-MP (0.6–1.6 μm). In this current study we tested three different silver species, and ionic Ag^+ being the most toxic to the biosensor tested. Similar results were found by Choi et al. [4] when testing the toxicity of silver species. In their studies, the

Table 1
IC_{50} (mg L^{-1}) values for light emission reduction by *P. putida* BS566::luxCDABE after 30, 90, 180 and 240 min. Values presented are an average of at least 3 independent experiments carried out with different batches, standard deviation between 1 and 15%.

	IC_{50} values (mg L^{-1})			
Time (min)	30	90	180	240
$AgNO_3$	0.44	0.18	0.25	0.30
Ag-NP	88	81	91.5	184
Ag-NP BSA	102	35	43	50
Ag-NP CA	147	126	136	149
Ag MP	715	530	765	1075
Ag MP BSA	375	256	308	330
Ag MP CA	700	240	300	337

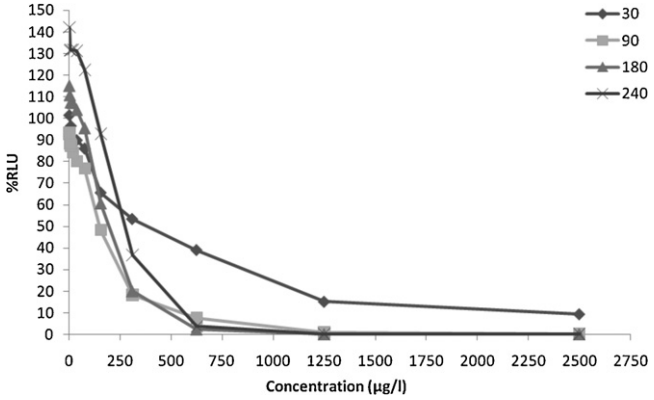

Fig. 1. Light output reduction by *P. putida* BS566::luxCDABE when challenged with 2500 µg L^{-1} silver ion after 30, 90, 80 and 240 min. Mean of three replicates with different batches.

ionic form Ag^+ was more toxic to heterotrophic *Escherichia coli* than 16 nm silver nanoparticles, while to autotrophic nitrifying bacteria Ag nanoparticles were more toxic than the ionic form.

It has been pointed out that the bactericidal effect of nanoparticles is dependent on the concentration of nanoparticles and the initial bacterial concentration [19]. In our study we used 35 nm silver nanoparticles which were dispersed in liquid cultures with an initial bacterial concentration of 10^8 UFC cells/ml for the toxicity tests. Using *E. coli* as a model, Sondi and Sondi-Salopeki [18] showed that the bacterial growth in LB liquid medium (with an initial bacterial concentration of 10^7 UFC cells/ml) was delayed by nanoparticles size 12 nm. However, other studies using the Microtox system [21] for bioluminescence testing have shown no toxicity on exposures to silver nanoparticles. In their study, however, these authors used a marine microrganism *Photobacterium phosphoreum* which requires a sodium chloride concentration of 22% and is not indicated for ecotoxicity of terrestrial systems [21]. Whole cell biosensors are of greater environmental relevance for luminescence-based testing in terrestrial systems compared to the Microtox system testing. Furthermore, whole cell biosensor have shown a greater sensitivity than Microtox system [16]. In this current study we used a whole cell biosensor *P. putida* BS566::luxCDABE, a terrestrial bacterium for the bioluminescence testing. This methodology has been used in many studies formerly [2,15,16,22–25]. Beaton et al. [22] showed that the biosensor *E. coli* HB101 pUCD607 is a sensitive indicator of changes in toxicity in a soil system spiked with 2,4-dichlorophenol; Shaw et al. [23] used the biosensor *lux* marked *Burkholderia* RASC c2 in bioluminesce inhibition studies and Boyd et al. [24] used *Burkholderia* RASC c2 and *Pseudomonas fluorescens* 10586 to assess the toxicity of chlorophenols. Sinclair et al. [25] showed the toxic response of the *lux*-marked biosensors as *Pseudomonas fluorencens* and *E. coli* to 2,4-dichlorophenol.

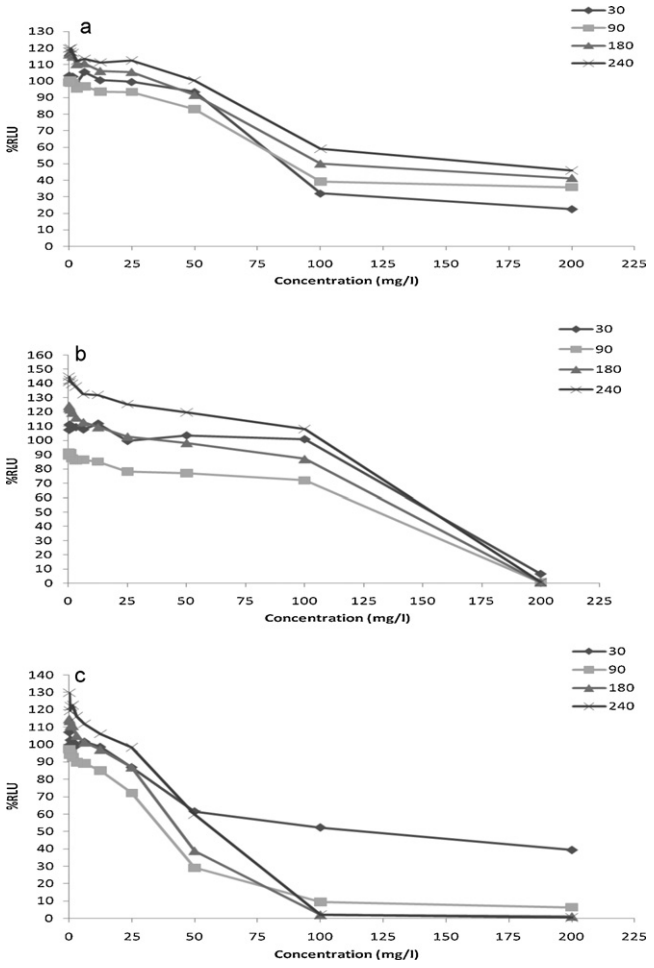

Fig. 2. Light output reduction by *P. putida* BS566::luxCDABE when challenged with 200 mg L^{-1} Ag nanoparticles: (a) without stabilizer, (b) with 0.1% citric acid, and (c) with BSA after 30, 90, 80 and 240 min. Mean of three replicates with different batches.

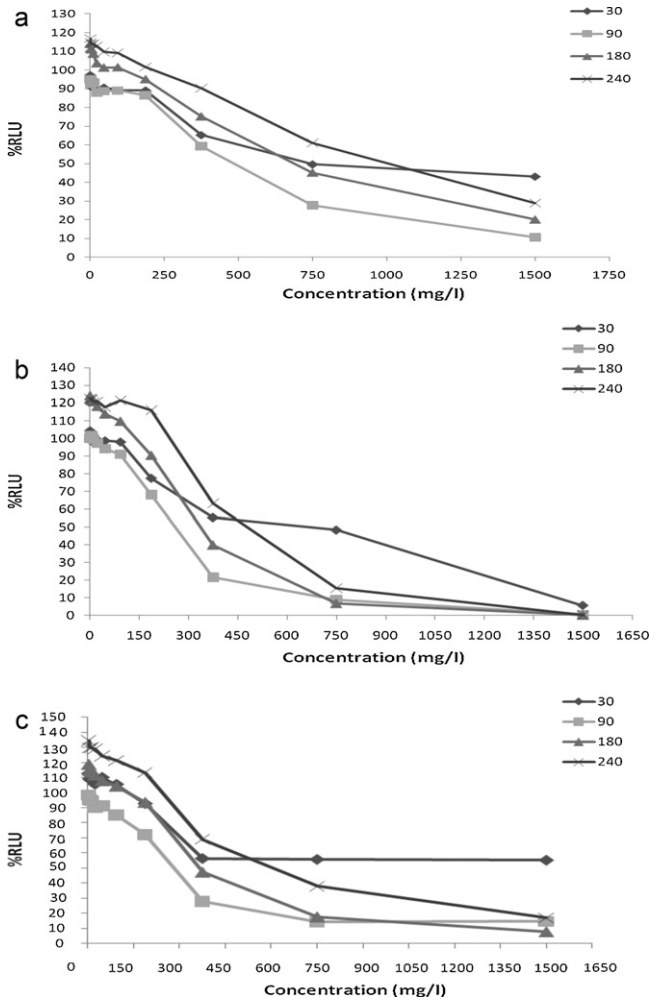

Fig. 3. Light output reduction by *P. putida* BS566::luxCDABE when challenged with 1500 mg L^{-1} Ag micro particles: (a) without stabilizer, (b) with 0.1% citric acid, and (c) with BSA after 30, 90, 80 and 240 min. Mean of three replicates with different batches.

Our biosensor responded within 90 min (as see in Table 1) to the presence of toxicants. Thus, this strain can be used for the rapid and sensitive detection of potentially toxic silver compounds. Overall, the toxicity of silver was found not to be dependent. Overtime, the IC_{50} values obtained over 90-min for ionic silver, Ag-NP and Ag-MP was about 1.5–2.0 times lower than the 240-min test. Therefore, according to our findings, a 90-min test should be taken when monitoring and evaluating wastewater treatment plants for silver toxicity. If we consider 90 min assay results, which shows the highest toxicity, to compare the data, it can be noticed that Ag-NP are nearly 200 times less toxic that Ag^+, with Ag-MP ~3000 times less toxic in non-stabilised systems ($P < 0.05$). The effect with Ag-NP was more pronounced in BSA systems with a calculated IC_{50} value ~4 times lower than Ag-NP with citric acid ($P < 0.05$). Addition of BSA seems to result in higher toxicity perhaps through better dispersion of the NP providing more surface area for Ag to have an effect. This however may not be the case as citric acid may be just as effective in preventing agglomeration. Citric acid is an effective chelating/complexing agent for metals in solution. The effect of citric acid on the toxicity value might be due to immobilization of Ag^+ released in solution or those on surfaces. On the one hand one may need to show a worst case toxic effect of well dispersed NP systems but on the other hand real systems will have a range of cheating/complexing agents that will affect the toxicity of substances released into its environment. Dissolution is likely to be a critical step for some metallic nanoparticles in determining fate in the environment and within the organism. In this study when particles were well dispersed using BSA as stabilizer, a higher toxicity was observed. Solubility strongly influences the toxicity and when no stabilizer was used the IC_{50} values were higher, probably indicating that less soluble compounds were available for the bacterial cells. Brunner et al. [26] observed that nanoparticles with a low solubility such as TiO_2 showed no toxicity to mammalian cells while more soluble nanoparticles like ZnO showed a higher toxicity. The use of different stabilisers and their effect on toxicity values must be assessed particularly if laboratory bioassay results are to be used to derive wastewater discharge consents.

There were no significant differences between the IC_{50} values obtained for Ag-MP with BSA and citric acid stabilisation ($P > 0.05$) and both values were ~2 times statistically lower than Ag-MP tested without stabilisation ($P < 0.05$).

Particle size does cause a toxicity difference. In the present study, if we compare the toxicity between nanoparticles and their micro size counterparts, we noticed that when Ag-NP were stabilized showed a higher toxicity when compared with the micro size counterparts ($P < 0.05$). Other studies have also showed no toxicity of micro scaled particles when compared with nano size counterparts. For instance, Jiang et al. [20] observed a higher toxicity of nanoparticles of Al_2O_3, TiO_2 and ZnO than their micro size scale counterparts which showed no or lower toxicity. Sinha et al. [27] have noticed that ZnO nanoparticles disintegrate Gram negative bacteria cell membrane and accumulate in cytoplasm, while when these cells were grown in micro particle counterparts the cell membrane and cytoplasm were intact.

In this study we used 35 nm spherical nanosilver particles. Smaller nanosilver particles are more active than larger ones because of their higher surface area. However, in this study we used relatively large size of silver nanoparticles (35 nm) which proved to have a higher level of toxicity against the biosensor tested. Silver nanoparticles surface area plays quite an important role for antibacterial activity which depends on its exposed surface area concentration. This dependency is originated from the released Ag^+ from the nanosilver surface. Recent studies [28,29] indicated that when nanosilver particles are small and release many Ag^+ ions, the antibacterial activity is dominated by these ions rather than the nanosilver particles. However, when larger sizes are used (as the ones used in this study) which have a low release of Ag^+ ions, the nanosilver particles themselves also influence the antibacterial activity as indicated by these results here presented. Metal nanoparticles as silver have a tendency to attach on the cell wall [20]. So, toxicity is not dependent only on release of Ag^+, but also it depends on other factors such as the attachment of particles on the cell surface, disruption of cell membrane and consequently accumulation of nanoparticles in the cytoplasm. Surprisingly, the counterpart micro sized particles showed a lower toxicity if we consider that smaller particles release more Ag^+ ions that the larger ones. However, one should consider other factors that affect toxicity such as the initial concentration of particles and bacteria, and particle bioavailability, among many other factors. So, although silver NPs have a higher surface area and probably a higher release of Ag^+, there was probably not enough contact in order to cause damage to cell membrane and cytoplasm since toxicity is dependent on contact and/or bacterial attachment to the particle as pointed by Jiang et al. [20] and Sinha et al. [27]. Furthermore, it has been demonstrated that small particles as the counterpart microsized are in suspension only in small numbers and are not able to attach to the bacterial surface as the nanoparticles do [20]. Then, in this case of the micro sized particles as the ones used in this study, they caused less harm to the bacterial cells than the nanoparticles ones due to their inability to attach on cell surface.

The antimicrobial effect is related to the amount and the rate of silver released by NP. Severe structural changes occur in the bacterial cell wall when ionised silver binds to cell membrane proteins which leads to protein distortion and cell death [30,31]. Silver is classified as the "soft" metal group [10] and it complexes with many organic or inorganic materials such as chloride, sulphide, thiosulphate [32]. In order to evaluate the impact of silver discharge in the environment it is important to understand the fate and transport of silver in wastewater treatment plants. The applicability of our sensor in wastewater treatment plants has been previously demonstrated [3,33]. The biosensor here tested, *P. putida* BS566::luxCDABE had accurately predicted toxicity shifts in wastewater treatment plants, with a high tolerance to a phenolic cocktail, thus demonstrating an effective biosensing in all treatment compartments [3]. Philp et al. [33] have tested immobilised *P. putida* BS566::luxCDABE in phenolic wastewater treatment plant. The biosensor tested proved to be able to discriminate toxicity of various zones within the waste treatment plant [33]. Further studies in our laboratories using wastewater samples affected by silver toxicity have being carried out using this biosensor and results will be published somewhere else. Nanowaste is likely to increase and therefore enter the wastewater treatment plants which are the final step to control silver discharge. Estimation of silver load in sewage sludge and its microorganisms growth inhibition has been predicted. Blaser et al. [10] predicted that an expected silver concentration in sewage treatment plant range from 2 $\mu g\,L^{-1}$ to 18 $\mu g\,L^{-1}$. Shafer et al. [34] reported a range of ~2–4 $\mu g\,L^{-1}$ of silver in sewage treatment plants treating common wastewater and a much higher load from industrial discharges (from 24 to 105 $\mu g\,L^{-1}$).

The removal of silver ion by chloride free sludge is dependent on the silver-sludge loading, the solution pH and the concentration of dissolved organic matter. Studying the interactions of silver with wastewater constituents, Wang et al. [35] showed that silver ion can be removed through chloride precipitation and sludge adsorption. However, the authors [35] pointed out that the formation of silver-ion-dissolved organic matter complexes, which is increased in alkaline conditions, reduces the silver ion adsorption by sludge.

4. Conclusions

In conclusion, our results demonstrate that the use of a bacterial biosensor as *P. putida* BS566::luxCDABE provides a robust, early warning system of acute toxicity which could lead to process failure. This strain is suitable for toxicity monitoring in a highly polluted industrial wastewater treatment streams. The information regarding the inhibition of microbial growth by different Ag compounds, especially in wastewater treatment systems, is valuable for operating planning and control. The presence and activity of microorganisms in biological wastewater treatment are vital to the process. One of the projected applications of such strains is its combined use as analytical panel for toxicant detection. An important advantage of using these organisms is that a positive response will not only indicate the presence of a toxicant but will also provide some idea as to its character.

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