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ORIGINAL PAPER

# In situ detection of aromatic compounds with biosensor *Pseudomonas putida* cells preserved and delivered to soil in water-soluble gelatin capsules

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**Abstract** While many types of bacteria have been engineered to produce an optical output in response to given analytes in a culture, their use for extensive, in situ monitoring of distinct chemical species in soil is hampered by a dearth of practicable spreading schemes. In this work, we report and validate a comprehensive system for the long-term preservation of *Pseudomonas putida* cells genetically designed for biosensing benzene, toluene, ethylbenzene, and xylenes (BTEX) in soil, along with a procedure to formulate, spread, and vigorously activate such bacteria at the desired site and occasion. To this end, various known lyoprotectants were tested for promoting the long-term maintenance of biosensor cells with quite variable outcomes. While a formulation of inositol and maltodextrines was optimal for preservation of freeze-dried BTEX-sensing bacteria, adsorption of *P. putida* cells to corncob powder (an abundant residue of the corn industry) endowed the resulting material with a lasting viability at ambient conditions. In any case, the thereby preserved bacterial biomass acquired physical and mechanical properties adequate for formulating the biosensor agent in water-soluble but otherwise hard dry gelatine capsules with a long shelf life. When such capsules were spread in a soil microcosm and subsequently liquefied with water or high humidity, the released microorganisms formed spots that gave an intense luminiscent signal upon exposure to effectors of the sensor circuit implanted in the chromosome of the *P. putida* strain. We argue that the procedures described here can facilitate implementation of wide-area biological detection strategies for revealing the location of toxic or perilous chemicals.

**Keywords** *Pseudomonas putida* · XylR · Biosensors · Desiccation · Encapsulation

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## Introduction

Bacteria have the ability to sense environmental chemicals by means of complex genetic regulatory circuits in which one master regulatory factor elicits a specific transcriptional response [1, 2]. The combination of such circuits with suitable reporter genes that allow optical readouts has facilitated the construction a large variety of whole-cell bacterial biosensors capable of detecting specific chemicals in the medium [3–5]. The one advantage of using in vivo sensors as compared with standard chemical analysis is the measure of the bioavailability of the compounds, which is distinct from their straight concentration [5, 6]. Alas, the use of live bacteria for this type of analytical applications is not without problems, as reporter readouts often depend on the physiological state and preservation conditions of the cells (see [7, 8] for reviews). One scenario, however, in which bacterial biosensors have an advantage with respect to other detection methods is the wide-area survey of given chemicals over extensive surfaces e.g. soil [9]. Under ideal conditions, sensor strains are spread evenly on the sites under study and the compounds of interest spotted because of an optical signal emitted by the engineered bacteria in contact with the chemical of interest [10]. One extreme scenario for such an application is the spreading of bacteria

bearing reporter fusions responsive to explosive residues (for example 2,4 dinitrotoluene) for detecting antipersonnel mines buried in soil [11].

Implementation of a wide-area bacterial biosensor system encompasses three separate aspects. Firstly, availability of transcriptional regulators and cognate promoters that respond to particular chemicals [2, 12]. Secondly, the choice of an appropriate strain as the stable host of the sensor circuit that is capable of withstanding harsh environmental conditions. And finally, the development of appropriate delivery systems for extensive application of the bacteria to the target site. The importance of the last requisite resides in that actively growing bacteria hardly fulfill the requirement to act as a portable biosensor, as they are not convenient to transport or deploy at user's will [13]. This highlights the value of establishing conditions for long-term maintenance of the bacteria and for their dissemination into the field in such a way that access to the target chemical and capture of optical signals are eased. Fortunately, recognition of visual readouts coming from the activity of the reporter genes, even low-level emissions, is possible with available technologies for remote detection of fluorescence or luminescence [14].

Although many bacterial strains have been engineered for identifying different classes of contaminants and toxic compounds [15–18], the problem of maintaining the long-term viability of such biosensors and their application to the target sites still entails a considerable difficulty [7]. Only in a few cases (mostly limited to a small number of Gram-positive strains) can biosensor bacteria be kept for long periods of time as dried spores and then easily regenerated to full vigor [19]. Since most available bacterial biosensors are based on Gram-negative bacteria (e.g., *Escherichia coli* and *Pseudomonas putida*, see below), much of the challenge is centered in non-sporulating microorganisms. In these cases, extension of the shelf life of bacterial cells is generally attempted by maintenance at low temperatures, often dried and combined with various types of preservants [20]. Out of the many drying techniques available to this end [21], lyophilization (freeze-drying) is the most extensively used as it allows numerous types of microorganisms to remain viable for longer periods [22]. Unfortunately, survival rates after freeze-drying and during the ensuing storage period change dramatically depending not only on the microbial species but also on ill-defined parameters such as culture media [23], growth phase [24], and the type of lyoprotectants used during the process [25]. A separate yet related issue involves the uniform spreading of such sensor microorganisms to the sites under surveillance for given chemicals. The literature contains examples, among others, of straight aerial spreading of cell suspensions on top soil, association of agar-grown bacteria to open-ended plastic or silicon tubings [26] and immobilizing microorganisms in alginate or polystyrene [27, 28]. Unfortunately, these procedures are either hazardous and/or unpractical for wide-area applications, as no specialized equipment has yet been developed for spreading bacteria in such physical formats.

The use of engineered *P. putida* for wide-area detection of aromatic compounds in soil, including nitro-derivatives [29, 30], has received a considerable attention in recent years. *P. putida* is a non-pathogenic member of the family *Pseudomonaceae* [31]; the natural habitats of which include soil and the plant rhizosphere. In particular, *P. putida* KT2440 is considered a safe and secure host for foreign gene cloning.[1] Later sequencing of its genome indicated that this strain is altogether devoid of virulence determinants or any other potentially hazardous traits [32, 33]. *P. putida* KT2440 is thus one strain of choice as the host of sensor genetic circuits aimed at detecting predetermined aromatics in the environment [10, 34]. Delivery of sensor *P. putida* cells to target sites is, however, afflicted by all the problems mentioned above, even exacerbated by the considerable sensitivity of this strain to air desiccation (Loza-Tavera et al., unpublished data).

This study attempts to overcome most difficulties mentioned above for in situ, wide-area application of *P. putida* cells engineered to act as biosensors of benzene, toluene, ethylbenzene, and xylenes (BTEX; see below). Our approach employs a simple and efficacious dry preservation procedure for optimal formulation of cells that allows maximal cell viability at ambient conditions while endowing microbial biomass with a coarse particulate texture. The resulting dry material had then optimal mechanical properties for large-scale dispensing in water-soluble but otherwise hard dry gelatin capsules amenable to long-term storage. These procedures shape the biosensors in a seed-like physical format suitable for extensive spreading with standard agronomical machinery. Furthermore, soil microcosm experiments showed that the capsules filled with the dry bacterial formulation are efficiently liquefied upon contact with water and the biosensors released in an active form able to produce the expected readouts upon encountering *m*-xylene in the target site.

## Experimental

### Bacterial strains and growth conditions

Bacterial cells were grown at 30 °C in an orbital shaker operated at 200 rpm in Luria-Bertani (LB) medium, or M9

[1] This strain was certified as early as in 1981 by the Recombinant DNA Advisory Committee of the NIH as the first host-vector biosafety system for gene cloning in Gram-negative soil bacteria (Federal Register of 1982, Appendix E, Certified Host-Vector Systems, vol. 47, 17197).

minimal medium containing 20-mM citrate. Where indicated, 1.5% agar plates were prepared with the same media and amended with potassium tellurite (50 μg/ml) or 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Xgal). The test *Pu-lacZ* strains used in this study were *P. putida* MAD1 and *P. putida* MAD2 [35], whereas the biosensor deployment strain (*P. putida* CPLUX) was constructed on purpose in two steps. Firstly, a transcriptional fusion of the *Pu* promoter to the *luxCDABE* reporter operon of *Photorhabdus luminescens* [36] was created by digesting plasmid p*attFRT*lux [10] with *Xma*I and *BamH*I and replacing the excised DNA by another *Xma*I/*Bam*HI segment from pMAD [35] containing the *Pu* promotor. This originated a plasmid (p*attPu*lux) carrying a *Pu-lux* transcriptional fusion in a 6,287 bp *Not*I insert. This fragment was then transferred to mini-transposon delivery vector pUT/mini-Tn5resLKm [10] thereby originating pTn*5attPu*Lux. The resulting mobile element was then randomly inserted into the chromosome of *P. putida* KT2440 with a mating technique as explained in de Lorenzo and Timmis [37]. In parallel, the 2.8 kb *Kpn*I-*Sac*I fragment of pBBXylR [10] containing *xylR* and its own constitutive promoter *Pr* was introduced in pUC18Not [37], resulting in plasmid pBX. This construct was digested with *Not*I to yield a 2,612-pb piece of DNA carrying the *Pr* → *xylR* module, which was then placed into pTn7-*FRT* [10] to generate the chromosomal delivery vector pTn7-BX. As the business part of this construct is placed in a mini-Tn*7* transposon vector, the mobile module is capable of recognizing a natural target site in the chromosome of *P. putida* (*P. putida attTn7*) and become inserted in a specific orientation [38–40]. The thereby engineered mini-Tn*7* transposon of pTn7-BX was then targeted to *P. putida* cells inserted with mini-Tn*5* *attPu*Lux (see above). One exconjugant of the filter-mating procedure [37] which displayed an optimal signal/noise ratio in luminescence emitted upon exposure to *m*-xylene, was labeled as *P. putida* CPLUX and kept as the reference biosensor strain (Fig. 1).

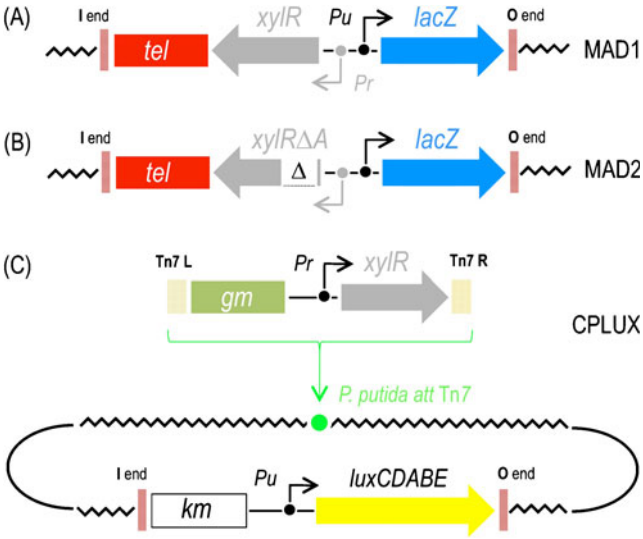


**Fig. 1** Organization of BTEX biosensing elements engineered in *Pseudomonas putida* strains MAD1, MAD2, and CPLUX. **a** The functional elements present in the mini-Tn*5* transposon of *P. putida* MAD1 are shown (not to scale), including the 19 bp I and O termini of transposon Tn*5*, the tellurite resistance gene (*tel*), the *xylR* gene of the TOL plasmid encoding a transcriptional factor that responds to toluene and structural analogs (like those present in BTEX), expressed through its native promoter *Pr* and the cognate promoter *Pu* placed upstream of a reporter *lacZ* gene. **b** The insert of *P. putida* MAD2 has the same organization, excepting that the *xylR* gene has been replaced by a constitutive allele named *xylRΔA*, the product of which activates *Pu* regardless of any aromatic inducer. **c** The BTEX sensor of strain CPLUX has also *xylR* and *Pu*, but each regulatory element is placed in a different configuration. *xylR* was targeted to the genomic *att*Tn*7* site of *P. putida* by means of a specialized gentamicin-resistant mini-Tn*7* transposon vector, whereas the reporter *Pu-luxCDABE* fusion was introduced in the chromosome by means of a kanamycin-resistant mini-Tn*5* system. Functional elements of the constructs (not to scale) are indicated in each case

## Lyophilization of bacteria

*P. putida* cells later employed for the various assays were cultured for 20 h in the media pointed out in each case. Prior to freeze-drying, the number of viable cells was counted in triplicate by spreading serial dilutions in LB agar medium. Bacterial cells were harvested by centrifugation and resuspended in one volume of a lyoprotectant solution (LB or minimal medium with the compound indicated) for 1 h at room temperature. Compounds used as lyoprotectants included sucrose, betaine, maltodextrins, inositol, mannitol, and skimmed milk at the concentrations specified in each instance. Sterile plastic tubes were filled with 2 ml of the bacterial suspensions containing the lyoprotectants, flash-frozen with dry-ice, covered with sterile cotton and then freeze-dried in a Virtis lyophilizer. The ensuing procedure involved an initial equilibration of samples at −85 °C for 30 min, application of vacuum down to ≤15 mtorr (30 mins) and then drying at 100 mtorr, −85 °C for the next 24 h. Samples were then recovered and maintained in the dark but otherwise exposed to ambient temperature and humidity. At least three independent samples were used to determine the number of viable bacteria after lyophilization. To this end, one volume of 150 mM of NaCl was added to cells, the suspension serially diluted and viable cells counted on LB plates. Bacterial survival is reported as the percentage of the number of bacterial cells present in the suspension after freeze-drying in respect to the number of viable cells before lyophilization.

## Immobilisation of *P. putida* cells with corncob powder

Corncob powder (kindly provided by J.R. van der Meer, University of Lausanne, Switzerland) was used as a carrier

for immobilization of *P. putida* MAD2. To this end, bacteria were cultured for 20 h in the media indicated and the number of viable counts determined as above. To adsorb cells, 0.6 ml of these cultures was mixed with 0.1 g of corncob powder in 15-ml Falcon tubes. A portion of the samples was kept in tightly closed tubes to maintain humidity, while the rest was subjected to spontaneous air-drying by leaving them covered with sterile cotton at room temperature for 15–30 days. At different times, samples were resuspended in 2 ml of 150 mM NaCl to calculate the number of viable bacteria. The colony-forming unit (CFU) count was averaged from triplicate samples by spreading serial dilutions on LB agar medium. Bacterial survival was followed as the percentage of the number of bacterial cells present in the suspension after immobilization in respect to the number of viable cells before immobilization with the corncobs. The humidity values are given as the percentage of the weight of the corncob mixtures on the day in which viability was measured as compared with the weight of the same material at the point of addition of the *P. putida* cultures.

Production and release of *P. putida* from gelatin capsules

Lyophilized and lyoprotected biomass of biosensor *P. putida* strains were formulated in hard plain-gelatin capsules size 3 (kindly provided by J. Pliego of OMCE S.A. http://www.omfe.es/). Capsules used in this work are composed of 14.5% *w*/*w* water and 85.5% *w*/*w* gelatin, with a total weight of 50 mg per unit. The equivalent of 200 μl of a freeze-dried culture in LB (nominally equaling a viable population of $\sim 3 \times 10^8$ cells) and lyoprotected with 15% maltodextrin and 5% inositol were deposited in the capsules, closed and placed on soil microcosms as indicated in each case. Such microcosms were assembled in Petri dishes with 20 g of Fluvisol-type standard soil collected from the Granada region in Spain, and mixed where indicated with 1.5% of agar in water. *m*-xylene was introduced in the microcosms by placing below the soil layer a 10-mm filter paper soaked with ~0.5 ml of a 1:10 dilution of the aromatic compound in DMSO. For detection of ß-galactosidase activity on a dark background, microcosms were sprayed with 0.2 ml of a fresh solution of 10 mg/ml of the fluorescent substrate 4-methylumbelliferyl-β-D-galactopyranoside in dimetil sulfoxide [41] and the emitted fluorescence recorded in Versadoc Imaging System Model 4,000 from Bio-Rad. Similarly, light emitted by luminescent *P. putida* cells in soil was recorded in the photon-counting charge-coupled device (CCD) camera of the same instrument.

## Results and discussion

The choice of engineered *P. putida* strains for in situ BTEX detection

BTEX is a mixture of benzene, toluene, ethylbenzene, and xylenes which is to be blamed for much contamination of soil and groundwater near petroleum production sites and gasoline stations [42]. The soil bacterium *P. putida* mt-2 harbors one plasmid (pWW0) encoding a complete set of genes for biodegradation of toluene, *m*-xylene, *p*-xylene, and 4-ethyltoluene [43]. The head transcriptional regulator of such a metabolic system is the protein XylR, which activates the first operon of the biodegradative route in response to a variety of aromatic inducers, both pathway substrates and structural analogs [43]. Since this regulator responds to all components of BTEX [44, 45], a number of whole-cell biosensor schemes have been proposed in which *Pu*, the promoter targeted by XylR, has been fused to a whole variety of reporter genes with measurable outputs, e.g., optical and others [17, 45–48]. In these cases, however, the BTEX detection protocol always implies bringing the environmental sample to a laboratory setup or to a dedicated piece of equipment. In contrast, no attempts have been made thus far for area-wide detection of the same compounds by means of spreading the sensor strain in the target site. The issues at stake in this case include not only the efficiency of the genetic circuits, but also the environmental robustness of the strains implanted with them, their biosafety upon release and the adoption of suitable procedures for their long-term storage and eventual dispersal.

Figure 1 shows the organization of the three genetic implants borne by the *P. putida* strain employed in this work for addressing the questions above. The host bacterium (originally an isolate from soil) is an extraordinarily robust microorganism able to endure many types of environmental stresses and amenable also to a whole range of genetic modifications. *P. putida* KT2440 also qualifies as *generally recognized as safe* (GRAS) by the American FDA and it is thus suitable for environmental release (see above). For the sake of this work, the strain lot includes two test variants (*P. putida* MAD1 and *P. putida* MAD2) and one deployment strain (*P. putida* CPLUX). The main features engineered in their genome are indicated in Fig. 1. *P. putida* MAD1 and *P. putida* MAD2 strains are inserted with genetic cassettes endowing resistance to potassium tellurite (which provide both a clear-cut selection determinant as well as a distinct visual marker [49]. $Tel^R$ genes are placed in these strains adjacent to a transcriptional *Pu-lacZ* reporter fusion along with either the wild-type *xylR* gene (*P. putida* MAD1) or the variant

*xylRΔA* (*P. putida* MAD2), which produces a constitutively active protein (i.e., triggers *Pu* activity without the need of an inducer, [35]). The deployment strain CPLUX is reminiscent of *P. putida* MAD1, but has a *Pu-luxCDABE* reporter fusion that emits light when it is activated by XylR/*m*-xylene (Fig. 1). Note that all three strains have their sensing circuits stably inserted in their genome rather than in plasmids, a must for bacteria destined for environmental release [50]. Granted that these engineered biosensor strains are environmentally robust, stable and safe, they were exploited for optimizing long-term preservation procedures and delivery methods explained below.

## Lyoprotectants increase viability and activity recovery of freeze-dried *P. putida* MAD2

The first step towards feasibility of area-wide application of whole-cell biosensors is that the biological agent could be prepared and preserved in bulk amounts to allow its safe storage and off-shelf availability. To this end, simple procedures for biomass recovery from a culture (centrifugation and filtration) could be followed by mere exposure to air until spontaneous dehydration resulting in powder or pellets of the virtually pure strain. Alas *P. putida* is very sensitive to sheer air-drying (Loza-Tavera et al., unpublished data), making this approach (which works well for other microorganisms, [21, 51]) unattainable in our case. As an alternative, we explored the possibility to mix *P. putida* cells with various lyprotectants and then to subject the mixture to a freeze-drying procedure. Lyophilization has been used for long-term preservation of numerous bacterial species [22, 51], including *P. putida* [24], as was thus a method of choice to our ends. The maintenance of viability of given microorganisms after freeze-drying depends on multiple factors including the initial concentration of cells [52], growth media [23], and especially the addition of protecting agents [25]. In order to test these conditions with the *P. putida* strain that hosts the BTEX biosensor element we employed *P. putida* MAD2, because its markers ($Tel^R$ and $LacZ^+$, see above) allows monitoring separately its viability and its transcriptional activity. In a first set of assays, cells destined for preservation were grown in synthetic, phosphate-buffered minimal medium. Since the survival of *P. putida* after freeze-drying is known to be higher as cells enter stationary phase [24], we collected cells of *P. putida* MAD2 from well-grown cultures (see "Experimental"). These stationary cells were resuspended in fresh medium added with different test compounds with known lyoprotective properties [21], including sugars (sucrose and maltodextrins), polyalcohols (mannitol and inositol), osmoprotectants (betaine), and skimmed milk. As shown in Fig. 2a, cells subjected to the freeze-drying process explained above failed to produce CFUs at any time after the completion of the procedure. In contrast, a

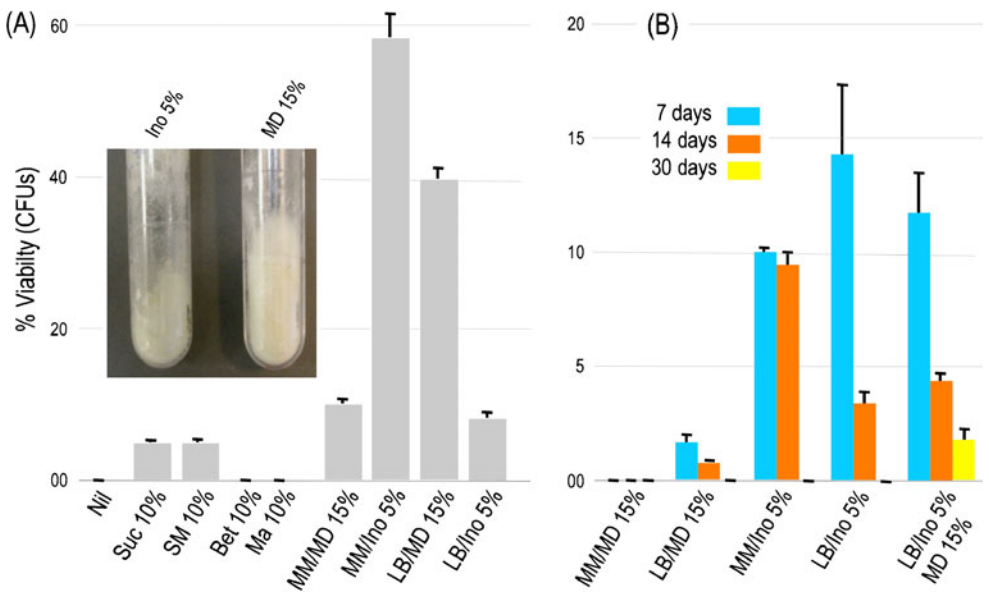


**Fig. 2** Effect of lyoprotectants on the survival of *Pseudomonas putida* MAD2 upon lyophylization. **a** Viability of stationary-phase *P. putida* MAD2 cells 1 day after lyophyllization in the presence of 10% sucrose (*Suc 10%*), 10% skimmed milk (*SM 10%*), 10% betaine (*Bet 10%*), 10% mannitol (*Ma 10%*), 15% maltodextrins (*MD 15%*), and 5% inositol (*Ino 5%*) in either minimal medium (*MM*) or LB as indicated. *Insert*, texture and consistency of freeze-dried *P. putida* cells lyoprotected with 5% inositol (*Ino 5%*) or MD 15%. **c** Viability *P. putida* MAD2 cells at 7, 14, and 30 days after lyophyllization in the presence of MD 15%, Ino 5%, or both (Ino 5% and MD 15%) when grown in MM or LB

good fraction of the cells lyophilized in the presence of various additives could be revived after complete desiccation. Figure 2a shows the result of the CFU count of the freeze-dried biomass kept at room temperature for 1 day and resuspended in a saline solution following the conclusion of the process. The data indicated that inositol was the agent that maintained the highest viability of *P. putida* cells. Other compounds (maltodextrins, sucrose, and skimmed milk) had a lesser, but still measurable protective effect, while betaine and mannitol had none. To check whether this state of affairs could be improved by pre-growing cells in a rich medium rather than minimal M9 [23, 53], we repeated the freeze-drying process with the otherwise best lyoprotectants (inositol and maltodextrins) added to *P. putida* MAD2 cells cultured in LB. Figure 2a shows the different effects of the growth medium in the ability of these compounds to promote viability of *P. putida* after desiccation. In this case, inositol treatment of LB-grown cells was not as beneficial for survival as the same in minimal medium. In contrast, maltodextrines multiplied by fourfold the CFU count of the sample coming from rich medium. While the molecular mechanisms behind these differences are beyond the scope of this article, these results indicated that both inositol and maltodextrines could definitely help to prepare *P. putida* cells as a dried material which could be later brought back to activity in a significant proportion. A bonus of these procedures was that addition of the lyprotectants to freeze-dried samples (in particular, maltodextrins), increased the physical consistency of the material carrying the biosensor bacteria (see insert of Fig. 2a) thereby facilitating its further manipulation as explained below.

The data of Fig. 2a was generated with cells tested after 1 day of the freeze-drying procedure, which basically reflected the survival of the biosensor bacteria to the desiccation process. The next question was, obviously, what was the viability of the same cells over longer periods of time. To examine this, lyophilized cells originated in minimal or LB cultures and amended with inositol, maltodextrins, or a mixture of both were kept in the darkness, under aerobic conditions, room temperature and ambient pressure. Samples were then taken at 7, 14, and 30 days and the viability tested as before. The results of Fig. 2b indicated that only the mixture of the two lyprotectants was able to maintain a significant portion of the dried cells (>2%) alive for periods beyond 1 month.

Although in a first sight this may look like a massive loss of the cell population, it should be noted that the high cell density of the freeze-dried material (~$10^9$ bacteria/g), makes elimination of 98% of them during storage to be still tolerable for application of such a fast-growing microorganism. As mentioned before, inclusion of the maltodextrins to the lyprotectant mixture endowed the resulting material with an optimal consistency for later encapsulation (see below). At the same time, the data above exposed the damage that complete desiccation does cause to *P. putida*. We thus wondered about alternative components of a formulation for maintaining certain degree of humidity while still preserving a manageable physical form for storage and off-shelf applications.

## Viability of *P. putida* MAD2 after adsorption to corncob

In order to examine whether a semi-dry formulation could keep the viability of biosensor *P. putida* cells for longer periods of time we resorted to utilization of corncob as the carrier material. Corncob is mainly composed of cellulose, hemicellulose, and lignine with small amounts of protein, starch and lipids [54]. This material has been used as carrier of molluscicides (pesticides against molluscs [55]) or in bioremediation field studies for immobilization of bacterial cells [54, 56]. Since corncobs could also hold a suitable consistency for later encapsulation (see below), we set out to test whether such a biological matrix could be a suitable alternative to lyoprotectants for longer-term viability of *P. putida* cells. To this end, we combined this material with an overnight culture of *P. putida* MAD2 (see "Experimental") and measured the viability of the samples along time (Fig. 3a). In this case, samples were sealed to maintain 65.4% of water saturation along the entire testing period. The evolution of the CFU count over time is shown in Fig. 3a. During the first day of the procedure there was a notable increase in the net number of viable bacteria associated to the corncob, due perhaps to the consumption of C sources leaked from the vegetal matrix. The CFU count then decreased down to approximately the initial levels by day 30, what was a considerably higher titer than that achieved with the additions to freeze-dried bacteria discussed above. The protective effect of corncob, however, quickly declined as the water-saturated formulation was let to air-dry at room temperature. Figure 3b shows how reduction of such humidity from 65% to 7% during just 1 day sufficed to bring down viability to <10% and then to <1.0% when samples were further dried to 0.47% humidity. The conclusion of these experiments is that semi-solid slurries of biosensor *P. putida* cells with corncobs can be maintained active and ready to use for periods of up to 30 days, beyond which there is little or no advantage in respect to the use of lyoprotectants to the same end.

## Formulation of *P. putida* MAD1 cells in hard gelatin capsules and in situ detection of BTEX

That some of preservation methods discussed above permitted the maintenance of biomass of *P. putida* cells for some time with a 2% viability allowed us to further

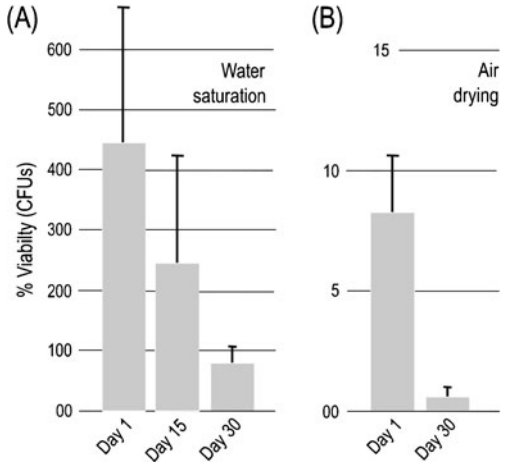


**Fig. 3** Survival of *Pseudomonas putida* MAD2 cells adsorbed to corncobs. **a** Viability of bacteria at 1, 15, and 30 days after adsorption to corncobs and kept saturated in water (65.40%) but otherwise under ambient conditions. **b** Survival of *P. putida* MAD2 adsorbed to corncobs after 30 days of open-air-drying

devise ways for delivery of such biosensors to the target sites in a manageable form. Since powered materials are prone to create dust and disperse in aerosols if simply spread in that form, it was clear that the desiccated bacteria were not the agent of choice as such for wide-area application. To tackle this, we explored the potential of hard (but otherwise water soluble) gelatin capsules as carriers. These capsules, which are widespread in the pharmaceutical industry are made of natural, biodegradable gelatin which can be melt with humidity and ultimately dissolved in water. Furthermore, they can be filled automatically with any powdered material for large-scale production, endowing lyoprotected bacteria with a physical carrier that is more tractable and undemanding to spread with standard agronomic techniques of e.g. seed dispersion. To see whether all these expectations could be fulfilled, we prepared hard gelatin capsules filled with *P. putida* MAD1 cells [35] prepared and lyoprotected as explained in the Experimental section. This $Tel^R$ strain (Fig. 1) bears a Tn*5* insertion carrying a *Pu-lacZ* fusion gene under the control of XylR, the main regulator of the TOL pathway [43]. To monitor the process of melting and release of *P. putida* MAD1 cells, the capsules were laid on water-saturated agar plates containing tellurite, and their progression recorded for the next 7 days. As shown in Fig. 4a, the capsules started to liquefy and release their content in the first day, followed by a clear growth of the bacteria around the leftover gelatin by day 4 (as detected with the black indicator of the $Tel^R$ marker) and complete colonization of the plate by day 7 (Fig. 4a). Since the reporter *xylR/Pu-lacZ* insert of *P. putida* MAD1 is responsive to BTEX, we run a similar experiment in which the agar plates were amended with Xgal and subject to vapors of the XylR effector *m*-xylene (one of the components of BTEX). To this end, the same capsules filled with the biosensor strain were hydrated for 1 week prior to *m*-xylene exposure, in order to let bacteria adopt an active physiological state. Follow-up of the plates revealed that those exposed to *m*-xylene vapors expressed sufficient β-galactosidase activity as to turn the plate blue, whereas controls without the aromatic compound remained white (Fig. 4b, c). These results confirmed that the encapsulation procedure not only allowed release of the bacteria but also the maintenance and activity of the biosensor circuit. Further experiments demonstrated that this ability was basically kept in capsules prepared with freeze-dried bacteria stored for various periods of time up to 1 month (Fig. 4c)

## Detection of BTEX in soil microcosms

The experiments with encapsulated *P. putida* above were made in conditions very far from actual application scenarios, in particular on nutrient-plenty, sterile LB agar plates. In order to examine whether the same outcome would hold in non sterile conditions and without any nutrient addition, we set up a pilot microcosm in which the same capsules with *P. putida* MAD1 were laid on top of water-saturated Fluvisol-type soil immobilized with agar and exposed or not to *m*-xylene. To reveal the activity of the *Pu-lacZ* fusion born by the bacteria on a dark background we resorted to the fluorescent substrate of β-galactosidase named 4-methylumbelliferyl-β-D-galactopyranoside (MUG). The results shown in Fig. 5 indicated that fluorescence only occurred in samples exposed to *m*-xylene, thereby confirming that the biosensor delivery and detection procedure was not damaged by contact with actual soil (at least soil of the type employed in the experiment).

The results above established the scenario for the ultimate test of feasibility of the various methods described for preservation and delivery of the biosensor strain. This involved the use of the deployment strain *P. putida* CPLUX (see "Experimental") rather than the test strains MAD1 and MAD2 and the application of the capsules directly on soil rather than on a more or less artificial setup. Moreover, the output of *P. putida* CPLUX is sheer light rather than color or fluorescence, because the *lux* genes engineered downstream of the *Pu* promoter (see "Experimental") endogenously generate luminescence in a fashion independent of any additional substrate [57]. Gelatin capsules containing *P. putida* CPLUX were prepared with desiccated samples of cells pre-grown in either minimal medium or LB and freeze-dried in the presence of lyoprotectants. The capsules were laid on plates filled with water-saturated soil exposed or not to *m*-xylene, and bioluminescence monitored for the next 82 h. The results shown in Fig. 6 indicated that only soil containing *m*-xylene brought about the production of light at

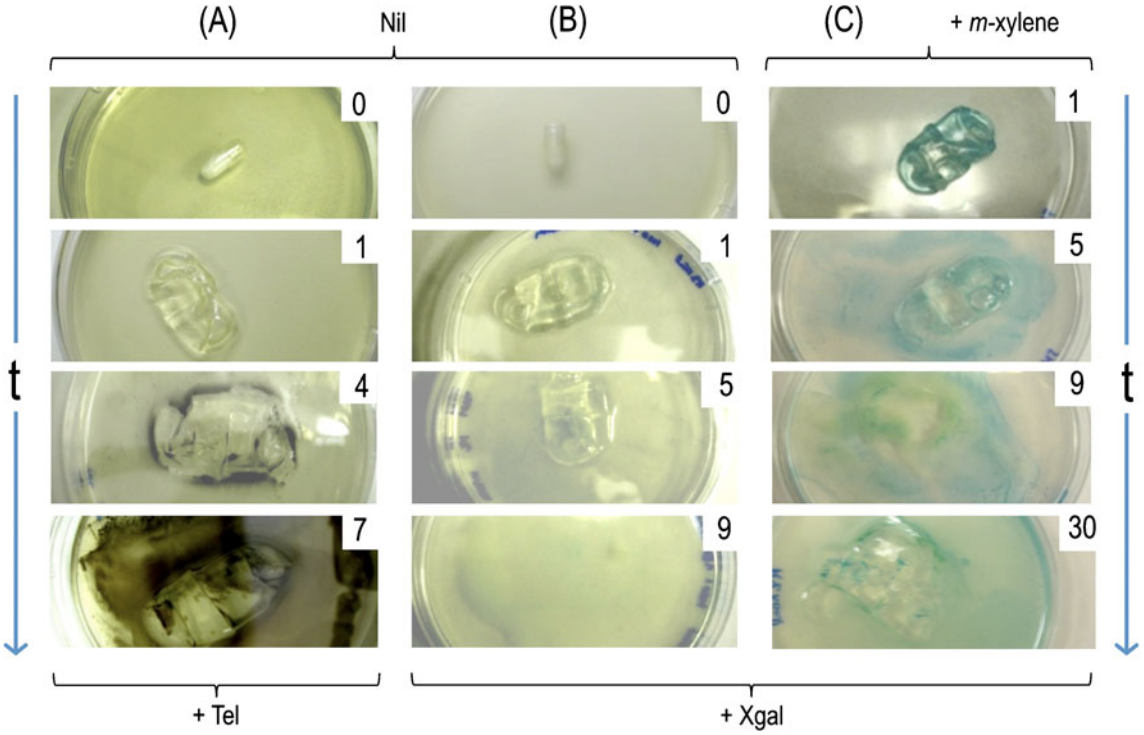


**Fig. 4** Release of *Pseudomonas putida* MAD1 from hard gelatin capsules upon watery melting. Capsules were prepared with fresh lyophilized cells. Monitoring liquefaction of capsules filled with freeze-dried *P. putida* MAD1 and placed on water-saturated minimal medium plates containing tellurite and Xgal and exposed to *m*-xylene, as indicated, for visualization of cell growth and activity of the *Pu-lacZ* fusion born by this strain. The numbers in upper right corner of each picture indicate the number of days following the start of the procedure for each plate. Note the virtually complete melting of the capsules by days 7–9 and the release of active bacteria (*A*). The specific response of the biosensor cells to *m*-xylene can be deduced from the lack of color of the Xgal plates not exposed to the aromatic compound (*B*) as compared with the blue dye of the counterparts with such XylR effector, a typical component of BTEX (*C*). The capsule of the *lower right panel* comes from cells freeze-dried in 15 maltodextrines/5% inositol kept dried for 30 days prior to application during the next 9 days. The presence of active *m*-xylene-responsive cells is apparent

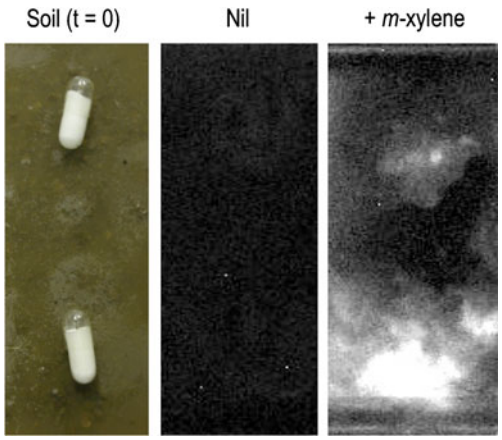


**Fig. 5** Detection of *m*-xylene in soil-agar plates with hard gelatin capsules containing freeze-dried *Pseudomonas putida* MAD1 cells. Capsules were laid on the surface of the microcosms with or without *m*-xylene and let to melt by the action of the saturating water conditions of the assay. After 1 week, the microcosms were spread with the fluorescent substrate of ß-galactosidase (MUG) to reveal the activity of the *Pu-lacZ* fusion borne by *P. putida* MAD1. Note the specificity of the response only in the sample treated with *m*-xylene.

significant levels. Out of the two formulations tested, the material derived from cells pre-grown in LB and then lyophilised with the lyoprotectant cocktail produced an earlier signal but also the light appeared to die off before. The corresponding capsule with cells pre-grown in minimal medium gave a later signal, which was still visible until the end of the experiment. The timing of light emission most likely reflects the peak of the active population that can be reached from the starting number of viable cells in each case. In any case, these experiments validated the viability of the process that goes from engineering a biosensor strain to their final application in an environmentally significant setup. The next logical steps (i.e., scaling of the capsule spreading procedure and the utilization of remote light detection devices) are beyond the scope of this work, but they are perfectly feasible within existing technologies.

## Conclusion: towards environmental Galenics

The possibility of using bacterial biosensors for wide-area applications has been often entertained in the literature [4,

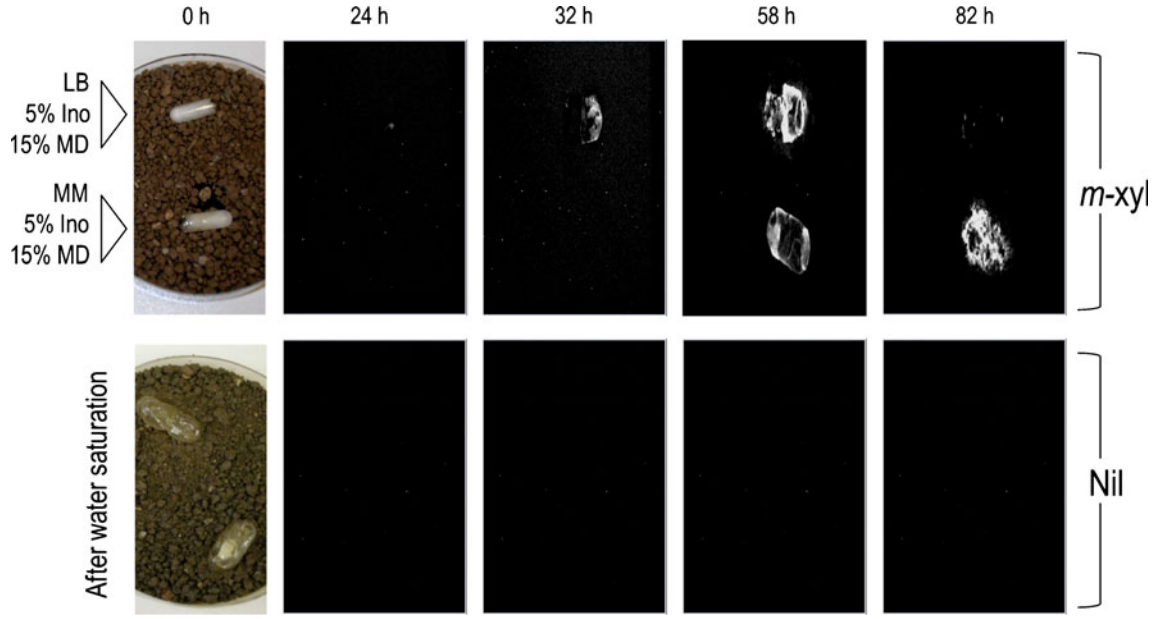


**Fig. 6** Detection of *m*-xylene in soil with the *Pseudomonas putida* biosensor strain CPLUX. Gelatin capsules filled with *P. putida* CPLUX pre-grown in either LB (*upper capsule*) or minimal medium (*lower capsule*) and freeze-dried with a lyoprotectant mixture of 5% inositol and 15% maltodextrins were laid on soil plates with or without *m*-xylene and let to melt by the action of the saturating water conditions of the assay (see rapid melting on lower plate). The presence or absence of *m*-xylene is faithfully reported after a few days through the light emitted by the reporter *Pu-luxCDABE* fusion engineered in the bacteria inoculated, which is not visible in the *m*-xylene-free microcosm. Pictures were captured with a CCD camera after 10 s of recording. Note the different kinetics of light emission dependent on the history of the cells prior to encapsulation

45, 47, 58–60] but hardly ever implemented. Fortunately, the choice of both a host (*P. putida*) and a reporter system (*lux* genes of *P. luminescens*), which naturally thrive in environmental scenarios and tolerate a range of harsh conditions ensures a reliable application in the field of engineered regulatory circuits. Still, bottlenecks in this respect include the preservation of the biosensor after its construction [7] and the implantation of engineered bacteria in an unfamiliar niche which might be afflicted by, e.g., predation by protozoa, a major cause of bacterial mortality [61]. Although freeze-drying is considered a method of choice for long-term preservation of bacteria [24, 51], including biosensors [13, 62, 63], the procedure is not devoid of problems, as the outcome depends on many factors [23–25, 52].

We found that inositol and maltodextrins extended significantly the shelf life of *P. putida* at ambient conditions. The effect of maltodextrins is intriguing, as it could originate in their isomerization and hydrolysis into trehalose [64]. One way or the other, we managed to find a formulation of lyoprotectants to maintain the viability of the sensor strain by 2% after storage for ≥30 days in a powdered form. This is much more than the suggested minimal (0.1%) of the original cell population that is sufficient to allow propagation of the dried bacteria as a starter in other biotechnological processes [65]. The alternative procedure with wet corncobs was also instrumental to maintain a high viability for some time, but the physical characteristics of the material did not allow us to proceed further with it. Slurries of *P. putida* made with this material could however find other applications in the delivery of live catalysts for remediation of polluted soil [54, 56].

Various strategies have been proposed to combine preservation methods with acquisition of a physical format for an easy handling of the bacteria at stake [66–73], including applications to soil [27, 28] or oral delivery of live bacterial cells for therapeutic purposes [74]. In fact, there is a definite parallelism between the problem of formulating and delivering medicines to the human body (which is the subject of Galenic science) and the issue of delivering bacteria to the environment for biosensing or bioremediation [75]. Alas, popular carriers for encapsulation of live bacteria [26, 76–79] are not suitable to be used for extensive applications because of their prize and/or their environmental persistence. In contrast, the use of low-cost water-soluble and biodegradable gelatin capsules might ease the implementation of such wide-area operations. Capsules of this type have a few extra bonuses in respect to other alternatives, such as the possibility of production in large numbers [80], and the incorporation of nutrients for maintaining bacteria active for longer periods of time after hydration. Also, it is possible to make cells dependent (e.g., auxotrophic) on a compound present only in the formula-

tion as to increase their biological containment. And last but not the least, the possibility of employing standard agronomic methods of seed dispersion for covering large test areas.

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