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Tracing explosives in soil with transcriptional regulators of *Pseudomonas putida* evolved for responding to nitrotoluenes

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Summary

Although different biological approaches for detection of anti-personnel mines and other unexploded ordnance (UXO) have been entertained, none of them has been rigorously documented thus far in the scientific literature. The industrial 2,4,6 trinitrotoluene (TNT) habitually employed in the manufacturing of mines is at all times tainted with a small but significant proportion of the more volatile 2,4 dinitrotoluene (2,4 DNT) and other nitroaromatic compounds. By using mutation-prone PCR and DNA sequence shuffling we have evolved *in vitro* and selected *in vivo* variants of the effector recognition domain of the toluene-responsive XylR regulator of the soil bacterium *Pseudomonas putida* that responds to mono-, bi- and trinitro substituted toluenes. Re-introduction of such variants in *P. putida* settled the transcriptional activity of the cognate promoters (*Po* and *Pu*) as a function of the presence of nitrotoluenes in the medium. When strains bearing transcriptional fusions to reporters with an optical output (*luxAB*, GFP) were spread on soil spotted with nitrotoluenes, the signal triggered by promoter activation allowed localization of the target compounds on the soil surface. Our data provide a proof of concept that non-natural transcription factors evolved to respond to nitroaromatics can be engineered in soil bacteria and inoculated on a target site to pinpoint the presence of explosives. This approach thus opens new ways to tackle this gigantic humanitarian problem.

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Introduction

Even assuming no further planting, the worldwide cost of landmine clearance and unexploded ordnance (UXO) using current technologies is estimated by the UN in the range of 30 billion dollars and hundreds of years of work (Bruschini and Gros, 1997; Rouhi, 1997; see also <http://www.sac-na.org>). In the meantime, large portions of agricultural land become abandoned, causing economic difficulties and loss of food stocks. Unfortunately, landmine installation was still surpassing landmine removal by a 30:1 ratio in the mid-1990s (Rouhi, 1997). Mines are difficult and dangerous to detect, because they are most often buried and camouflaged. Moreover, spotting of such explosives is still an archaic practice, as there is not yet any rapid, cost-effective technique, wide-area scanning system available. Traditional de-miners use metal detectors to hand probe the land being examined (although many types of mines have plastic construction to reduce uncovering), dogs for sniffing explosive vapours, and vegetation cutters (Group, 1996). This is hazardous, time-consuming and costly. New principles have been proposed to address this phenomenal problem, including nuclear quadrupole resonance (Suits *et al.*, 1998), solid-state NMR, (Garroay, 1999), surface-enhanced Raman spectroscopy (Sylvia *et al.*, 2000), neutron activation analysis (Csikai *et al.*, 2004), measure of scattered photons (gamma- and X-rays; Hussein and Waller, 2000) and others (for reviews see Group, 1996; Bruschni and Gros, 1997). Many of these methods rely on detection of metals (with the inherent problem of false positives) or bulk chemical detection.

A large number of anti-personnel mines are based on the commodity explosive 2,4,6 trinitrotoluene (TNT) and small amounts of this chemical frequently leak from unexploded mines and get into contact with the surrounding microbial communities. Although explosive-grade TNT is often > 90% pure, its industrial manufacture leaves significant amounts of 1,3 dinitrobenzene (1,3 DNB) and 2,4 dinitrotoluene (2,4 DNT) as impurities (George *et al.*, 1999; Sylvia *et al.*, 2000). All these chemicals permeate through the plastic landmine components, as well as cracks or pores in the mine casing, and migrate to the surface. In addition, some of the leaked TNT degrades

to 4-amino-2,6-dinitrotoluene. The occurrence of these by-products in soil is thus a descriptor of the presence of explosives in a given site. In particular, 2,4 DNT is environmentally more stable, more soluble and more concentrated in the vapour phase than TNT (George *et al.*, 1999; Sylvia *et al.*, 2000). These compounds do have biological activities and can be degraded totally or partially by a number of microorganisms (Esteve-Nunez *et al.*, 2000; Halasz *et al.*, 2002; Lewis *et al.*, 2004); Van Aken *et al.*, 2004). As a result, such biological responses have a potential for detection purposes.

A large collection of strains of soil bacteria (predominantly *Pseudomonas putida* and similar species) have been engineered to produce an optical signal (luminescence, fluorescence) when exposed to specific chemicals (Daunert *et al.*, 2000; van der Meer *et al.*, 2004). In the most elaborated constructs [those for detection of naphthalene (Heitzer *et al.*, 1994; Ripp *et al.*, 2000) or BTEX (Bundy *et al.*, 2000; Daunert *et al.*, 2000; Phoenix *et al.*, 2003)], reporter genes with an optical output (*lux* or *gfp*) were assembled downstream of promoters of the catabolic operons for degradation of such compounds. As these biodegradative pathways are inducible by transcriptional regulators that respond to their natural substrates or to some of their metabolic intermediates (Diaz and Prieto, 2000; Tropel and Van Der Meer, 2004) the reporter genes are turned on in their presence. Unfortunately, there are not TNT-degrading pathways known thus far which are genetically characterized, let alone specific promoters or regulators which respond directly to either TNT or 2,4 DNT (Lonneborg *et al.*, 2007). This may be related to the very poor solubility of TNT, which prevents accumulation of enough bio-available substrate as to induce any intracellular transcriptional regulator (normally in the μM range: Ramos *et al.*, 1990; Salto *et al.*, 1998; van der Meer *et al.*, 2004).

Despite these limitations, microbial-based detection of UXO is still a promising possibility and various experimental avenues have been recently explored to this end. In one case, Looger and colleagues (2003) were able to redesign computationally the ligand specificity of a ribose-responding periplasmic protein of *Escherichia coli* to bind TNT. The engineered receptor was then coupled to a synthetic bacterial signal transduction pathway which was claimed to trigger reporter gene expression in response to extracellular trinitrotoluene. In a second instance, an olfactory receptor expressed in yeast was set to respond to 2,4 DNT (Radhika *et al.*, 2007). However, the microorganisms that hosted these sensors are not robust enough for an extensive application in the field.

In this work we have exploited state-of-the-art combinatorial approaches (Galvao and de Lorenzo, 2005b; Galvao *et al.*, 2007) for evolving *in vitro* prokaryotic transcriptional regulators derived from the XylR protein

encoded by the *P. putida* TOL plasmid pWW0 that activate the cognate promoter *Pu* in response to nitrotoluenes, i.e. the predominant components of a major class of anti-personnel mines. The resulting regulatory elements were then assembled in *P. putida* cells fused to either *lux* or GFP reporters and spread on a target experimental soil microcosm containing 2,4 DNT. Our results show the production of an optical output upon contact of the sensor bacteria with the target chemical. The data thus validate for the first time the capacity of engineered microbial bioreporters to reveal trace amounts of explosives that are typical of landmines and may migrate to the surface of the ground.

Results and discussion

Rationale for the search of 2,4 DNT and TNT-responding variants of XylR

The ultimate basis of a bacterial-based biosensor for detection of specific chemicals is the use of transcriptional regulators which, by themselves or implanted in a genetic circuit, respond to such molecules (Daunert *et al.*, 2000; Wise and Kuske, 2000; van der Meer *et al.*, 2004). To the best of our knowledge, scientific literature has not reported so far any naturally existing bacterial transcription factors responding to TNT or 2,4 DNT. The one instance that comes close to this is the LysR-type regulatory DntR protein from a *Burkholderia* isolate able to degrade 2,4 DNT (Smirnova *et al.*, 2004). Unfortunately, this protein does not respond directly to 2,4 DNT (but to salicylates) and the attempts to redesign its specificity for binding productively this nitroaromatic compound have been quite suboptimal (Lonneborg *et al.*, 2007). In view of this, we turned our attention to the XylR protein, which controls the activity of the σ^{54} -dependent *Pu* promoter of the TOL plasmid pWW0 of the soil bacterium *P. putida* mt-2 for biodegradation of toluene, *m*-xylene and *p*-xylene (Perez-Martin and de Lorenzo, 1996a; Ramos *et al.*, 1997). XylR has a modular structure (Fig. 1A) that includes an N-terminal, signal-reception region (A domain). This A module interacts directly with an inducer molecule (e.g. toluene, *m*-xylene), an event that leads to the conversion of the regulator into a form able to promote transcription from the *Pu* promoter (Fernandez *et al.*, 1995; Perez-Martin and de Lorenzo, 1996b). We have in the past successfully isolated XylR variants bearing changes in the A domain that respond to non-native effectors, including nitroaromatics (Garmendia *et al.*, 2001; Galvao and de Lorenzo, 2005b; Galvao *et al.*, 2007). This allowed us to use two XylR mutant libraries produced in our Laboratory in the search of a protein variant best suited for the type of final application (e.g. *in situ* detection of 2,4 DNT) pursued in this work.

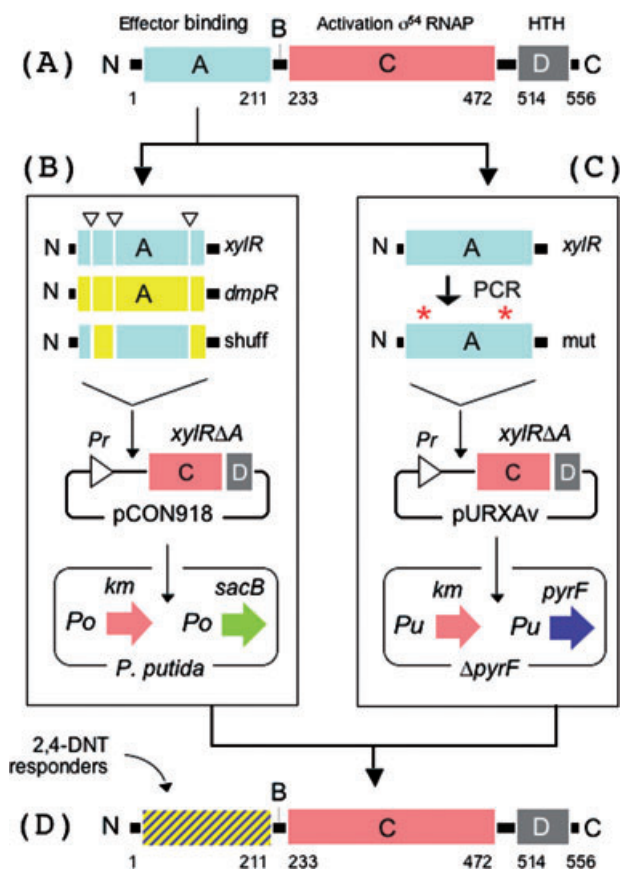


Fig. 1. Strategies for experimental evolution of the XylR protein. A. Domain organization of XylR. Relevant portions of the protein include the signal reception (effector binding) N-terminal A domain (amino acid residues 1–211), the B linker (211–233), the central C module involved in NTP binding and RNAP- σ^{54} activation (233–472), and the D domain at the C-terminus, with a helix–turn–helix (HTH) motif for DNA binding (514–556).

B. Shuffling between the DNA sequences of the A domains of XylR and DmpR. The procedure (Garmendia *et al.*, 2001) implies the generation and mix of DNA segments covering the sequences of interest and their rescue in pCON918, a broad-host-range vector designed for cloning the products of shuffling the A domain sequences of the NdeI–SnaBI fragments. The resulting plasmids are then passed to a *Po*→*km*/*Po*→*sacB* *P. putida* strain for selection of clones able to activate *Po* in the presence of Km and 2,4 DNT, and counterselection of constitutive *xylR* mutants in plates with sucrose. C. Mutagenic PCR. Following error-prone amplification of the DNA sequence of the *xylR* A domain, the resulting PCR products were prepared as an EcoRI–AvrII fragment and cloned in the corresponding sites of plasmid pURXAv, thereby reconstituting full-length *xylR*. The ligation pool is then passed to *P. putida* TEC3 that bears chromosomal *Pu*→*pyrF*-*lacZ* and *Pu*→*km* transcriptional fusions. This allowed selection of the clones responsive to 2,4 DNT by means of the conditionally expressed Km resistance and *pyrF*. D. The outcome of either of these two procedures is the isolation of *xylR* variants encoding A domains that respond to 2,4 DNT.

Breeding and selection of XylR types responsive to nitrotoluenes

Two experimental strategies were employed to generate combinatorial libraries of XylR and selection of variants responsive to nitrotoluenes. In one case (Fig. 1B) we

produced a pool of A domain sequences by shuffling the corresponding DNA segment of XylR (Garmendia *et al.*, 2001) with that of the homologous N-terminal domain of the phenol-responding regulator DmpR (Shingler and Moore, 1994; see *Experimental procedures*). The second approach (Fig. 1C) involved the production of single-amino-acid changes (rather than extensive shuffling) through the sequence of the A domain of XylR by means of error-prone PCR, as explained in detail in Galvão and colleagues (2007).

The results of these two alternative selection procedures are shown in Fig. 2A. The four XylR variants verified to produce the desired phenotypes had mutations previously recognized in other mutagenesis experiments

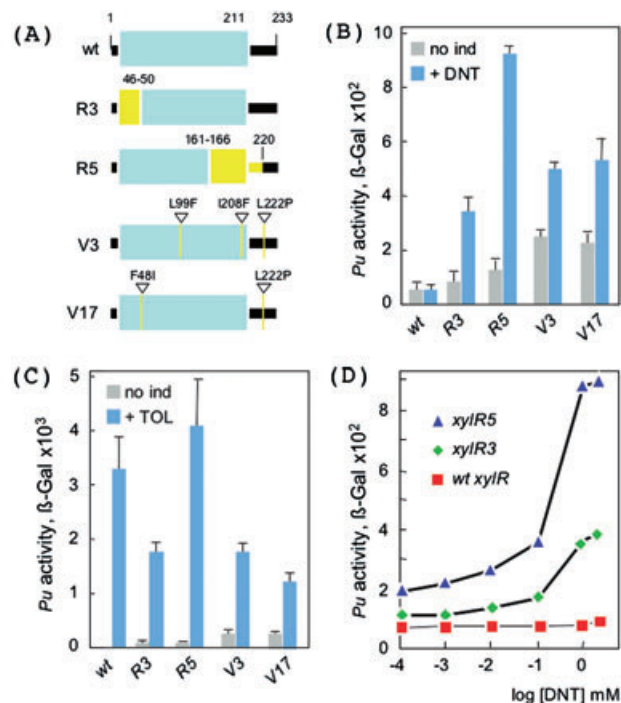


Fig. 2. Characterization of XylR variants responsive to 2,4 DNT.

A. Localization of the amino acid changes found in individual variants of the A and B XylR domain that cause sensitivity to 2,4 DNT.

B. Response of XylR mutants to 2,4 DNT. Cultures of the *Pu*→*lacZ* strain *P. putida* SF05 carrying plasmids encoding each of the *xylR* variants indicated were grown in LB medium to an $A_{600} \sim 1.2$ and then added with 2 mM 2,4 DNT. Following a further 3 h incubation, accumulation of β -galactosidase was measured as described in *Experimental procedures*. Values given represent the average of at least three independent experiments, each of which was conducted in duplicate samples, with deviations being less than 15%. ind, inducer.

C. Response of *xylR* variants to the natural XylR inducer toluene. Experiments were made as before, excepting that the cultures were exposed for 3 h to saturating vapours of the volatile inducer in an airtight flask.

D. Dose–response patterns of XylR, XylR3 and XylR5 to varying concentrations of 2,4 DNT. Induction conditions were identical with those described above excepting for the different concentrations of the inducer.

(Garmendia *et al.*, 2001; Galvao *et al.*, 2007) to expand the range of aromatic compounds that triggered *Pu* activity upon XylR activation. When passed to the reporter *Pu*→*lacZ* strain *P. putida* SF5 (Fig. 2B) and their relative activities compared, it became clear that the variant named XylR5 was the one that originated a better signal/noise ratio in response to 2,4 DNT, followed by XylR3. The other two (V3 and V17 mutants) had a considerable response to 2,4 DNT and a lesser sensitivity to toluene, the natural XylR effector (Fig. 2C). However, these desirable properties occurred together with a greater basal expression level. This made us concentrate in the two A domain shuffled proteins (XylR3 and XylR5) and leave behind the XylR mutants with point mutations. These are certainly useful for understanding basic mechanisms of specificity maturation through XylR evolution (Galvao *et al.*, 2007) but impractical for the *in situ* detection of 2,4 DNT pursued in this work. On the contrary, we judged the dose–response curves of XylR3 and XylR5 to 2,4 DNT (Fig. 2D) in the assay system to be satisfactory enough to go ahead with the pilot experiments for detecting this compound in soil described below. All XylR variants found to respond to 2,4 DNT triggered a strong induction of the *Pu*→*lacZ* fusion when cells were exposed to each of the mono-substituted nitrotoluenes (not shown).

Responses of XylR3 and XylR5 to 2,4 DNT under non-saturating water conditions

In order to assess further the ability of XylR3 and XylR5 for detection of explosive-related compounds, we next compared the responses of *P. putida* cells bearing plasmids encoding each of these proteins in liquid versus agar media. Water limitation and matric stress, a frequent condition of bacteria living in soil, is known to dramatically alter both overall physiology and the structure and activity of specific proteins (Cytryn *et al.*, 2007). In order to compare responses in excess water (liquid medium) with those in non-saturating water conditions (growth on 1.5% agar plates) we streaked out strain *P. putida* *Po*→*luxAB* (Table 1), bearing plasmids pCON916 (*xylR*⁺), pCON922 (*xylR3*⁺) and pCON924 (*xylR5*⁺) on agar plates amended with 2,4 DNT. In this case, the cells were endowed with a luminiscent *luxAB* reporter fusion that can be visualized non-disruptively (*Experimental procedures*). Following growth, plates were exposed to traces of *n*-decanal and placed on X-ray film. The results of such a procedure for the *xylR5*-bearing strain are shown in Fig. 3A (nearly identical results were obtained with the *xylR3* counterpart, not shown). The signal brought about by *luxAB* as reporter basically reproduced the β -galactosidase data of liquid culture of Fig. 2B. These results indicated that the induction properties of the XylR mutants revealed in liquid cultures were kept in a medium not saturated with water.

Furthermore, the data of Fig. 3A suggested that *luxAB* was a suitable visual reporter for revealing the presence of the nitroaromatics under study.

Pinpointing 2,4 DNT spots on a surface with Po→*luxAB P. putida* cells

On the basis of the data shown above, we used strain *P. putida* *Po*→*luxAB* (pCON924 *xylR5*⁺) as a test strain to assess the use of the 2,4 DNT-responsive XylR variants for pinpointing the location of 2,4 DNT on a flat surface. To this end, we run the experiment shown in Fig. 3B, in which we spread a homogeneous suspension of the reporter *P. putida* cells on an agar plate (*Experimental procedures*) in the centre of which a small sample of 2,4 DNT had been laid. Incubation of the plates followed by a brief exposure to *n*-decanal traces triggered production of luminiscent, which could be even detected with the naked eye (Fig. 3C). As a control, the same plates inoculated with *P. putida* *Po*→*luxAB* (pCON916) expressing wild-type XylR did not produce any significant signal. Although these were promising results in a first sight, we noticed also that the optical output of the *lux* genes was quite diffuse and that the luminiscent decreased quickly at higher inducer concentrations. This was surely due to intrinsic toxicity of 2,4 DNT (Galvao *et al.*, 2007) and the likely decrease of intracellular ATP available to the light-emitting reaction (Jansson, 2003). We therefore explored the GFP – rather than *lux* – as the reporter of choice for designing bacteria indicative of the presence of residues of explosives in soil.

Conditional fluorescence of P. putida Pu→GFP with DNT-responsive XylR variants

The same plasmids pCON916 (*xylR*⁺), pCON922 (*xylR3*⁺) and pCON924 (*xylR5*⁺) encoding the various *xylR* alleles discussed above were passed to strain *P. putida* *Pu*→GFP (Table 1) in which a hyperfluorescent GFP variant is expressed under the control of the *Pu* promoter. Each of the strains was grown on agar plates amended or not with a positive induction control (3-methylbenzylalcohol, 3MBA) or the target chemical 2,4 DNT. Figure 4 shows the results of such a test when grown colonies were illuminated with blue light. Consistent with the precedent behaviour of equivalent strains with *lacZ* (Fig. 2) and *luxAB* (Fig. 3) reporters, cells bearing XylR3 and XylR5 variants displayed a strong fluorescence in the presence of 2,4 DNT, which was not seen in the case of those with wild-type XylR. However, we noticed also that, under such conditions, *xylR3* caused a non-neglectable fluorescent signal in the absence of any inducer – perhaps reflecting the same basal activity

Table 1. Strains and plasmids.

Strains/plasmids	Relevant genotype/phenotype/characteristics	Reference
<i>Escherichia coli</i>		
HB101	<i>rpsL</i> (Sm ^r), <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>leu</i> , <i>hsdR</i> <i>hsdR</i> [*] (<i>E. coli</i> K12/ <i>E. coli</i> B hybrid)	Sambrook <i>et al.</i> (1989)
XL1-blue	<i>F</i> ::Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ M15/ <i>recA1</i> <i>endA1</i> <i>gyrA96</i> (<i>Nal</i> ^r) <i>thi</i> <i>hsdR17</i> (<i>rk</i> ⁻ <i>mk</i> ⁻) <i>supE44</i> <i>relA1</i> <i>lac</i>	Lab collection
CC118 λ pir	Δ (<i>ara-leu</i>), <i>araD</i> , Δ <i>lacX</i> 74, <i>galE</i> , <i>galk</i> , 17 <i>phoA</i> , <i>thi</i> -1, <i>rpsE</i> , <i>rpoB</i> , <i>argE</i> (<i>Am</i>), <i>recA</i> lysogenized with λ pir	de Lorenzo and Timmis (1994)
S17-1 λ pir	Tp ^r , Sm ^r , <i>recA</i> , <i>thi</i> , <i>hsdR</i> M ^r , RP4::2-Tc::Mu::Km::Tn7, λ pir lysogen	de Lorenzo and Timmis (1994)
<i>Pseudomonas putida</i>		
KT2440	KT2440 inserted with mini-Tn5 Km2 <i>Po</i> → <i>luxAB</i> transcriptional fusion	Pavel <i>et al.</i> (1994)
<i>Po</i> → <i>luxAB</i>		
KT2440	KT2440 inserted with mini-Tn5 Sm <i>Po</i> → <i>km</i> transcriptional fusion	Pavel <i>et al.</i> (1994)
<i>Po</i> → <i>km</i>		
KT2440	KT2440 inserted with mini-Tn5 Sm <i>Po</i> → <i>km</i> and Tc <i>Po</i> → <i>sacB</i> transcriptional fusions	Garmendia <i>et al.</i> (2001)
<i>Po</i> → <i>km</i> / <i>Po</i> → <i>sacB</i>		
KT2442	KT2442 inserted with mini-Tn5 Km1 <i>Pu</i> → <i>GFP</i> transcriptional fusion from pCON926	This study
<i>Pu</i> → <i>GFP</i>		
SF05	KT2442, prototrophic, rifampicin-resistant derivative of reference strain <i>P. putida</i> KT2440, <i>hom.fg.</i> inserted with mini-Tn5 Sm/Sp <i>Pu</i> → <i>lacZ</i> transcriptional fusion	Fernandez <i>et al.</i> (1994)
TEC3	KT2442, Δ <i>pyrF</i> , Rif ^r Sm ^r Tel ^r ; insertions of mini-Tn5s with <i>Pu</i> → <i>Km</i> and <i>Pu</i> → <i>lacZ-pyrF</i> fusions	Galvao <i>et al.</i> (2007)
Plasmids		
pRK600	Cm ^r , <i>oriColEI</i> , <i>mobRK2</i> , <i>traRK2</i> , helper for mobilization of <i>oriT</i> <i>RK2</i> ⁻ -containing plasmids	Kessler <i>et al.</i> (1992)
pV1567	Cb ^r , RSF1010 replicon, derived from pMMB66HE Δ inserted with 660 bp fragment spanning 1–660 bp <i>xyIR</i> sequence	Skärftstad <i>et al.</i> (2000)
pCON916	Cb ^r , pV1567 derivative expressing the wild-type <i>xyIR</i> sequence under the control of its native <i>Pr</i> promoter.	Garmendia <i>et al.</i> (2001)
pCON918	Cb ^r , pV1567-derived vector for cloning <i>xyIR-dmpR</i> shuffling DNA products.	Garmendia <i>et al.</i> (2001)
pCON922	Cb ^r , equivalent to pCON916 but encoding the <i>xyIR3</i> allele sequence (<i>DmpR</i> 1–45-ASFRR- <i>XylR</i> 46–220)	Garmendia <i>et al.</i> (2001) and this study
pCON924	Cb ^r , equivalent to pCON916 but encoding the <i>xyIR5</i> allele sequence (<i>XylR</i> 1–160-SAFMGR- <i>DmpR</i> 167–220)	Garmendia <i>et al.</i> (2001) and this study
pGreenTir	Ap ^r , pUC derivative carrying the <i>gfp</i> ^{**} gene (double mutant F64L/S65T)	Miller and Lindow (1997)
pMAD	Ap ^r , pUJ9 vector inserted with a 312 bp EcoRI–BamHI fragment spanning the entire <i>Pu</i> promoter sequence	Cases <i>et al.</i> (1996)
pURXAv	Cb ^r ; pJB655 derivative (Blatny <i>et al.</i> , 1997) expressing a <i>xyIR</i> sequence in which the A domain can be excised as EcoRI–AvrII fragment	Galvao <i>et al.</i> (2007)
pURXV3	Cb ^r ; equivalent to pURXAv plasmid expressing <i>xyIRV3</i> variant with mutations L99F, I208F and L222P	Galvao <i>et al.</i> (2007) and this study
pURXV17	Cb ^r ; equivalent to pURXAv plasmid expressing <i>xyIRV17</i> variant with mutations F48I and L222R	Galvao <i>et al.</i> (2007) and this study
pGFP-MAD	Ap ^r , pMAD carrying a 700 bp BamHI fragment spanning the <i>GFP</i> gene from pGreenTir	This study
pCON926	Ap ^r , pUT/mini-Tn5 Km carrying a 5 kb NotI fragment spanning the <i>Pu</i> → <i>GFP</i> transcriptional fusion	This study

detected with the *Pu*→*lacZ* reporter of Fig. 2B. On this basis, we concentrated on the XylR5 variant, as this protein was the one that produced a more robust and consistent 2,4 DNT-responsive phenotype with each reporter systems tested and growth media assayed.

Visual detection of 2,4 DNT in soil amended with 2,4 DNT

Once strain *P. putida* *Pu*→*GFP* (pCON924 *xyIR5*⁺) was recognized as a candidate 2,4 DNT indicator strain, we set out to examine its performance in a small-scale simulation with many of the elements at play in soil tainted with residues of explosives. For this, we immobilized a layer of

a characteristically agricultural fluvisol-type soil in a Petri dish by addition of a top soft agar (*Experimental procedures*), on which a suspension of the reporter cells was unevenly sprinkled. Such plates were then either exposed to saturating vapours of 2,4 DNT or randomly dotted with crystals of the same chemical. As a control, the same set-up was assembled with *P. putida* *Pu*→*GFP* (pCON916) strain which bears the wild-type *xyIR* gene. After overnight incubation, soil plates were examined for fluorescence upon illumination with blue light. The pictures of Fig. 5 clearly show the optical output of GFP in *xyIR5*-bearing cells exposed to 2,4 DNT – in contrast with those with the wild-type regulator. The strongest fluorescence was observed in spots of the soil plate where confluent

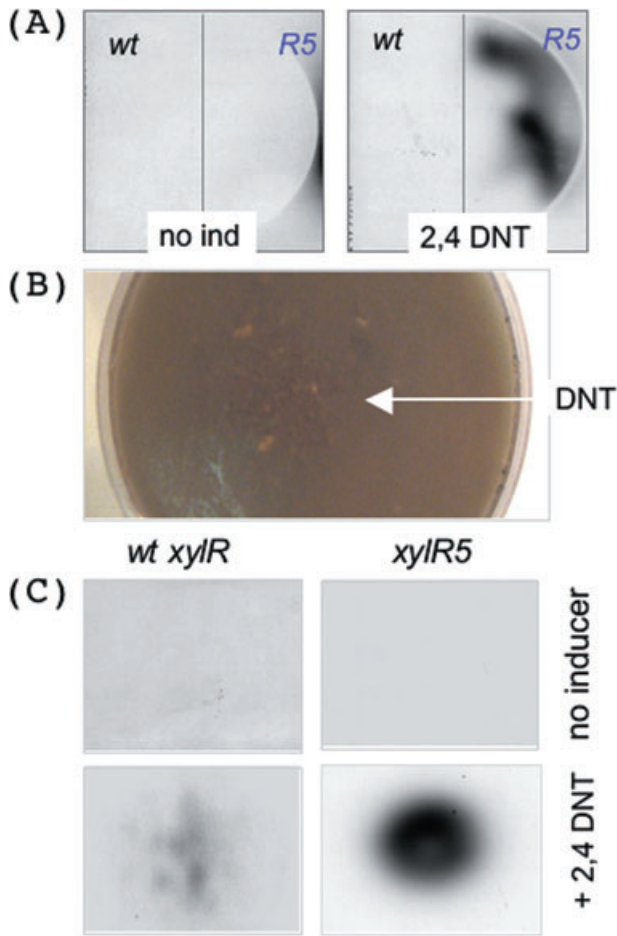


Fig. 3. 2,4 Dinitrotoluene-dependent light emission by *P. putida* *Po*→*luxAB* bearing *xyIR* variants.
 A. 2,4 Dinitrotoluene-dependent luminisence test of *P. putida* *Po*→*luxAB* transformed with either pCON16 (*xyIR*⁺) or pCON924 (*xyIR5*⁺). Strains were streaked out on plates with or without 2 mM DNT and the light recorded on an X-ray film. Note the excellent signal to noise ratio caused by *xyIR5* under these conditions.
 B. Light emission in response to high 2,4 DNT concentrations. An agar LB plate was overlaid with a suspension of approximately 10⁶ colony-forming units (cfu) of the same strains, on the centre of which an approximately 1 mg crystal of 2,4 DNT was deposited in the point indicated with the arrow.
 C. The X-ray film record of such an assay, in which a strong light emission concentrates in the close proximity of the inducer in contact with *P. putida* *Po*→*luxAB* (pCON924.*xyIR5*) cells. Similar results were observed with the equivalent strain bearing *xyIR3*.

growth of the reporter strain overlapped with dots of solid 2,4 DNT crystals. However, vapours of the same compound sufficed to trigger a perfectly detectable optical signal. It is likely that direct exposure of the cell surface to the airborne aromatic compound causes a stronger induction than the equivalent concentration in liquid culture, as 2,4 DNT has to go through an additional phase transfer event in aqueous medium. These pilot experiments demonstrated the viability of the whole-cell sensor approach for detection of 2,4 DNT in soil, the scale-up of

which and the application to real scenarios will be subject of future efforts.

Detection of TNT with 2,4 DNT-responsive XylR mutants

The XylR variants discussed before were evolved and selected to respond to 2,4 DNT. Yet, we have shown previously that changes in the XylR A domain that alter effector specificity broaden also the permissiveness of the resulting protein towards bulkier inducers (Garmendia *et al.*, 2001; Galvao *et al.*, 2007). Although our attempts to set up genetic traps for TNT-responsive *xyIR* mutants similar to those of Fig 2B did not yield any shuffled or mutated variants (data not shown), we wondered whether the ones that responded to 2,4 DNT had simultaneously acquired some sensitivity to TNT (as they had to mono-

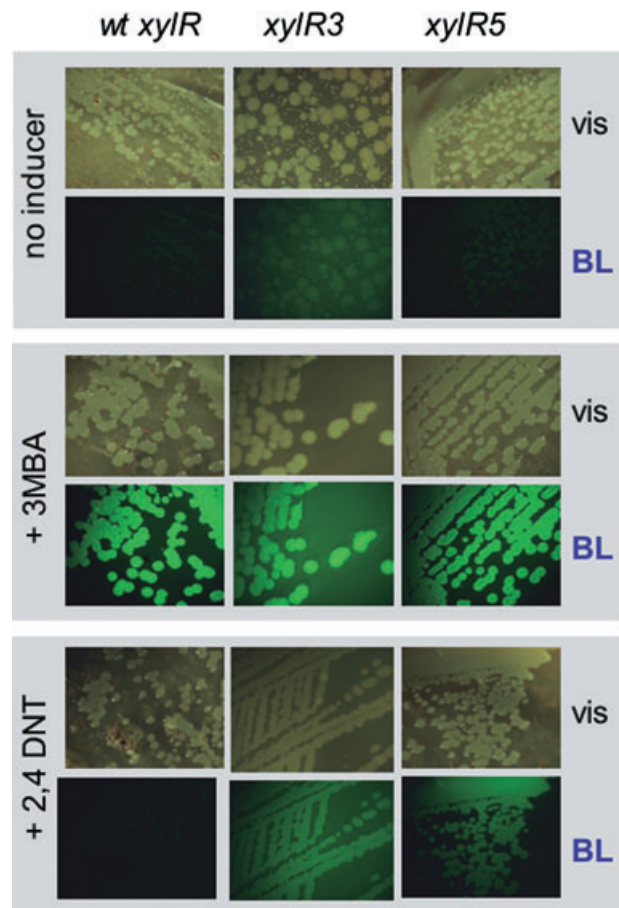


Fig. 4. Emission of green fluorescence by *P. putida* *Pu*→*GFP* bearing *xyIR* variants. pCON916 (*xyIR*⁺), pCON922 (*xyIR3*⁺) and pCON924 (*xyIR5*⁺) encoding various *xyIR* alleles were passed to strain *P. putida* *Pu*→*GFP* (Table 1) and streaked out on agar plates amended with 2 mM of either 2,4 DNT or the natural XylR effector 3-methylbenzylalcohol (3MBA). After overnight growth, plates were illuminated either with white visible light (vis) or with blue light (BL) for exciting the fluorescence of GFP, as indicated in each case.

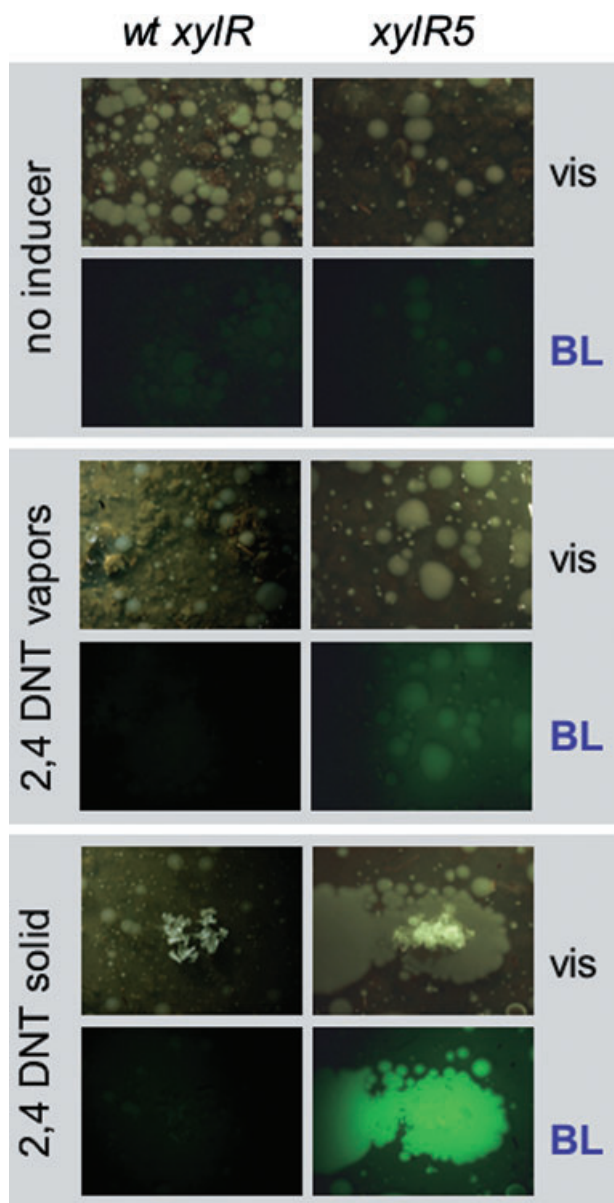


Fig. 5. Detecting 2,4 DNT in soil-agar microcosms spread with reporter bacteria. *Pseudomonas putida* *Pu*→*GFP* cells transformed with pCON924 (*xylR5*⁺) were unevenly sprinkled on soil plates immobilized with soft agar and either exposed to 2,4 DNT vapours or blotted with small dots of the solid compound. As a controls, the *P. putida* *Pu*→*GFP* strain transformed with pCON916 encoding wild-type *xylR* was tested under the same conditions. Note a vigorous fluorescent signal in the parts of the plate where bacterial growth has direct contact with the inducer.

substituted nitrotoluenes, see above). To this end, we examined not only *xylR5*, but also the two other mutants *xylR3* and *xylRV17*, which were selected in the earlier mutagenesis procedures (see above).

Pseudomonas putida *Pu*→*lacZ* cells carrying separately plasmids pCON916 (*xylR*⁺), pCON922 (*xylR3*⁺), pCON924 (*xylR5*⁺) and pURXAV17(*xylRV17*⁺) were

subject to TNT induction experiments in liquid medium in the same conditions as those used for examining the response to 2,4 DNT, the results being shown in Fig. 6A. Although not as pronounced as in the case of 2,4 DNT, we could systematically record an increase of the β -galactosidase levels in cells bearing *xylR5* and *xylRV17* which had been grown in cultures with TNT. In contrast, strains with the wild-type *xylR* or the *xylR3* variant did not react to the presence of the compound. In order to investigate the potential of this result for *in situ* visualization of TNT in soil, we passed plasmids pCON916 (*xylR*⁺), pCON922 (*xylR3*⁺), pCON924 (*xylR5*⁺) and pURXAV (*xylRV17*) to strain *P. putida* *Pu*→*GFP* and reproduced the experiments of Fig. 5 using TNT instead of 2,4 DNT as

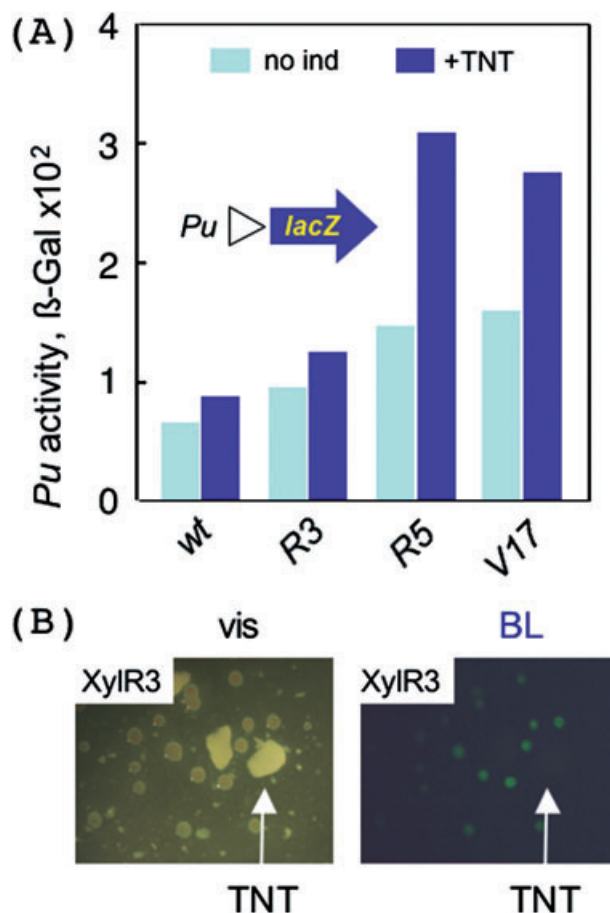


Fig. 6. Responses of 2,4 DNT-sensitive variants of XylR to 2,4,6 trinitrotoluene (TNT).

A. Liquid medium assays. *Pu*→*lacZ* strain *P. putida* SF05 transformed with plasmids encoding each of the *xylR* variants indicated were grown in LB medium and added with 1 mM TNT in the same conditions explained in the legend to Fig. 2B. β -Galactosidase was recorded after 3 h of induction.

B. Plate assay. Strain *P. putida* *Pu*→*GFP* (Table 1) bearing plasmid pCON922 (*xylR3*⁺) was spread on an agar plate sprinkled with solid TNT. Note fluorescence only in the close proximity of the compound. No other XylR variant or the wild-type regulator was observed to produce a significant signal in the same conditions.

the test explosive residue. In contrast to the data of Fig. 6A, neither *xyIR5* nor *xyIRV17* brought about any significant fluorescent signal in colonies grown in the proximity of TNT (not shown). However, cells bearing the *xyIR3* mutant increased its fluorescent output quite above the basal level without inducer (Fig. 6B). That the response of the mutants to TNT depends on whether the assays are made on water-saturating conditions (liquid cultures of Fig. 6A) or cells grown under matrix stress (Fig. 6B) is intriguing. Although high-purity TNT was used in the assays, we cannot altogether rule out that the XylR mutants detect small amounts of contaminating 2,4 DNT instead of sensing *bona fide* TNT. Should this be the case, the contaminant would be in a much lower concentration than the levels found in actual explosives and therefore the strain would still be useful as a biosensor. While this issue deserves some clarification, we argue that the A domain of the XylR protein is a suitable scaffold for developing whole-cell bioindicators for a large variety of chemicals (Galvao and de Lorenzo, 2005b).

Conclusion

The work presented in this article proves that bacterial bioreporters can be utilized to find trace amounts of explosives that are typical of landmines and may migrate to the surface. To this end, we have exploited state-of-the-art combinatorial approaches to generate prokaryotic transcriptional regulators that activate one cognate promoter in response to the predominant components of anti-personnel mines, i.e. nitrotoluenes. These regulatory elements were then engineered in a soil bacterium that is spread on the site under scrutiny and produces an optical output upon contact with the target chemicals. While the concept has been entertained in the scientific and technical literature since at least 1999 (Burlage *et al.*, 1999; see Habib, 2007 for a recent review), to the best of our knowledge, this is the first time that a *bona fide* transcriptional regulator is deliberately produced and characterized to respond to an explosive descriptor such as 2,4 DNT – and shown to work upon spreading in a model soil setup. Other reported attempts have relied on periplasmic ligand-binding proteins (Looger *et al.*, 2003) or olfactory receptors engineered in yeasts (Radhika *et al.*, 2007) but, unfortunately, the biological materials engineered with the sensor system (*E. coli*, yeasts) are not suited for an extensive environmental spreading.

Biological approaches for UXO detection have been often proposed but very poorly documented – if at all – in the scientific literature (Habib, 2007). These include honeybees (Bromenshenk *et al.*, 2003), algae (Altamirano *et al.*, 2004), transgenic plants bearing fusions to putative TNT-responsive promoters (Mentewab *et al.*, 2005) or responsive to NO₂ (http://www.aresa.dk/landmine_plant_

[project_english.html](#); Habib, 2007). Despite the public attention occasionally given to these procedures, their actual value is difficult to assess, as most specific details are hitherto unavailable to a critical inspection. Under these circumstances, we believe that this report is the first in the peer-reviewed literature that rigorously substantiates the capacity of bacterial sensors for *in situ* detection of explosives. Our data provide a first proof of concept that non-natural transcription factors can be engineered in soil bacteria and spread on given sites to pinpoint the presence of explosive traces. Yet, whether such constructs can function for revealing target compounds in real scenarios is to be verified. To this end, the principle shown in this work can be improved in a number of ways (sensitivity, specificity, strain robustness, inoculation protocols, remote detection etc.). We argue that this approach can be applied for the detection and mapping of other dangerous substances and the development of large-scale processes for area reduction. Furthermore, we expect these advances to benefit from the new conceptual frame of Synthetic Biology (Endy, 2005). Specifically, the modularity and orthogonality of the various parts that compose genetic circuits will be implemented through a separate assembly of the sensor parts, the downstream signal-emitting devices and the genetic *chassis* of the host soil bacterium. These are all efforts currently undergoing in our Laboratory.

Experimental procedures

Strains, plasmids and general methods

Recombinant DNA manipulations were carried out according to published protocols (Sambrook *et al.*, 1989). The bacterial strains and plasmids used in this work are listed in Table 1. The characteristics of *E. coli* strains XL-1, S17-1 λ *pir* and HB101 have been published previously (de Lorenzo and Timmis, 1994). The reference *P. putida* strains KT2440 (Nelson *et al.*, 2002) and its rifampicin-resistant variant KT2442 have also been explained elsewhere (Herrero *et al.*, 1990). *Pseudomonas putida* SF05 (Fernandez *et al.*, 1994) is a derivative of *P. putida* KT2442 which bears a *Pu*→*lacZ* transcriptional fusion recombined in its chromosome. *Pseudomonas putida* *Po*→*luxAB* carries a chromosomal fusion of the XylR/DmpR-responsive promoter *Po* (Fernandez *et al.*, 1994) fused to promoterless, luminescent reporter genes from *Vibrio harveyi*. The *Po*→*km*/*Po*→*sacB* *P. putida* strain used as the host for products of the shuffling procedure is described in Garmendia and colleagues (2001). The *Pu*→*GFP* *P. putida* strain was constructed as follows. The 5.0 kb NotI fragment of plasmid pGFP-MAD (Table 1), bearing a fusion between the *Pu* promoter and the promoterless double mutant F64L/S65T of the *GFP* gene was re-cloned at the corresponding site of the transposon delivery vector pUT/mini-Tn5Km (de Lorenzo and Timmis, 1994), thereby generating pCON926. The resulting mini-transposon was inserted into the chromosome of *P. putida* KT2442 by triparental mating of this strain with donor *E. coli* CC118 λ *pir* (pCON926)

and *E. coli* HB101 (pRK600) as helper (de Lorenzo and Timmis, 1994). Selection of exconjugants was made in minimal medium M9 (Sambrook *et al.*, 1989) with 5 mM benzoate as the only carbon source, and 100 µg ml⁻¹ kanamycin. *Bona fide* insertions of the mini-Tn5Km [*Pu*→*GFP*] transposon were verified by inspecting their sensitivity to carbenicillin (Cb, 1 mg ml⁻¹), followed by PCR of relevant sequences to ensure the presence of the desired insert and the loss of the delivery plasmid. Finally one of such insertions was kept for further use. Other plasmids and strains are briefly referred to in Table 1.

Growth and induction conditions

Unless otherwise indicated, *P. putida* strains were grown overnight at 30°C in LB medium amended with suitable antibiotics prior to any procedure. For induction experiments, cultures were diluted 100-fold in fresh medium and grown with vigorous shaking until an absorbance of 1.2 was reached at 600 nm (A_{600}). For inducers tested in their airborne form, the samples were then exposed to saturating vapours of the aromatic compounds under scrutiny in airtight flasks. These were further incubated for 3 h and β -galactosidase levels measured as explained below. Alternatively, once the cultures had reached an $A_{600} = 1.2$, the desired effector was added with 2 mM of the inducer (pre-dissolved in dimethylsulfoxide, except TNT that was pre-dissolved in methanol) and incubated in the same airtight flasks. The activity of *lacZ* fusions was quantified by assaying the accumulation of β -galactosidase in *P. putida* SF05 (Table 1) transformed with the plasmids encoding the *xylR* variants of interest and grown in a liquid culture. β -Galactosidase assays were made with cells permeabilized with chloroform and sodium dodecyl sulfate as described by Miller (1992) under the conditions specified in each case. The linearity of the assay within the range of cell densities and the time of reaction with *o*-nitrophenyl- β -D-galactoside was verified in all cases. Alternatively, cells were grown on the agar surface of airtight Petri dishes with media amended with dissolved inducers as before or exposed, where indicated, to saturating vapours of the same inducers. Chemicals used for induction experiments were purchased from Aldrich, Fluka or Merck and were always of superior analytical purity ($\geq 99\%$).

Generation and selection of 2,4 DNT-responsive *XylR* variants

The two strategies pursued to produce mutants of the transcription factor *XylR* that had acquired the ability to respond to 2,4 DNT are sketched in Fig. 1. In one case (Fig. 1B), the method was based on the shuffling (Stemmer, 1994) of the DNA sequences of the similar A domains of homologous proteins *XylR* and *DmpR*, followed by both positive selection of responders through a *Po*→*km* positive selection cycle and *sacB*-based counterselection of constitutive clones (Skärfs-tad *et al.*, 2000; Garmendia *et al.*, 2001). The second procedure (Fig. 1C) was based on the error-prone PCR of the DNA sequence of the A domain of *xylR* only (Galvão *et al.*, 2007), followed by selection of the reconstructed *xylR* sequences in

a strain bearing a *Pu*→*pyrF* fusion (Galvão and de Lorenzo, 2005a; Galvão *et al.*, 2007).

In situ monitoring of *Pu* activity

Different reporter systems were employed for direct visualization of transcription from *Pu* in response to various aromatic effectors. In one case, *P. putida* KT2440 *Po*→*luxAB* cells containing plasmids with *xylR* variants were grown overnight at 30°C on the agar surface of Petri dishes. After this, plates were exposed to traces of *n*-decanal and the light emission produced by the *luxAB* reporter of the host strains grossly recorded by direct contact of the plates with an X-ray film. Alternatively, strains endowed of *Pu*→*GFP* fusions were grown on Petri dishes as before and examined and photographed under the blue-filtered light of a tunable lighting system coupled to a Leica M7 FLIII lens instrument. For the soil-agar plates, 5 g of sterile soil (Fluvisol type, the kind gift of J.L. Ramos) was evenly laid on the bottom of an empty Petri dish and immobilized with 5 ml of lukewarm soft (0.5%) agar prepared with a mineral-citrate M9 medium (Miller, 1992). Indicator strains and test compounds were then added to such plates as indicated in *Results and discussion*. The fluorescent emission of the cell growth was then recorded as before after overnight incubation.

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