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The construction of a whole-cell biosensor for phosphonoacetate, based on the LysR-like transcriptional regulator PhnR from *Pseudomonas fluorescens* 23F

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Summary

The *phnA* gene that encodes the carbon-phosphorus bond cleavage enzyme phosphonoacetate hydrolase is widely distributed in the environment, suggesting that its phosphonate substrate may play a significant role in biogeochemical phosphorus cycling. Surprisingly, however, no biogenic origin for phosphonoacetate has yet been established. To facilitate the search for its natural source we have constructed a whole-cell phosphonoacetate biosensor. The gene encoding the LysR-type transcriptional activator PhnR, which controls expression of the phosphonoacetate degradative operon in *Pseudomonas fluorescens* 23F, was inserted in the broad-host-range promoter probe vector pPROBE-NT, together with the promoter region of the structural genes. Cells of *Escherichia coli* DH5α that contained the resultant construct, pPANT3, exhibited phosphonoacetate-dependent green fluorescent protein fluorescence in response to threshold concentrations of as little as 0.5 μM phosphonoacetate, some 100 times lower than the detection limit of currently available non-biological analytical methods; the pPANT3 biosensor construct in *Pseudomonas putida* KT2440 was less sensitive, although with shorter response times. From a range of other phosphonates and phosphonoacetate analogues tested, only phosphonoacetaldehyde and arsonoacetate induced green fluorescent protein fluorescence in the *E. coli* DH5α (pPANT3) biosensor, although at much-reduced sensitivities (50 μM phosphonoacetaldehyde and 500 μM arsonoacetate).

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Introduction

Phosphonates are compounds that contain a direct, highly stable, carbon-phosphorus bond; they are frequently of biogenic origin and are ubiquitous in the environment (Ternan *et al*., 1998). The significance of phosphonates in global biogeochemical phosphorus cycling is increasingly recognized (Gilbert *et al*., 2009), especially in marine environments in which P is often the limiting nutrient (Dyhrman *et al*., 2006), yet our understanding of the routes by which they are mineralized is far from complete. Four mechanisms for the microbial cleavage of the C-P bond are known to date: a broad-specificity multi-enzyme system, C-P lyase and three C-P hydrolases that are specific, respectively, to phosphonoacetaldehyde, phosphonopyruvate and phosphonoacetate. Homologues of the genes that encode all four of these activities have been identified in the sequenced genomes of a wide diversity of prokaryotes, and in a range of environmental metagenomic libraries (Quinn *et al*., 2007).

It was originally thought that all of the enzymes involved in the microbial uptake and catabolism of phosphonates were under the control of the *pho* regulon, and hence inducible only under conditions of phosphate limitation (McGrath *et al*., 1997, Ternan *et al*., 1998). The identification of phosphonoacetate hydrolase in a soil *Pseudomonas fluorescens* isolate, which used the compound as sole carbon and energy source (McMullan and Quinn, 1994; McGrath *et al*., 1995), was of particular significance, however, as production of the enzyme was shown to be independent of the phosphate status of its host. Our subsequent structural and functional analysis of the phosphonoacetate hydrolase (*phnA*) gene region in *P. fluorescens* 23F (Kulakova *et al*., 1997; 2001) instead showed that expression of the genes encoding the hydrolase and an associated phosphonate transporter (*phnB*) was inducible by phosphonoacetate, the sole substrate of the enzyme. Induction of phosphonoacetate hydrolase was also shown to be dependent on the presence of the product of an adjacent divergently transcribed regulatory gene, *phnR*. The latter is a member of the group of transcriptional regulators that comprise the LysR (LTTR)

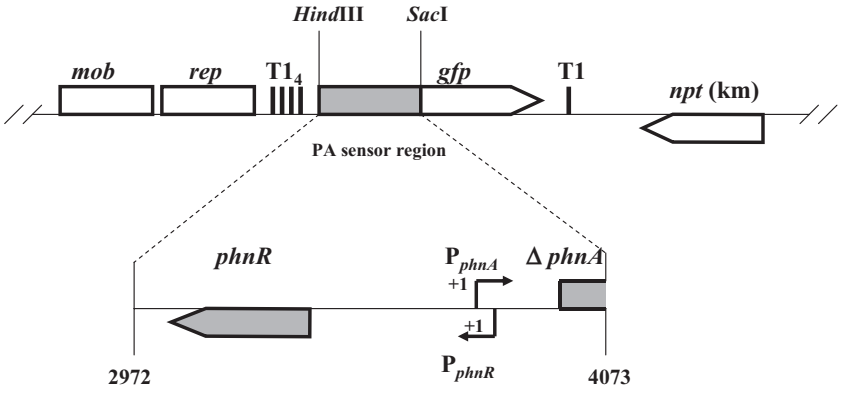

Fig. 1. Construction of the PA biosensor plasmid pPANT3. A 1108 bp fragment from the genome of *P. fluorescens* 23F containing the *phnR* gene together with the *phnA* regulatory region was amplified by PCR and cloned into the HindIII-SacI sites of the promoter probe vector pPROBE-NT. *km*, kanamycin-resistance gene of pPROBE-NT; T1 and T4, *rrnB1* transcriptional terminators of *E. coli*. Δ*phnA*, first 29 nt of the *phnA* gene.

family; these are now thought to be the largest family of prokaryotic DNA binding proteins (Zaim and Kierzek, 2003).

The unique substrate specificity of phosphonoacetate hydrolase, its inducibility by that sole substrate and the widespread distribution of *phnA* homologues are all puzzling, given that phosphonoacetate has not been identified as a natural product (Fields, 1999); it must be regarded as highly likely that biogenic production of the compound does in fact occur. Attempts to identify any such sources have been hindered, however, by the insensitivity of currently available chemical methods for phosphonoacetate detection, which have a lower limit of 50 μM (Klimek-Ochab *et al*., 2003; Panas *et al*., 2006).

To address this difficulty, and thus facilitate the identification of possible biogenic sources of phosphonoacetate, we have exploited the phosphonoacetate-responsive LTTR (PhnR) from *P. fluorescens* 23F (Kulakova *et al*., 2001) to construct a whole-cell bacterial biosensor based on the stable, broad-host-range, promoter probe vector pPROBE-NT (Miller *et al*., 2000). A suitable bacterial host containing this construct might therefore be expected to develop a fluorescent response to the presence of the inducer, phosphonoacetate. Biosensors based on such microbial sensing and signalling systems are increasingly used in industrial and environmental applications because of their sensitivity, simplicity and robustness (Huang *et al*., 2005, Garmedia *et al*., 2008); several have exploited the interaction between members of the LTTR family and their cognate (frequently aromatic) small-molecule effectors (e.g. Cebolla *et al*., 1997; Smirnova *et al*., 2004; Huang *et al*., 2006).

Results

Construction of the biosensor plasmid pPANT3 and creation of phosphonoacetate sensor strains

A 1108 bp fragment of the phosphonoacetate degradative gene cluster from *P. fluorescens* 23F (Kulakova *et al*., 2001), consisting of the entire LTTR regulatory gene *phnR*, the promoter regions for *phnR* and its associated structural genes (*phnA* and *phnB*), and the first 29 5′-end nucleotides of the phosphonoacetate hydrolase gene (*phnA*), was amplified and cloned into pPROBE vectors as described in *Experimental procedures*. This led to the creation of a *phnR–ΔphnA–gfp* transcriptional fusion (Fig. 1). Transformation of cells of *Escherichia coli* DH5α-T1R (further designated as DH5α) with pPROBE::*phnR–ΔphnA* constructs allowed for the selection of clones showing an elevated, statistically significant, fluorescence response to the presence of phosphonoacetate in the medium when compared with phosphonoacetate-free controls. Analysis of these strains demonstrated that the fluorescence values produced by pPROBE-NT-based constructs were approximately two times higher than those based on pPROBE-TT under similar conditions. In the light of this finding, a pPROBE-NT::*phnR–ΔphnA* plasmid designated pPANT3 was used in subsequent biosensor optimization studies.

Effect of growth conditions on biosensor response to phosphonoacetate

It is known that growth conditions affect the expression of *gfp* in bacterial hosts. We found that *gfp* induction ratios were approximately three times higher after incubation with phosphonoacetate at 28°C when compared with 37°C (results not shown). All subsequent experiments were therefore conducted at 28°C. Medium composition is also thought to exert an effect, while in addition the system has been shown to be especially well suited for quantification of promoter activity in cells grown on solidified, agar-based media (Lissemore *et al.* 2000). We therefore compared phosphonoacetate-induced GFP production by cells of DH5α (pPANT3) grown on mineral salts medium, Luria–Bertani broth (LB) and quarter-strength and one-tenth strength LB. As *gfp* induction ratios in cells grown on mineral salts medium were no higher than those grown on LB, the latter was used for further experiments (results not shown). The lowest concentration of phosphonoacetate (0.5 μM) was detectable by DH5α (pPANT3)

Table 1. Induction of GFP expression in DH5α (pPANT3) by phosphonoacetate.

Growth/induction medium	Induction time (h)	GFP induction ratio (mean ± SD)[a] by PA (μM)[b]					
		0.5	1.0	2.5	5.0	10.0	25.0
Solidified (agar):							
LB	24	1.0 ± 0.1[a]	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.4	5.1 ± 1.1
LB	72	1.0 ± 0.1	1.6 ± 0.5	2.0 ± 0.4	2.2 ± 0.6	4.7 ± 0.9	18.1 ± 2.6
0.25 LB	24	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.8 ± 0.2	4.4 ± 1.1	10.3 ± 1.6
0.25 LB	48	1.0 ± 0.1	1.4 ± 0.4	1.3 ± 0.1	1.3 ± 0.1	3.6 ± 0.7	7.7 ± 17
0.25 LB	72	1.0 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	2.5 ± 0.3	5.9 ± 2.2
Liquid (broth):							
LB	24	1.9 ± 0.2	2.1 ± 0.3	ND	18.6 ± 1.9	28.2 ± 3.0	ND
LB	48	2.4 ± 0.3	3.2 ± 0.7	14.5 ± 4.3	23.9 ± 1.3	34.2 ± 3.0	ND
0.25 LB	24	1.0 ± 0.1	2.0 ± 0.3	ND	ND	ND	ND
0.25 LB	48	1.0 ± 0.2	2.0 ± 0.4	2.0 ± 0.3	2.1 ± 0.4	2.1 ± 0.3	2.5 ± 0.4
0.1 LB	24	1.5 ± 0.5	2.4 ± 0.2	ND	2.5 ± 0.1	3.3 ± 0.3	3.5 ± 0.5
0.1 LB	48	1.5 ± 0.5	2.5 ± 0.3	ND	3.2 ± 0.7	3.7 ± 0.9	4.0 ± 0.4

a. Induction ratios were calculated as SFUx/SFUo, where SFUx is the specific fluorescence of the sample in the presence of the inducer and SFUo is the specific fluorescence of the uninduced control sample at the same time point. A value of 1.0 therefore corresponds to no fluorescence being detected.
b. Values represent means of three independent experiments.
Underlining of values shows that there is a statistically significant increase in fluorescence ($P < 0.05$) over the no-inducer control (based on a paired *t*-test).
ND, not determined.

grown in full-strength LB (Table 1). Growth on more dilute medium generally resulted in lower levels of induction, especially at higher phosphonoacetate concentrations (10–25 μM). Prolonged induction (48–72 h) led to a significant increase in biosensor sensitivity only on agar-solidified LB. Broadly similar findings were obtained by Stiner and Halverson (2002) in their study of a whole-cell toluene biosensor based on a *PtbuA1–gfp* transcriptional fusion. Accordingly, biosensor cells for all further experiments were produced by overnight growth at 28°C in full-strength LB.

Specificity of the pPANT3 biosensor construct

To determine whether transcription of *phnA* can be induced by other compounds, GFP expression levels in *E. coli* DH5α (pPANT3) were studied in the presence of 17 organophosphonates and 5 phosphonoacetate structural analogues (see full list in *Experimental procedures*). Exposure of the DH5α (pPANT3) cells to the majority of these compounds did not result in detectable levels of GFP fluorescence. Control experiments were set up in which each of these compounds was added to growth media at a concentration of 2 mM. These, with the exception of phosphonoacetaldehyde, did not affect the growth of the sensor strain.

Apart from phosphonoacetate, only phosphonoacetaldehyde and arsonoacetate were found to induce fluorescence by the *E. coli* DH5α (pPANT3) whole-cell biosensor. Its response to a broad range of concentrations (0.05 μM–50 mM) of these effectors was further tested in liquid LB and compared with that of phosphonoacetate (Fig. 2). These data confirm that phosphonoacetate induces detectable biosensor fluorescence at concentrations of as little as 0.5 μM, and that the response reaches saturation in the presence of approximately 500 μM. By contrast, the biosensor reacts to phosphonoacetaldehyde with some 100-fold reduced sensitivity, while the maximum response is only some 40% of that obtained using phosphonoacetate (Fig. 2). The decreased level of biosensor response above 500 μM phosphonoacetaldehyde is most likely due to the inhibition of the host's cellular activities by this compound [this was also demonstrated by the almost complete inhibition of growth of *E. coli* DH5α (pPANT3) on solidified LB medium containing 50 mM phosphonoacetaldehyde]. Arsonoacetate was found to be an even less effective inducer than phosphonoacetaldehyde; it was detected by the DH5α (pPANT3) biosensor at threshold concentrations of between 0.5 and 50 mM – a decrease in sensitivity relative to phosphonoacetate of greater than 1000-fold – while the maximum level of GFP expression reached only 50% of that produced by phosphonoacetate (Fig. 2).

Expression of pPANT3 in different bacterial hosts

The experiments described above demonstrate that the *E. coli* DH5α (pPANT3) whole-cell sensor can respond to low concentrations of phosphonoacetate. In order to investigate whether the same plasmid can function in other γ- and β-proteobacteria, pPANT3 was introduced into *Pseudomonas putida* KT2440, *Variovorax* sp. Pal2 and *Achromobacter* sp. Pal29. Five pPANT3 transformant

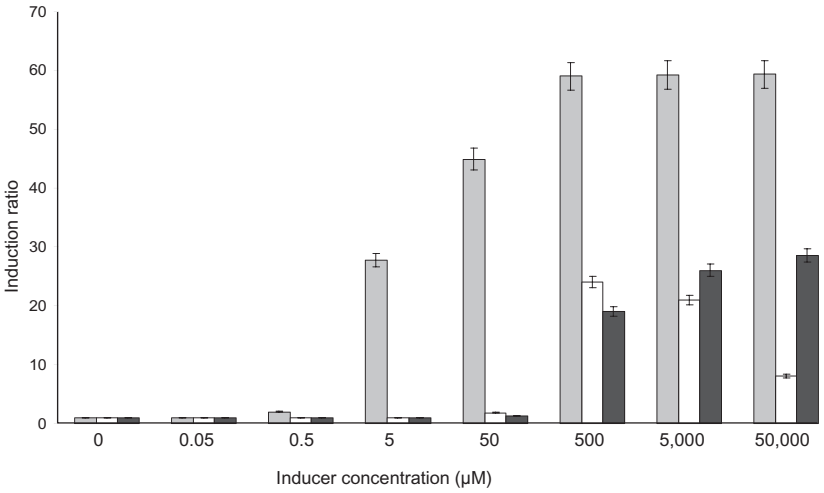

Fig. 2. Induction of *gfp* in *E. coli* DH5α (pPANT3) in the presence of PA (light grey), phosphonoacetaldehyde (white) and arsonoacetate (dark grey). Induction ratio was calculated as SFUx/SFUo, where SFUx refers to the sample containing the inducer and SFUo to the control sample at the same time point: a value of 1.0 corresponds to no fluorescence being detected. Specific fluorescence (SFU) was calculated as described in *Experimental procedures*. All measurements were made in triplicate. Error bars represent a standard deviation of the mean ($n = 3$).

colonies of each strain were then tested for expression of GFP after exposure to up to 10 mM phosphonoacetate. It was shown that neither of the β-proteobacterial strains (*Variovorax* sp. Pal2 and *Achromobacter* sp. Pal29) containing pPANT3 could serve as whole-cell phosphonoacetate sensors. However, the γ-proteobacterium *P. putida* KT2440 (pPANT3) showed GFP fluorescence when induced by phosphonoacetate, although detectable levels of induction required phosphonoacetate concentrations of 10 μM and greater. It has been shown previously that for some promoters (especially weak ones) production of a GFP response is species-dependent and more pronounced in *E. coli* cells (Leveau and Lindow, 2001).

In contrast to the comparative insensitivity of the *P. putida* KT2440 (pPANT3) biosensor, its expression of GFP in the presence of 100 μM phosphonoacetate was more rapid than that of the *E. coli* DH5α-based reporter; a detectable response was produced after 2 h incubation (Fig. 3), and peak induction levels after 20 h were consistently 2.0–2.5 times higher than in the case of *E. coli* DH5α (pPANT3).

Discussion

The pPANT3 plasmid construct has proved to be an effective basis for a whole-cell biosensor for phosphonoacetate in both *E. coli* and *P. putida* host strains, and is capable of detecting concentrations of as little as 0.5 μM. The chromatographic assay techniques currently employed, by contrast, have a lower detection limit of 50 μM phosphonoacetate (Klimek-Ochab *et al*., 2003; Nowack 2003; Panas *et al*., 2006). Importantly, even greater biosensor sensitivity might be achieved by the introduction of pPANT3 into an *E. coli* B host as this strain, unlike *E. coli* K-12 (i.e. DH5α-T1R), where *phnE* gene is inactivated by an 8 bp insertion (Makino *et al*., 1991), has a fully functional phosphonate uptake system.

It was found that two analogues of the target compound (phosphonoacetaldehyde and arsonoacetate) could also induce GFP fluorescence by the pPANT3-based biosensor, although only when present in much higher concentrations. This latter finding raises the possibility that site-specific mutagenesis and/or directed evolution of the *phnR* component of pPANT3 might lead to the development of biosensors for a wider range of phosphonates of environmental or clinical importance, for example, glyphosate. In terms of our more immediate objective, however, the pPANT3 biosensor construct will be central to a screening programme that will use a variety of enrichment criteria in an effort to detect phosphonoacetate production by environmental microorganisms. A possible route would be through catabolism of the predominant biogenic phos-

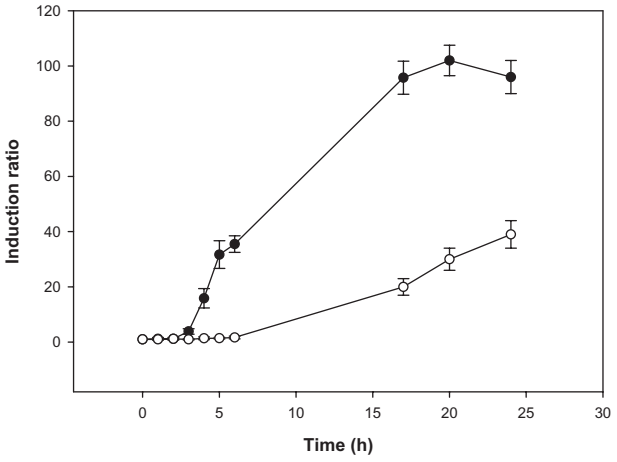

Fig. 3. Kinetics of GFP expression in *E. coli* DHα (pPANT3) and *P. putida* KT2440 (pPANT3). The concentration of phosphonoacetate was 100 μM. Induction ratios were calculated as described in Fig. 2 and in *Experimental procedures*. (-■-), GFP induction ratio in *P. putida* KT2440 (pPANT3); (-○-), GFP induction ratio in *E. coli* DH5α (pPANT3). Error bars represent a standard deviation of triplicate measurements.

phonate, 2-aminoethylphosphonic acid (2-AEP) (Ternan and Quinn, 1998; Quin and Quin, 2001); the analogous production of sulfoacetate from taurine, the sulfonate analogue of 2-AEP, has been documented (Denger *et al*., 2004). It would be possible, for example, to conveniently screen large numbers of 2-AEP-metabolizing environmental isolates growing on solid medium using phosphonoacetate biosensor cells immobilized in an agar overlay.

Experimental procedures

Bacterial strains, vectors, chemicals and media

The phosphonoacetate-degrading isolate *P. fluorescens* 23F (McGrath *et al*., 1995) was the source of the *phnR* and *phnA* genes. The other bacterial strains used in this study were *P. putida* KT2440 with a published genome sequence (Nelson *et al*., 2002), *Variovorax* sp. Pal2 (Kulakova *et al*., 2003) and *Achromobacter* sp. Pal29 (laboratory collection). Competent cells of *E. coli* DH5α-T1R (Invitrogen) were also used. The LB medium (liquid or solidified with 1.5% agar) was used for general cultivation of *E. coli* and *Pseudomonas* strains and for induction studies. For culture of *Variovorax* sp. Pal2 and *Achromobacter* sp. Pal29 minimal medium (Kulakova *et al*., 2001), or 5- or 10-fold diluted LB medium, were used.

Antibiotics were added to media where appropriate at the following final concentrations: kanamycin – 50 μg ml^{-1} for *E. coli*, 100 μg ml^{-1} for *P. putida* KT2440 and 300 μg ml^{-1} for *Variovorax* sp. Pal2 and *Achromobacter* sp. Pal29; tetracycline – 20 μg ml^{-1} for *E. coli*.

All chemicals studied as potential inducers (18 phosphonates and 5 phosphonoacetate homologues; see below) were of highest commercially available purity (> 99.5%). All corresponding aqueous stock solutions were filter-sterilized with 0.2 μm pore-size filters.

Molecular biology techniques and materials

Standard methods for DNA manipulation were used throughout this work (Sambrook and Russel, 2001). Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen). Polymerase chain reaction (PCR) products and restriction DNA fragments were purified from agarose gels with the Concert Rapid Gel Extraction system (Gibco BRL, Life Technologies). All restriction enzymes and T4 ligase were obtained from Promega. *Pfu*-Turbo DNA polymerase was from Stratagene. The PCR and sequencing primers were synthesized by Sigma-Genosys (Sigma-Aldrich). Nucleotide sequences were determined by the Sequencing Service of Dundee University (School of Life Sciences, Dundee, Scotland).

Preparation of competent cells

Electrocompetent cells of *P. putida* KT2440, *Variovorax* sp. Pal2 and *Achromobacter* sp. Pal29 were prepared as follows: bacterial cultures grown to OD_{600} = 0.35–0.4 were precipitated by centrifugation at 1000 *g* and washed subsequently with ice-cold deionized water (two times), 10% glycerol (four times) and once with GYT medium (Tung and Chow, 1995). Cells were finally resuspended in GYT medium (1/100 of the initial culture volume). This preparation was divided into 40 μl aliquots, which were frozen in liquid nitrogen and stored at –80°C until required. Electroporation of the pPANT3 plasmid into bacterial cells was performed using a Model 2510 electroporator (Eppendorf) in accordance with the manufacturer's recommendations.

Construction of the phosphonoacetate whole-cell sensor

The GFP promoter probe vectors pPROBE-NT and pPROBE-TT (Miller *et al*., 2000) were employed to produce the phosphonoacetate biosensor plasmid. Both are based on the broad-host-range pBBR1 replicon (Antoine and Locht, 1992); they differ only in that the former confers resistance to kanamycin, the latter to tetracycline. Gene fusions in the pPROBE suite of promoter probe vectors are flanked by T1 transcriptional terminators from the *E. coli rrnB1* operon that prevent readthrough transcription from the cloned promoters (Miller *et al*., 2000). The primers JQ158 (forward): 5′-GGG AAG CTT AAA TAC CGG CAC CAA TAT CTA and JQ165 (reverse): 5′-CGC GAG CTC ACG CTG ATA AGT TGT CGT A with introduced HindIII and SacI restriction sites (underlined) were used for PCR amplification of the *phnR-phnA* promoter region. The PCR conditions were as follows: 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, followed by extension at 72°C for 5 min. The PCR product was purified from agarose gel and after digestion with appropriate restriction enzymes was directionally cloned into both pPROBE-NT and pPROBE-TT vectors. Ligated DNAs were transformed into *E. coli* DH5α-T1R competent cells. Transformants were selected on LB plates with kanamycin (or tetracycline) and phosphonoacetate (10 mM). The GFP fluorescence was assessed both visually and by use of a TECAN microplate reader (Maennedorf, Switzeland) following 48 h of colony growth at 28°C. The pPROBE-NT-derived plasmid present in an individual transformant that was selected for further analysis was designated pPANT3.

Analysis of GFP fluorescence induced by phosphonates

The phosphonoacetate-sensing properties of pPANT3 in different bacterial hosts were investigated using cells grown in liquid and solid media in the presence of kanamycin as selective marker for the sensor plasmid. Induction levels and effector range were tested in liquid media as follows: overnight cultures grown at 28°C were diluted 100 times with LB medium and incubated with aeration on an orbital shaker (100 r.p.m.) at 28°C for 2 h. Phosphonoacetate, or other effectors to be tested, were added at this point and incubation was continued under the same conditions. At specific time points 300 μl samples were taken, cells were precipitated by centrifugation and resuspended in the same volume of 0.9% NaCl to avoid elevated background fluorescence levels. Samples were then placed into 96-well microplates and fluorescence was immediately measured.

Cultures grown on solid medium were tested as follows: 50 μl of mid-log phase culture was plated on LB agar plates containing the effector to be tested and were incubated overnight at 37°C, or for 2 days at 28°C. Cells were

then washed from plates with 0.9% NaCl, the suspension was adjusted to $OD_{600} \approx 0.5$ and fluorescence was immediately measured. In addition to phosphonoacetate, the following phosphonates or phosphonate analogues were tested as potential inducers of GFP expression in the whole-cell biosensors: phosphonoacetaldehyde, glyphosate, 2-aminoethanephosphonate, 2-phosphonopropionate, phenylphosphonate, 3-phosphonopropionate, ethanephosphonate, diethylmethanephosphonate, hydroxymethanephosphonate, methanephosphonate, 3-hydroxymethanephosphonate, 3-aminopropanephosphonate, phosphonoformate, phosphonoalanine, 2-phosphonobutyrate, 4-aminobutanephosphonate, aminomethanephosphonate, malonate, oxalate, acetylphosphate, arsonoacetate and sulfoacetate. Acetate and phosphate were also tested. The above compounds were added to growth media to final concentrations of 1 and 2 mM.

All experiments were performed in triplicate. Fluorescence measurements were taken by setting the excitation wavelength to 485 nm and measuring emission at 535 nm. Specific fluorescence unit (SFU) was calculated by dividing the relative fluorescence value obtained by the cell density (RFU/OD_{600}) to allow normalization. The induction ratio was calculated as SFUx/SFUo, where SFUx is the SFU of the sample in the presence of the inducer and SFUo is the SFU of the uninduced control sample at the same time point.

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