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A *luxCDABE*-based bioluminescent bioreporter for the detection of phenol

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A bioluminescent reporter strain, *Acinetobacter* sp. DF4-8, was constructed for the detection of phenol by inserting a *mopR*-like promoter upstream of the *Vibrio fischeri* bioluminescent *luxCDABE* gene cassette in a modified mini-Tn5 construct. When introduced into the chromosome of *Acinetobacter* sp. DF4, the bioreporter produced a sensitive bioluminescent response to phenol at concentrations ranging from 2.5 to 100 ppm. This response was linear (R^2=0.986) in the range from 20 to 90 ppm. A significant bioluminescent response was also recorded when strain DF4-8 was incubated with slurries from aged, phenol-contaminated soil.
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Introduction

Phenols are distributed in various environmental sites either as natural or man-made aromatic compounds. Their existence as major pollutants in industrial wastewater treatment plants, such as oil refineries, petrochemical plants, coking plants, and phenol resin industry plants, has been well established [22,27]. Phenol and its homologs exhibit environmental toxicity and are among the most frequently found pollutants in rivers, industrial effluents, and landfill runoff waters [21]. They are also problematic toxicants in waste treatment systems [5]. Consequently, inexpensive and real-time methods for detecting and monitoring phenol in the environment are warranted. Potentially, this could be achieved through the use of bioreporter organisms. Bioreporters refer to intact, living microbial cells that have been genetically engineered to produce a measurable signal in response to a specific chemical or physical agent in their environment. The genetic construct consists of an inducible promoter gene fused to a reporter gene such as *lacZ*, *gfp*, *luc*, *luxAB*, or *luxCDABE*. The *luxCDABE* reporter gene has been used extensively in bioreporter constructs since it produces an easily measured signal (bioluminescence) that requires no extraneous addition of substrate for activation. Therefore, *luxCDABE*-based bioreporters operate autonomously to provide real-time, on-line assessments of target analyte bioavailability [3,13,15,17].

Microbes belonging to the genus *Acinetobacter* have been isolated from various environmental sites and are capable of growing on phenol as a sole carbon and energy source. For example, *Acinetobacter calcoaceticus* strain NCIB8250 metabolizes phenol to catechol *via* a chromosomally encoded multicomponent phenol hydroxylase located in the *mop* operon [10]. Specific regulation occurs at the *mopR* locus, where MopR activates phenol hydroxylase expression upon binding with phenol, as well as 3-chlorophenol, *o*-cresol, and *m*-cresol [25]. MopR-regulated transcriptional activation is not established in response to any of the dimethylphenols or catechols, and to only a few of the dichlorophenols. Due to such specificity, *Acinetobacter* strains are prime candidates for whole cell bioreporter monitoring of phenol.

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Materials and methods

Isolation and characterization of the phenol-degrading strain DF4

Strain DF4 was isolated from industrial wastewater samples obtained from Misr Fine Spinning and Weaving (Alexandria, Egypt) by plating on MP agar plates (1^{-1} H_2O: 2.75 g of K_2HPO_4, 2.25 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2{\cdot}6H_2O$, 0.1 g of NaCl, 0.02 g of $FeCl_3{\cdot}6H_2O$, 0.01 g of $CaCl_2$, pH 7.0) containing 3 mM phenol as sole carbon source [28]. A single colony type appeared after incubation at 30°C for 72 h. Total genomic DNA was prepared from the isolate by the method of Olsen *et al* [19]. 16*S* rRNA analysis was then performed using the eubacterial universal primers 27F and 1492R [16], generating a PCR fragment of approximately 1.4 kb. The fragment was purified using QIAquick spin columns (Qiagen, Valencia, CA), sequenced on an Applied Biosystems 373 DNA sequencer (Foster City, CA), and analyzed using the CHECK_CHIMERA and SIMILARITY_RANK databases from the Ribosomal Database Project and the BLAST program from the National Center for Biotechnology Information [1]. The 16*S* rRNA sequence has been deposited in the GenBank database under accession no. AF356748.

Construction of a bioluminescent bioreporter for phenol

mopR is a regulator gene involved in the phenol degradation pathway of *A. calcoaceticus* NCIB8250 (accession no. Z69251) [25]. A 0.72-kb *mopR*-like promoter segment was amplified from strain DF4 using two primers specific for the *mopR* promoter of *A. calcoaceticus* NCIB8250. The first primer,GCGGCCGCTCCCCAGCCAAAGATGTGAAGG-3, targeted the region from 275

to 296 bp from the *mopR* sequence and contained an introduced unique *Not*I site on its 5′ end (underlined). The second primer,-TCTAGACTTTAATTGATTGAGTTCCGCCTG-3, was targeted to the region from 935 to 958 bp and contained an introduced unique *Xba*I site on its 5′ end (underlined). PCR was performed in a PT200 thermocycler (MJ Research, Watertown, MA) using the following protocol: initial 5-min denaturation at 95°C, 25 cycles in which the denaturing, annealing, and extension times and temperatures were 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min, respectively, and a final extension cycle at 72°C for 7 min. The resulting PCR product was cloned into the pCR2.1-TOPO vector using a TOPO TA cloning kit from Invitrogen (Carlsbad, CA). The resulting plasmid, DF4P, was digested with *Not*I and *Xba*I to yield the 0.72-kb *mopR*-like promoter fragment, which was then ligated into a promoterless *lux* cassette (*luxCDABE*) from *Vibrio fischeri* carried on the modified mini-Tn5 vector pUTK215 [3] that had previously been digested with *Not*I and *Xba*I (Figure 1). The ligation mix was transformed into electrocompetent *Escherichia coli* SV17 (*λpir*) [7] and resulting transformants selected on Luria–Bertani (LB) plates containing 50 mg l^{-1} kanamycin. Plasmid DNA was isolated from the transformants and sequenced to confirm the presence and orientation of the promoter fragment, resulting in the plasmid designated pUTK258 (Figure 1). *E. coli* SV17 pUTK258 was mated with strain DF4 and selected on LB plates containing ampicillin and kanamycin at 50 mg l^{-1} each [2]. Ten colonies were present after overnight incubation at 30°C. These colonies were transferred to LB plates containing 3 mM phenol and examined for light production visible by the naked eye. The brightest colony, designated DF4-8, was chosen for further analysis.

Bioluminescent assays

Bioluminescent assays using the DF4-8 bioreporter were performed essentially as described by Heitzer *et al* [14]. Strain DF4-8 was grown overnight at 30°C in yeast extract–peptone–glucose (YEPG) medium [14]. The next day, cultures were diluted 1:10 in YEPG medium and incubated with shaking at 30°C to an optical density of 0.38 at 546 nm. The culture was washed two times in a minimal salts medium (MSM) [26] and resuspended in an equal volume of MSM. Two milliliters of the culture was then added to 20-ml scintillation vials containing 2.0 ml of MSM supplemented with phenol-saturated MSM at final concentrations ranging from 0 to 160 ppm. Aliquots of 200 μl were removed from each scintillation vial and transferred to low-fluorescence black 96-well Microfluor microtiter plates (Dynex Technologies, Chantilly, VA) to produce eight replications of each dilution. Well-to-well crosstalk in these plates was previously shown to be minimal [4,24]. An additional eight replications consisting of both positive (a constitutively bioluminescing strain DF4 constructed *via* insertion of the previously described pUTK2 plasmid [17]) and negative (wild-type strain DF4) controls were also incorporated into each plate. Wells were covered with transparent plate sealer and placed in a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer/Wallac, Gaithersburg, MD) for luminescence detection (2 s integration time) at room temperature.

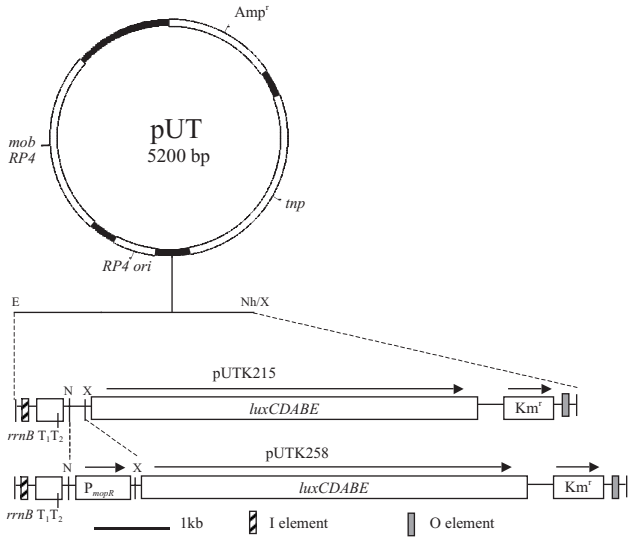

Figure 1 Genetic constructs used for construction of the DF4-8 bioluminescent bioreporter. For descriptions of the suicide vectors pUT and pUTK215, see de Lorenzo *et al* [8] and Applegate *et al* [3], respectively. pUTK258 contains the *mopR*-like promoter isolated from strain DF4 ligated to the *V. fischeri luxCDABE* cassette. E, *Eco*RI; X, *Xba*I; Nh, *Nhe*I; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

Substrate specificity

To determine substrate specificity of the DF4-8 bioreporter, microtiter plate assays were performed essentially as described above using the phenol derivatives *o*-cresol, *m*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 3,4-dichlorophenol, 2,3-dimethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, and catechol. Since strain DF4-8 cannot utilize these compounds as sole sources of carbon, cultures were maintained in YEPG rather than MSM.

Bioluminescent assay in phenol-contaminated soil slurries

To assess the response of strain DF4-8 under more environmentally relevant conditions, bioluminescent assays were also performed using aged, phenol-contaminated soil obtained from Misr Fine Spinning and Weaving, which has been located at its current site for 40 years. Five grams of soil was washed in 5 ml of MSM and shaken (250 rpm) at room temperature for 24, 48, or 120 h. The slurries were then centrifuged at 8000×*g* for 10 min to remove larger soil particles. Two milliliters of the slurry supernatant was added to 2 ml of a DF4-8 culture resuspended in MSM as described above. DF4-8 cells were also added to a soil slurry void of phenol contaminants (negative control) as well as to an MSM solution containing 80 ppm phenol (positive control). Bioluminescent assays were performed in microtiter plates as described above. Phenol concentrations in the slurry supernatants were determined using the 4-aminoantipyrene method [9].

Results

Identification of strain DF4

The 1.4-kb 16*S* rRNA sequence of strain DF4 was compared to all sequences in the GenBank database, revealing a high-sequence similarity ($\geq$99%) to the previously isolated phenol-degrading strain *A. calcoaceticus* A2 (accession no. AF159045). We have, therefore, placed strain DF4 in the genus *Acinetobacter*.

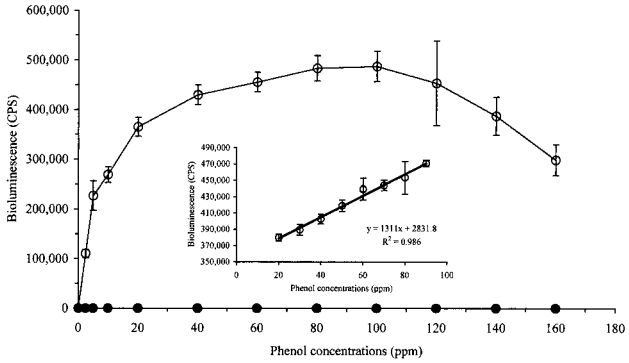

Figure 2 Bioluminescent response of the DF4-8 bioreporter (◯) and the parental DF4 *Acinetobacter* strain (●) to various concentrations of phenol. Positive control (constitutive *lux* bioreporter) not shown since data points exceeded graph's scale. Inset shows linearity of the curve occurring between 20 and 90 ppm phenol. CPS, luminescence counts s^{-1}. Error bars represent standard error of the mean ($n=8$).

Bioluminescent response kinetics of the DF4-8 bioreporter

The DF4 *mopR*-like promoter was ligated to the *luxCDABE* cassette to form the bioluminescent bioreporter DF4-8, which was then exposed to varying concentrations of phenol (0–160 ppm) to determine overall response kinetics. Resulting bioluminescent responses are plotted in Figure 2. Typical Michaelis–Menten kinetics was observed. Bioluminescence induction occurred at phenol concentrations as high as 100 ppm, after which toxicity effects led to a rapid decrease in optical density and a consequent decrease in bioluminescence. The lower limit of detection was determined to be 2.5 ppm (3σ above background). Linearity of the bioluminescent response was observed at phenol concentrations ranging from 20 to 90 ppm ($R^2=0.986$) (Figure 2). At phenol concentrations within this range, bioluminescence was typically initiated 4 h after exposure and increased exponentially thereafter up to approximately 12.5 h, whereupon maximum bioluminescence occurred at an average of 480,000±16,000 counts s^{-1} (CPS) (Figure 3). Bioluminescence rapidly decreased beyond 12 h due to cell death, as determined by reductions in optical density.

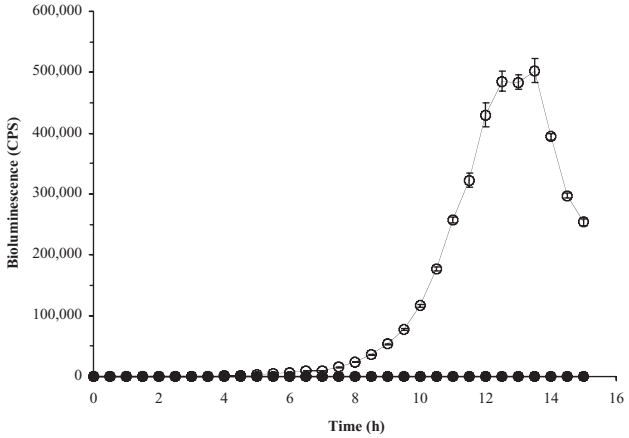

Figure 3 Bioluminescent response of strain DF4-8 in MSM containing phenol at approximately 50 ppm (◯) and MSM control (●). Error bars calculated as for Figure 2.

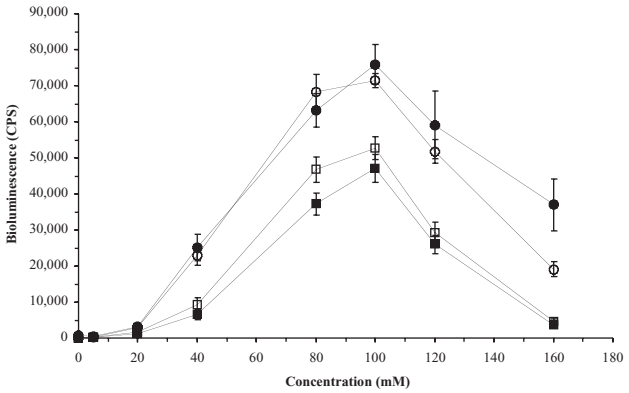

Figure 4 Bioluminescence induction of the DF4-8 bioreporter to phenol (◯) and the phenol derivatives 3-chlorophenol (●), *o*-cresol (□), and *m*-cresol (■). Exposures to 2-chlorophenol, 4-chlorophenol, 3,4-dichlorophenol, 2,3-dimethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, and catechol yielded only background levels of bioluminescence (<5000 CPS). Graphs shown are representative exposures of each chemical at concentrations of approximately 50 ppm. Error bars calculated as for Figure 2.

Substrate specificity

To determine substrate specificity of the DF4-8 bioreporter, microtiter plate assays were also performed using various phenol derivatives (*o*-cresol, *m*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 3,4-dichlorophenol, 2,3-dimethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, and catechol) (Figure 4). Significant bioluminescent responses (*t*-test, $P=0.05$) were produced only upon exposure to *o*-cresol, *m*-cresol, and 3-chlorophenol.

Bioreporter response to aged, phenol-contaminated soils

As an indication of the potential for using strain DF4-8 as an environmental bioreporter, laboratory tests were performed to determine bioluminescent response kinetics upon exposure to aged, phenol-contaminated soils obtained directly from Misr Fine Spinning and Weaving. Contaminated soil was washed in MSM medium for 24, 48, or 120 h to form a soil slurry that was then exposed to the DF4-8 bioreporter. The 24- and 48-h washes failed

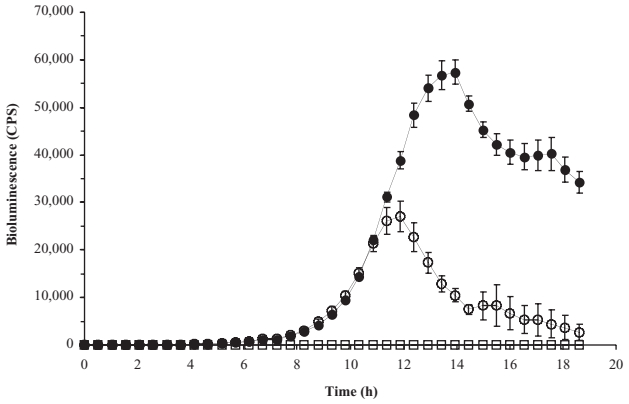

Figure 5 Bioluminescent response of strain DF4-8 to an aged, phenol-contaminated soil slurry (◯), MSM containing 45 ppm phenol (●), and soil void of phenol contaminants (□). Error bars calculated as for Figure 2.

to invoke a significant bioluminescent response. Phenol concentrations in these samples were shown to be below 1 ppm, indicating that washings up to 48 h were inadequate for these particular aged soils. However, bioreporter exposure to the 120-h soil washing, which yielded a phenol concentration of 45 ppm, generated a significant bioluminescent response (t-test, $P=0.05$). The response was observed 5.6 h after bioreporter addition and continued for up to 12 h, reaching a maximum light intensity of approximately 27,000 CPS (Figure 5).

Discussion

The DF4-8 bioreporter, containing a *mopR*-like promoter fused to a *V. fischeri luxCDABE* cassette, was able to autonomously detect aqueous phase phenol at concentrations ranging from 2.5 to 100 ppm within approximately 4 h. When exposed to phenol-contaminated soil washings obtained from an environmentally contaminated site, however, bioluminescence levels were reduced by over an order of magnitude and response times increased by almost 2 h. The longer lag period and lower bioluminescence levels in the soil slurries (Figure 5), compared to those observed in the aqueous samples (Figure 3), were somewhat expected based on similar results obtained by Hay *et al* [13], using a bioluminescent bioreporter for the detection of 2,4-D in soil, and King *et al* [15], using a bioluminescent bioreporter for the detection of naphthalene in soil. The longer lags and decreased bioluminescence are likely the result of reduced bioavailability due to soil adsorption and partitioning as well as scattering and/or absorption of bioluminescent signals by soil particles [14,18]. Although proven functional in phenol-contaminated soils, the DF4-8 bioreporter is likely better suited for phenol monitoring in aqueous environments.

Considering that EPA concentration limits for phenol in water are established at 3.5 ppb, strain DF4-8 cannot be regarded as a highly sensitive detector. However, its ease of use, low-cost, minimal sample pretreatment requirements, specificity, and ability to measure phenol bioavailability rather than just phenol presence may somewhat compensate for this lack in sensitivity. Perhaps, its most useful role will be in the detection and monitoring of industrial wastewater phenols, where concentrations typically fall well within or greatly exceed the established range of the DF4-8 bioreporter [11]. In cases where phenol concentrations are excessive and toxic to the bioreporter, serial dilutions could be performed to diminish toxicity, resulting in increased bioluminescence in more dilute samples [12]. Strain DF4-8 could also serve as an on-line process monitoring and control tool when linked to light accessing equipment such as photomultiplier tubes or fiber optic cables [23]. Realizing that this may require introduction and long-term survival within the contaminant profile, strain DF4-8 was constructed with a chromosomally encoded *lux* element rather than a simpler plasmid-based reporter system. This negates the requirement for selective pressure and potentially deleterious environmental use of antibiotics [6].

The induction spectrum of the DF4-8 *mopR*-like/*lux* bioreporter was in agreement with other findings regarding utilization by *Acinetobacter* spp. of phenol and its derivatives, with efficiencies of effectors being highest for phenol and 3-chlorophenol, followed by *o*-cresol and *m*-cresol (Figure 4) [20,25]. Due to the limited sensitivity of strain DF4-8, all other phenol derivatives tested could not initiate a bioluminescent signal above background. The DF4-8 *mopR*-like promoter/*lux* construct was also inducible by phenol in *E. coli* SV17, indicating that activity was triggered by phenol binding (data not shown). This paralleled the results obtained by Schirmer *et al* [25] regarding the introduction of an *A. calcoaceticus* NCIB8250 *mopR* promoter/*lacZ* fusion into *E. coli* DH5α. Despite these similarities, however, the putative *mopR* gene described here had no significant homology to the *mopR* gene of *A. calcoaceticus* NCIB8250. Further studies to identify and characterize the role of this clearly unique *mopR*-like gene in the phenol degradation pathway of *Acinetobacter* are currently underway.

Acknowledgements

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References

1 Altschul SF, W Gish, W Miller, EW Myers and DJ Lipman. 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
2 Applegate B, C Kelley, L Lackey, J McPherson, S Kehrmeyer, F-M Menn, P Bienkowski and G Sayler. 1997. *Pseudomonas putida* B2: a *tod-lux* bioluminescent reporter for toluene and trichloroethylene co-metabolism. *J Ind Microbiol Biotech* 18: 4–9.
3 Applegate BM, SR Kehrmeyer and GS Sayler. 1998. A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing. *Appl Environ Microbiol* 64: 2730–2735.
4 Arias RS, G Mochizuki, R Fukui and AM Alvarez. 1996. Most probable number method to enumerate a bioluminescent *Xanthomonas campestris* pv. *campestris* in soil. *Soil Biol Biochem* 28: 1725–1728.
5 Boyd SA, DR Shelton, D Berry and JM Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. *Appl Environ Microbiol* 46: 50–54.
6 Christensen FM. 1998. Pharmaceuticals in the environment — a human risk? *Regul Toxicol Pharmacol* 28: 212–221.
7 de Lorenzo V, S Fernandez, M Herrero, U Jakubzik and KN Timmis. 1993. Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene* 130: 41–46.
8 de Lorenzo V, M Herrero, JM Sanchez and KN Timmis. 1998. Mini-transposons in microbial ecology and environmental biotechnology. *FEMS Microbiol Ecol* 27: 211–224.
9 Eaton AD, LS Clesceri and AE Greenberg. 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association/American Water Works Association, Washington, DC.
10 Ehrt S, F Schirmer and W Hillen. 1995. Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. *Mol Microbiol* 18: 13–20.
11 Guerin TF. 1999. Bioremediation of phenols and polycyclic aromatic hydrocarbons in creosote contaminated soil using *ex-situ* land treatment. *J Hazard Mater, B* 65: 305–315.
12 Harkey GA and TM Young. 1999. Effect of soil contaminant extraction method in determining toxicity using the Microtox assay. *Environ Toxicol Chem* 19: 276–282.
13 Hay AG, BM Applegate, NG Bright and GS Sayler. 2000. A bioluminescent whole-cell reporter for detection of 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenol in soil. *Appl Environ Microbiol* 66: 4589–4594.
14 Heitzer A, OF Webb, JE Thonnard and GS Sayler. 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability by

using a bioluminescent catabolic reporter bacterium. *Appl Environ Microbiol* 58: 1839–1846.
15 King JMH, PM DiGrazia, B Applegate, R Burlage, J Sanseverino, P Dunbar, F Larimer and GS Sayler. 1990. Rapid, sensitive bioluminescence reporter technology for naphthalene exposure and biodegradation. *Science* 249: 778–781.
16 Lane DJ. 1991. 16*S*/23*S* rRNA sequencing. In: Stackebrandt E and M Goodfellow (Eds), Nucleic Acid Techniques in Bacterial Systematics. Wiley, New York, NY, pp. 115–148.
17 Layton AC, M Muccini, MM Ghosh and GS Sayler. 1998. Construction of a bioluminescent reporter strain to detect polychlorinated biphenyls. *Appl Environ Microbiol* 64: 5023–5026.
18 Mandoli DF, GA Ford, LJ Waldron, JA Nemson and WR Briggs. 1990. Some spectral properties of several soil types: implications for photomorphogenesis. *Plant Cell Environ* 13: 287–294.
19 Olsen RH, G DeBusscher and WR McCombie. 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J Bacteriol* 150: 60–69.
20 Paller G, RK Hommel and H Kleber. 1995. Phenol degradation by *Acinetobacter calcoaceticus* NCIB8250. *J Basic Microbiol* 35: 325–335.
21 Prasad S and BE Ellis. 1978. *In vivo* characterization of catechol ring-cleavage in cell cultures of *Glycine max*. *Phytochemistry* 17: 187–190.
22 Rebhun M and N Galil. 1988. Inhibition by hazardous compounds in an integrated oil refinery. *J Water Pollut Control Fed* 60: 1953–1959.
23 Ripp S, DE Nivens, Y Ahn, C Werner, J Jarrel, JP Easter, CD Cox, RS Burlage and GS Sayler. 2000. Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. *Environ Sci Technol* 34: 846–853.
24 Ripp S, DE Nivens, C Werner and GS Sayler. 2000. Bioluminescent most-probable-number monitoring of a genetically engineered bacterium during a long-term contained field release. *Appl Microbiol Biotechnol* 53: 736–741.
25 Schirmer F, S Ehrt and W Hillen. 1997. Expression, inducer spectrum, domain structure, and function of MopR, the regulator of phenol degradation in *Acinetobacter calcoaceticus* NCIB8250. *J Bacteriol* 179: 1329–1336.
26 Stanier RY, NJ Palleroni and M Doudoroff. 1966. The aerobic Pseudomonades: a taxonomic study. *J Gen Microbiol* 41: 159–271.
27 Watanabe K, S Hino and N Takahashi. 1996. Responses of activated sludge to an increase in phenol loading. *J Ferment Bioeng* 82: 522–524.
28 Watanabe K, M Teramoto, H Futamata and S Harayama. 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl Environ Microbiol* 64: 4396–4402.