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Characterization of a new solvent-responsive gene locus in *Pseudomonas putida* F1 and its functionalization as a versatile biosensor

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Summary

A new gene cluster, designated *sepABC* and a divergently transcribed *sepR*, was found downstream of the two-component *todST* phosphorelay system that regulates toluene degradation (the *tod* pathway) in *Pseudomonas putida* F1 (*Pp*F1). The deduced amino acid sequences encoded by *sepABC* show a high homology to bacterial proteins known to be involved in solvent efflux or multidrug pumps. SepA, SepB and SepC are referred to be periplasmic, inner membrane and outer membrane efflux proteins respectively. Effects on growth of various *Pp*F1 mutants compared to that of the wild type in the presence of toluene indicated a possible protective role of the solvent efflux system in a solvent-stressed environment. Growth tests with the complemented mutants confirmed the involvement of the Sep proteins in conferring solvent tolerance. The *sepR* gene encodes a 260-residue polypeptide that is a member of the *E. coli* lclR repressor protein family. The repressor role of SepR was established by conducting tests with a *sep-lacZ* transcriptional fusion in *Escherichia coli* and *Pp*F1, expression of SepR as a maltose-binding fusion protein in a DNA binding assay, and mRNA analysis. Southern hybridization experiments and analysis of the *P. putida* KT2440 genome sequence indicated that *sepR* is a relatively rare commodity compared to homologues of the *sepABC* genes. We developed a whole-cell bioluminescent biosensor, *Pp*F1G4, which contains a chromosomally based *sep-lux* transcriptional fusion. The biosensor showed significant induction of the *sepABC* genes by a wide variety of aromatic molecules, including benzene, toluene, ethylbenzene, and all three isomers of xylene (BTEX), naphthalene, and complex mixtures of aliphatic and aromatic hydrocarbons. *Pp*F1G4 represents a second-generation biosensor that is not based on a catabolic promoter but is nonetheless inducible by aromatic pollutants and moreover functional under nutrient-rich conditions.

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Introduction

Pseudomonas putida F1 (*Pp*F1) is a fluorescent soil bacterium that is best known for its toluene degradation (*tod*) pathway characterized by its dioxygenase enzyme complex (Gibson *et al*., 1990) and the prototypical two-component phosphorelay system, designated TodS and TodT, that controls it (Lau *et al*., 1997). Besides the ability to grow on toluene, ethylbenzene or benzene as the sole source of carbon and energy, *Pp*F1 is also capable growing on *p*-cymene (*p*-isopropyltoluene) and its acid derivative, *p*-cumate (Eaton, 1996; 1997). The metabolism of these compounds, however, employs a separate set of genes that consists of an 'upper' pathway (designated *cym*) responsible for the oxidation of *p*-cymene to *p*-cumate, and a 'lower' pathway (designated *cmt*) that takes *p*-cumate to isobutyrate, pyruvate and acetyl coenzyme A (Eaton, 1996; 1997). These pathways are regulated by a repressor, *cymR*, whose specificity does not include toluene (Eaton, 1997; Patel *et al*., 1997; unpublished results, this laboratory). The *cym*/*cmt* and the *tod* pathway genes are less than 3 kb apart in the *Pp*F1 chromosome. Proximity aside, the recruitment of two separate pathways in a single organism for the degradation of two related aromatic hydrocarbons is a rarity among known degradative pathways (Diaz *et al*., 2001).

Although the molecular basis is not known, *Pp*F1 is chemotactic to toluene in an inducible manner. Other attractants include benzene and ethylbenzene which are also growth substrates, as well as non-growth substrates such as isopropyl benzene, naphthalene and trichloroethylene (TCE) (Parales *et al*., 2000). Interestingly, regulatory mutants of *Pp*F1 that lack either of the TodS histidine kinase or the TodT response regulator are no longer chemotactic to toluene. In selecting bacterial strains to be

used in bioremediation, it has been suggested that the trait of chemotaxis be considered a desirable feature besides the usual biodegradative properties (Marx and Aitken, 2000).

In this study, we uncovered another attribute of *Pp*F1 by the localization of a new solvent-responsive locus found downstream of the *todST* genes. Properties of this new gene cluster, designated *sepABC* and a divergently transcribed *sepR*, as well as a distally located integrase sequence are described. A preliminary report of this work has been presented (Phoenix *et al*., 2000).

Results

Cloning and sequence analysis

The DNA sequencing of a previously generated clone containing the *todST* genes of *Pp*F1 indicated the presence of a partial open reading frame (*todU,* Wang *et al*., 1995) downstream of the *todT* response regulator showing a high sequence identity with the C-terminus of the OprM of *Pseudomonas aeruginosa* PAO1. Subsequently, the plasmid pPF9SX (Fig. 1, Table 1) was obtained and sequenced entirely. This established the complete sequence of *todU*, which we renamed *sepC* and the accompanying open reading frames (ORF), *sepA* and *sepB*. The *sep* nomenclature was adopted in consideration of the extensive sequence homology observed with the solvent resistant pump (*srpABC*) system of *P. putida* S12 (Kieboom *et al*., 1998a). In the pPF9SX clone, the *sepA* was missing at least the first two codons. Consequently, pGEM-3.8PB was obtained to complete this sequence. On the opposite strand we were able to identify a divergently transcribed ORF, designated *sepR*. Moreover, at the distal end, separated by 1106 bp, there is an integrase-encoding (*int-F1*) sequence that is found on the same strand as the *sepABC* genes (Fig. 1).

Characteristics of the deduced amino acid sequences of SepA, SepB, SepC, SepR and Int-F1 are summarized in Table 2. The most significant alignment produced by the 'protein-specific scoring matrices' (PSSMs) in the NCBI Conserved Domain Database (CDD) is given. The calculated molecular masses of the SepABC proteins agree quite well with those determined in the *E. coli* T7 polymerase/promoter system (Fig. 2). SepABC are members of the bacterial efflux pump proteins, along with the solvent resistant pump (*srpABC*) system from *P. putida* S12 (Kieboom *et al*., 1998a), and the toluene tolerance systems (*ttgABC, ttgDEF* and *ttgGHI*) of *P. putida* DOT-T1E (Mosqueda *et al*., 1999; Mosqueda and Ramos, 2000; Rojas *et al*., 2001; Duque *et al*., 2001) that belong to the resistance-nodulation-division (RND) family of proteins (Johnson and Church, 1999). The SepABC proteins are tentatively called periplasmic, inner membrane and outer membrane efflux proteins, respectively, following the nomenclature of Johnson and Church (1999). Among the three Ttg systems of strain DOT-T1E, TtgDEF is most related to SepABC: SepA is 100% identical to TtgD; SepB is two amino acids shorter than TtgE; and SepC has four amino acid substitutions from TtgF. Notably, the multidrug resistance proteins such as those of the MexA-MexB-OprM system found in *P. aeruginosa* PAO1 are early members of the RND family (Poole *et al*., 1996).

The predicted amino acid sequence of SepR is most closely related to the putative regulatory protein, TtgV (63% identity) of strain DOT-T1E (AF299253), and SrpS (62%) of strain S12 (AF061937), as well as to a hypothetical protein (Rv 1719; 39%) present in the *Mycobacterium tuberculosis* H37Rv genome (emb Z81360). These proteins are members of the IclR (isocitrate lyase regulator) family of transcriptional regulators that include some of the regulators in aromatic metabolism (Diaz and Prieto, 2000). The N-terminal portion of SepR is predicted to contain a helix–turn–helix DNA-binding motif whereas the C-terminal most likely binds to effector molecules.

The predicted 395-amino acid integrase sequence (Int-F1) was found to belong to the Int family of site-specific recombinases (Nunes-Duby *et al*., 1998). In particular, it is a 'phage-type' integrase, and known catalytic residues of the Cre recombinase of phage P1 (Arg173, His289, Arg292 and Tyr324; CD:pfam00589) are conserved in the Int-F1 sequence (Arg229, His324, Arg327, and Tyr361). In the following, except for discussion, no additional characterization of Int-F1 is available.

Phenotypic characterization of null mutants of sepBC *and* sepR *genes*

In order to determine the possible involvement of the s*epABC* genes and their putative regulatory gene *sepR* in solvent tolerance, we constructed the following null mutants: *Pp*F1(*sepC*::Km^r), *Pp*F1(*sepB*::Km^r), and *Pp*F1(*sepR*::Km^r) as described in *Experimental procedures.* To investigate the difference in growth between the wild type and *sep* mutants, we conducted a time study in which we monitored the growth of *Pp*F1, *Pp*F1(*sepC*::Km^r) and *Pp*F1(*sepR*::Km^r) in minimal medium with toluene as sole carbon source, at a nominal concentration of 9.5 mM (higher than the aqueous solubility of toluene = 6 mM = 526 p.p.m.). Figure 3A shows that the growth of the *sepC* mutant was inferior to that of the wild type and the *sepR* mutant. This result, coupled with the fact that the *sepR* mutant exhibited an apparent shorter lag period compared to the wild type or *sepC* mutant, suggests the involvement of the *sep* genes in conferring toluene tolerance to *Pp*F1.

To further correlate the possible role of the *sep* genes with toluene tolerance in *Pp*F1, we evaluated the short-

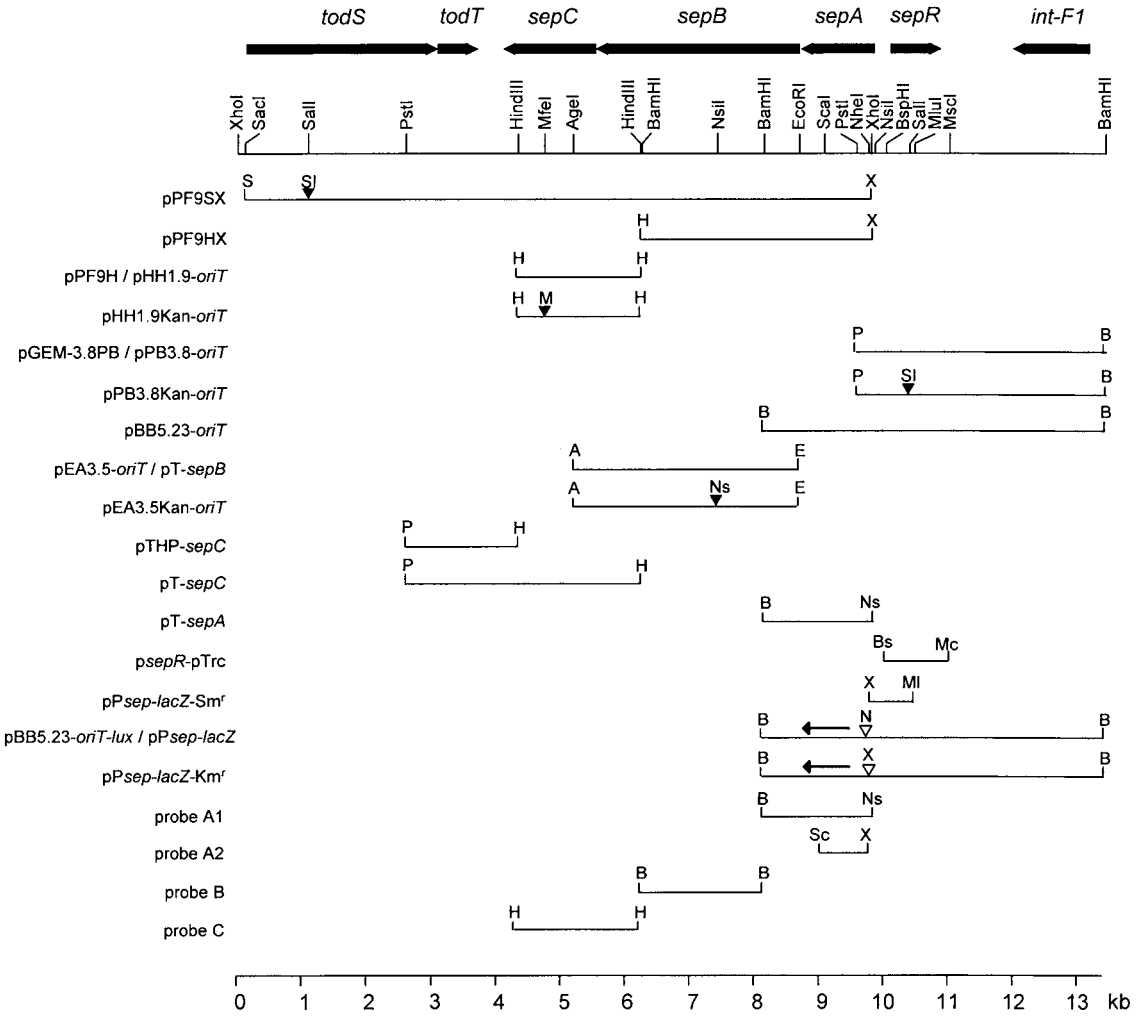

Fig. 1. The gene context of the solvent efflux pump (*sep*) in *Pp*F1 and its restriction map and plasmid derivatives. The restriction sites are not necessarily unique; A: *Age*I, B: *Bam*HI, Bs: *Bsp*HI, E: *Eco*RI, H: *Hin*dIII, N: *Nhe*I, Ns: *Nsi*I, M: *Mfe*I, Mc: *Msc*I, Ml: *Mlu*I, P: *Pst*I, S: *Sac*I, Sc: *Sca*I, Sl: *Sal*I, X: *Xho*I. The plasmid names indicated on the left are described in Table 1. The symbol ▼, indicates sites of kanamycin (Km) resistance gene disruptions, ∇ indicates sites of *luxCDABE* and *lacZ*-Km^r cassette insertion, and the adjacent arrows show the orientation of the reporter genes.

term survival of the wild type and the three *sep* mutants in response to sudden toluene shock, according to the method described by Mosqueda and Ramos (2000). After pre-growing cells in LB medium at 30°C, with or without toluene supplied in the gas phase, the cultures were spiked with toluene (28.5 mM), and then the number of viable cells was assessed over time. When cells were pre-exposed to toluene, both the *sepC*::Km^r and *sepB*::Km^r mutants were less tolerant than the wild type *Pp*F1 (Fig. 3C). However, growth of these cells approximated that of the wild type when these mutants were complemented with plasmid pEX-31 or pPE-31 (Table 1) that contains the wild-type *sepB* or *sepC* gene, respectively (data not shown). In assays when cells were not pregrown with toluene, the *sepR*::Km^r mutant had higher viable cell counts compared to the wild type (Fig. 3B). The survival of the *sepR*::Km^r mutant may be due to derepressed *sep* gene expression, indicating that the solvent efflux system of *Pp*F1 plays a protective role in regard to solvent tolerance.

On the other hand, the *sep* genes were found not to be involved in antibiotic efflux when we compared the antimicrobial susceptibility of *Pp*F1 and the *Pp*F1(*sepR*::Km^r) and *Pp*F1(*sepC*::Km^r) mutants (data not shown). In tests conducted in LB and minimal media containing glucose, the minimal inhibitory concentration (MIC) of tetracyclin, ampicillin, chloramphenicol, streptomycin, and novomycin was determined for the wild type and the mutants. Both *sepR* and *sepC* mutants showed no difference in their antibiotic susceptibility compared to the wild type.

Evidence that SepR is a repressor of sepABC *gene expression*

In order to show that SepR can repress *sepABC* gene

Table 1. Strains and plasmids used in this study.

Plasmid or strain	Relevant genotype or characteristics	Source or reference
Strains		
E. coli		
DH5α	F$^-$ λ$^-$ *endA1 hsdR17*($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA96*(Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 spoT1 deoR*(φ80 *dLac*Δ(*lacZ*)*M15*)	Life Technologies
S17-1	*rec*A *pro hsdR thi chr*::RP4-2(tc::mu Km::Tn7); Tp^r, Sm^r	Simon *et al.* (1983)
UT5600	F$^-$ *ara-14 leuB6 azi-6 lacY1 proC14 tsx-67* Δ(*ompT-fepC*)266*entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*	New England Biolabs
K38	HfrC T2R *relA1 pit-10 spoT1 tonA22 ompF627 phoA4* λ$^-$	Tabor (1990)
RFM443	*rpsL galK2* Δ*lac74*	Drolet *et al.* (1995)
P. putida		
F1	wild type, toluene$^+$, *p*-cymene$^+$	Gibson *et al.* (1968)
F1G4	*Pp*F1 containing a *sep-lux* insertion in the chromosome; Ap^r	This study
TVA8	*Pp*F1 containing a mini-Tn*5*Km*tod-lux* insertion in the chromosome; Km^r	Applegate *et al.* (1998)
F1APR1	*Pp*F1 containing a *sep-lacZ*-Km^r insertion in the chromosome; Km^r, Ap^r	This study
F1L2.1	*Pp*F1 containing a *sep-lacZ* insertion in the chromosome; Ap^r	This study
F1(*todS*::Km^r)L2.1	*Pp*F1(*todS*::Km^r) containing a *sep-lacZ* insertion in the chromosome; Ap^r	This study
F1(*todS*::Km^r)	*Pp*F1 with a Km insertion in the *Sal*I site of *todS*; Km^r	Lau *et al.* (1997)
F1(*sepR*:: Km^r)	*Pp*F1 with a Km insertion in the *Sal*I site of *sepR*; Km^r	This study
F1(*sepB*:: Km^r)	*Pp*F1 with a Km insertion in the *Nsi*I site of *sepB*; Km^r	This study
F1(*sepC*:: Km^r)	*Pp*F1 with a Km insertion in the *Mfe*I site of *sepC*; Km^r	This study
Plasmids		
pBluescriptKS(–)	Cloning vector with MCS *Kpn*I-*Sac*I; Ap^r	Stratagene
pUC4K	pUC4 derivative containing a Km resistance cassette; Ap^r, Km^r	Pharmacia
pPF9SX	11-kb *Sac*I-*Xho*I fragment from the chromosome of strain *Pp*F1(*todS*:: Km) inserted into pBluescript KS(–); Km^r	This study and Lau *et al.* (1997)
pPF9HX	3.0-kb *Hin*dIII-*Xho*I fragment from pPF9SX inserted intopBluescript KS(–); Ap^r	This study
pPF9H	1.9-kb *Hin*dIII fragment from pPF9SX inserted intopBluescript KS(–); Ap^r	This study
pHH1.9-*oriT*	1.9-kb *Hin*dIII fragment from pPF9H inserted into pUC13-*oriT*; Ap^r	This study
pHH1.9Kan-*oriT*	Same as pHH1.9-*oriT*, but containing a Km^r cassette from pUC4Kon 1.25-Kb *Eco*RI fragment inserted into the *Mfe*I site of s*epC* gene; Ap^r, Km^r	This study
pUC13-*oriT*	Mobilisable pUC-derived plasmid; Ap^r	Wang *et al.* (1995)
pGEM3Zf	Cloning vector; Ap^r	Promega
pGEM-3.8PB	3.8-kb *Pst*I-*Bam*HI fragment from the chromosome of strain *Pp*F1inserted into pGEM3Zf; Ap^r	This study
pPB3.8-*oriT*	3.8-kb *Pst*I-*Bam*HI fragment from pGEM-3.8PB inserted into pUC13-*oriT*; Ap^r	This study
pPB3.8Kan-*oriT*	Same as pPB3.8-*oriT* but containing a Km^r cassette from pUC4K on a 1.25-kb *Sal*I fragment, inserted into the unique *Sal*I site of *sepR*; Ap^r, Km^r	This study
pBB5.23-*oriT*	1.6-kb *Nhe*I-*Bam*HI fragment from pT-s*epA* ligated to the 3.6-kb *Bam*HI-*Nhe*I fragment of pPB3.8-*oriT* inserted into pUC13-*oriT*; Ap^r	This study
pEA3.5-*oriT*	3.5-kb *Eco*RI-*Age*I fragment from pPF9SX inserted into *Eco*RI-*Xma*I sites of pUC13-*oriT*; Ap^r	This study
pEA3.5Kan-*oriT*	Same as pEA3.5-*oriT*, but containing a Km^r cassette from pUC4K on a 1.25-kb *Pst*I fragment inserted into the *Nsi*I site of *sepB*; Ap^r, Km^r	This study
pVLT31	Broad host range expression vector with a *tac* inducible promoter; Tc^r	de Lorenzo *et al.* (1993b)
pEX-31	3.5-kb *Eco*RI-*Xba*I fragment from pEA3.5-*oriT* inserted into correspon-ding sites of pVLT31; contains the complete *sepB* gene; Tc^r	This study
pPE-31	1.99-kb *Pvu*II-*Eco*RV fragment containing the complete *sepC* gene, inserted into *Sma*I site of pVLT31; the *sepC* gene is under the control of the *tac* promoter; Tc^r	This study
pT7-5	Expression vector carrying an *Eco*RI-*Hin*dIII MCS downstream of a phage T7 strong promoter; Ap^r	Tabor (1990)
pT7-6	Same as pT7-5 but with an *Hin*dIII-*Eco*RI MCS.	Tabor (1990)
pTHP-s*epC*	1.73-kb *Hin*dIII-*Pst*I fragment from pPF9SX inserted into the *Hin*dIII-*Pst*I site of pT7-6; Ap^r	This study
pT-s*epC*	1.9-kb *Hin*dIII fragment from pPF9HX ligated to the *Hin*dIII digested pTHP-s*epC*; contains the complete s*epC* gene; Ap^r	This study
pT-s*epB*	Same as pEA3.5-*oriT*, but inserted into the *Xma*I-*Eco*RI sites of pT7-5; Ap^r	This study
pT-s*epA*	1.7-kb *Nsi*I-*Bam*HI fragment from a PCR amplified fragment from the chromo-some of *Pp*F1inserted into *Pst*I-*Bam*HI sites of pT7-6; Ap^r	This study
pMAL-c2X	Vector for inducible cytoplasmic expression of genes as fusion with maltose binding protein(MBP); Ap^r	New England Biolabs
pMBP-s*epR*	0.787-kb PCR fragment containing s*epR* inserted in frame into the *Bam*HI site of pMAL-c2X; produces a MBP fusion protein with SepR; Ap^r	This study
p*Trc*99A	Expression vector with a strong IPTG inducible *Trc* promoter; lacI^q; Ap^r	Pharmacia
ps*epR*-p*Trc*	0.972-kb *Bsp*HI-*Msc*I fragment from pGEM-3.8PB inserted into *Nco*I-*Sma*I sites of pTrc99A; contains a promoterless s*epR* fragment; Ap^r	This study

Table 1. *cont.*

Plasmid or strain	Relevant genotype or characteristics	Source or reference
pHRP317	Cohort vector with Sm^r/Sp^r cassette for promoter cloning; Sm^r/Sp^r, Km^r	Parales and Harwood (1993)
pHRP309	Broad-host range, low-copy l*acZ* transcriptional fusion vector; Gm^r	Parales and Harwood (1993)
pHRP311	Promoterless Sm^r *lacZ* transcriptional fusion vector; used as a negative control; Sm^r, Gm^r	Parales and Harwood (1993)
pPs*ep*-Sm^r	0.642-kb *Mlu*I-*Xho*I fragment from pGEM-3.8PB inserted into the *Mlu*I-*Xho*I sites of pHRP317 cohort plasmid; Sm^r, Km^r	This study
pPs*ep-lacZ*-Sm^r	2.75-kb *Sma*I-*Xho*I fragment from pP*sep*-Sm^r (where *Xho*I site was blunted with Klenow), inserted into the *Sma*I site of pHRP309; contains the Sm^r cassette and the s*ep* promoter fused with *lacZ*; Sm^r, Gm^r	This study
pMR149	P*todX-lacZ* fusion vector in pHRP309; Sm^r, Gm^r	Wang *et al*. (1995)
pKOK6.1	pBR322 derivative containing a promoterless *lacZ*-Km^r cassette (a modified version of pKOK6 with the cassette inserted in the inverted direction, carrying 3 stop codons in all reading frames upstream of reporter gene); Ap^r, Tc^r, Km^r	Kokotek and Lotz, 1989
pP*sep-lacZ*-Km^r	4.73-kb *Sal*I fragment containing *lacZ*-Km^r cassette from pKOK6.1, inserted into the *Xho*I site of *sepA* in pBB5.23-*oriT*, in the orientation that yields a *sepABC-lacZ* transcriptional fusion; Km^r, Ap^r	This study
pP*sep-lacZ*	3.6-kb *Sma*I-*Xmn*I fragment from pHRP309 containing *lacZ* gene inserted into the *Nhe*I site (blunted with Klenow) of pBB5.23-*oriT*, in the orientation that yields a *sepABC-lacZ* transcriptional fusion; Ap^r	This study
pCGLS-11	pUC18/19 plasmid containing *luxCDABE* genes from *Photorhabdus luminescens*; Ap^r	Frackman *et al*. (1990)
pBB5.23-*oriT-lux*	6.7-kb *Eco*RI fragment from pCGLS-11, end-blunted with Klenow, inserted into the *Nhe*I site (also blunted with Klenow) of pBB5.23-*oriT*; contains a s*ep-lux* transcriptional fusion; Ap^r	This study
pGP1-2	plasmid with p15A origin of replication harbouring a T7 RNA polymerase gene under the control of lP_L promoter, which is repressed by a temperature sensitive repressor *c*I857; Km^r	Tabor (1990)

expression, we compared the β-galactosidase (*lacZ*) activity of a s*epABC-lacZ* transcriptional fusion (pP*sep-lacZ*-Sm^r, Table 1) to that of the promoterless *lacZ* fusion vector pHRP311, in *E. coli* strain RFM443 (*lac*$^-$). As well, we used a previously constructed plasmid pMR149 that contained a *todX-lacZ* transcriptional fusion (Wang *et al*., 1995). The TodX-encoding gene is the first gene of the *tod* operon that encodes a membrane protein with the possible function of facilitating toluene transport. Previously, we established the location of a toluene-inducible promoter to which the response regulator TodT binds to the '*tod* box' in front of the *todX* gene (Lau *et al*., 1997). In the present set of experiments, either SepR was overexpressed *in trans* (p*sepR*-p*Trc*) in the various strains, or the p*Trc*99A vector alone was used as a negative control. Figure 4 shows that when SepR was provided in *trans*, *sepABC* gene expression was completely repressed in *E. coli*; i.e. β-galactosidase activity returned to the basal level of the promoterless *lacZ* fusion in pHRP311. On the other hand, the *todX-lacZ* fusion in pMR149 was unaffected by the overexpression of SepR, even though the basal level of β-galactosidase activity was higher than the other strains. Similar results were obtained when SepR was overexpressed as a maltose binding protein (MBP) fusion protein (see *Experimental procedures*).

In order to give direct *in vitro* evidence for the binding of SepR to the promoter region of *sepABC*, we carried out gel retardation assays using varying amounts of the purified MBP-SepR protein. Figure 5 shows the DNA binding activity to a 120 bp fragment that is proximal to the *sepA* gene. Specific binding of MBP-SepR was also observed with fragment C that corresponds to the complete 241 bp intergenic region and fragment N that extends to the *Nsi*I restriction site located 3′ to the predicted −10 promoter element and within a potential 6 bp hairpin structure that could serve as an operator sequence (not shown). Mal-

Table 2. Properties of the predicted gene products.

Gene	Suggested functional name	Amino acids	Predicted mol. mass [a]	Homology in NCBI conserved domain database	Score (bits)	E value
sepA	periplasmic efflux pump	382	41, 525 (42)	pfam00529 HlyD family secretion protein	128	6e-31
sepB	inner membrane efflux protein	1046	113, 359 (116)	pfam00873 ACR_tran ACR B/D/F family	1085	0.0
sepC	outer membrane efflux protein	480	52, 813 (52)	pfam02321 outer membrane efflux protein	107	1e-24
sepR	repressor protein	260	27, 894 (29)	pfam01614 LYR bacterial transcriptional regulator	76.1	1e-15
int-F1	integrase	365	45, 153	pfam00589 phage integrase	53.1	3e-8

a. Experimentally determined molecular masses in kDa are in parentheses.

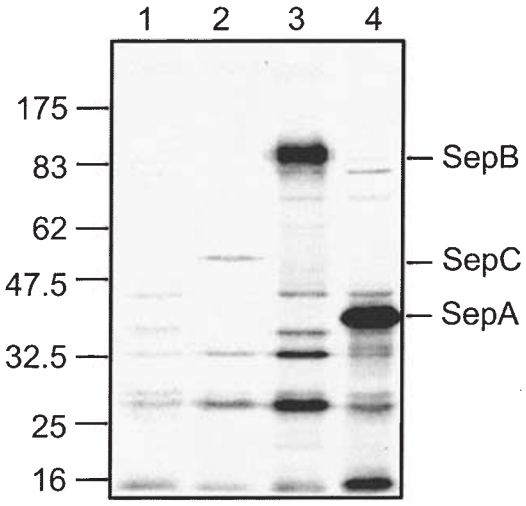

Fig. 2. Expression of (^{35}S) methionine-labelled polypeptides of SepA, SepB and SepC in *E. coli* K38/pGP1-2 strain. Lane 1, control vector pT7-6; lane 2, plasmid pT-*sepC*; lane 3, plasmid pT-*sepB*; and lane 4, plasmid pT-*sepA*. The sizes of the protein markers (kDa) are indicated on the left.

tose binding protein alone was found not to bind to any of the fragments mentioned (not shown).

Inducibility of the sep *genes*

The repressor role of SepR was further supported by measurement of β-galactosidase activity when a *sepABC-lacZ* transcriptional fusion (pP*sep-lacZ*-Sm^r) was mobilized in the *Pp*F1 strain derivative *Pp*F1(*sepR*::Km^r). In this case, β-galactosidase activity showed a modest 1.5-fold increase compared to the wild type; the actual Miller units in one experiment were 10 160 versus 6680. On the other hand, a rather low basal level of β-galactosidase activity (300 Miller units) was observed when the control pHRP311 plasmid was used in both the wild type and *Pp*F1(*sepR*::Km^r). Furthermore, measurement of specific *sepA* mRNA by dot-blot analysis indicated that *sepA* was not expressed constitutively in the absence of toluene but instead was inducible (Fig. 6). Upon toluene induction, the mRNA level increased very rapidly (within 10 min), reaching a maximum level which was maintained for at least the next 20 min.

According to the mRNA study, expression of *sepABC* in *Pp*F1 was under positive regulation in response to toluene. We determined the relationship between toluene concentration (0.45, 0.95, 4.5, 9.5 and 14 mM) and *sep* gene induction, by measuring the β-galactosidase activity of the *sepABC-lacZ*-Km^r fusion (pP*sep-lacZ*-Km^r) in *Pp*F1APR1. It was found that toluene can induce expression of *sepABC-lacZ*-Km^r at 0.45 mM (= 40 p.p.m.), providing an activity of 6 Miller units. This response almost

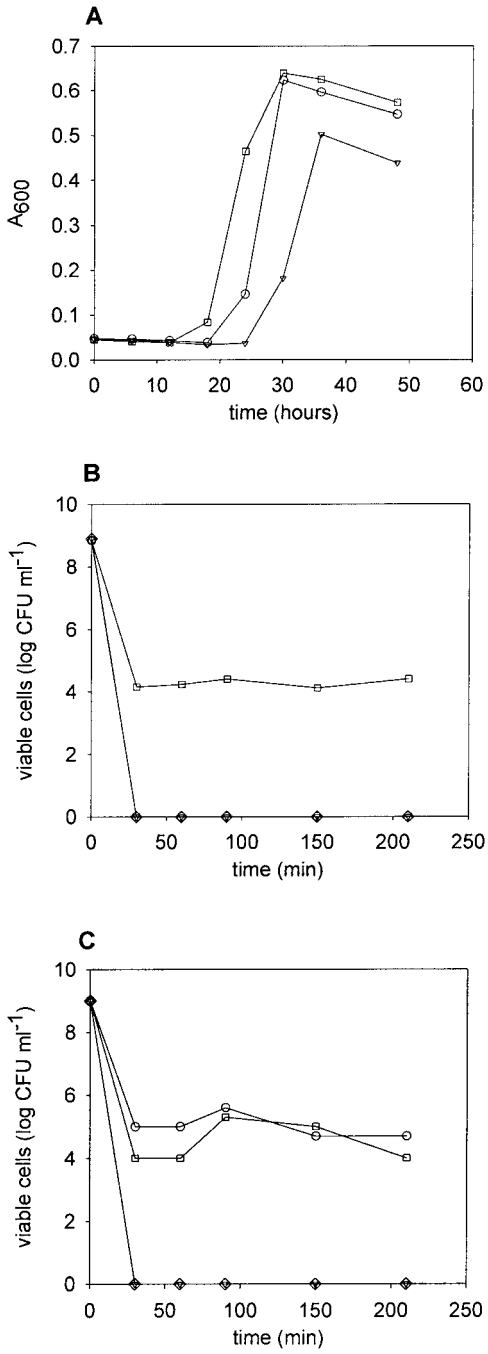

Fig. 3. A. Shows growth of wild-type *Pp*F1 and some of its derivatives in the presence of toluene as sole carbon source (9.5 mM). The following two panels depict survival in response to toluene shock of *Pp*F1 and some of its derivatives in LB media.
B. Represents the number of viable cells after the sudden addition of toluene for cells that had not been exposed to toluene before the shock.
C. Shows the survival of cells that had been pre-exposed to toluene in the vapour phase before being spiked with 28.5 mM toluene. The number of viable cells was determined just before toluene addition and at 30 minute intervals for a period of 3.5 h. Legend key: ○ F1; ◇ F1(*sepB*::Km^r); ▽ F1(*sepC*::Km^r); □ F1(*sepR*::Km^r).

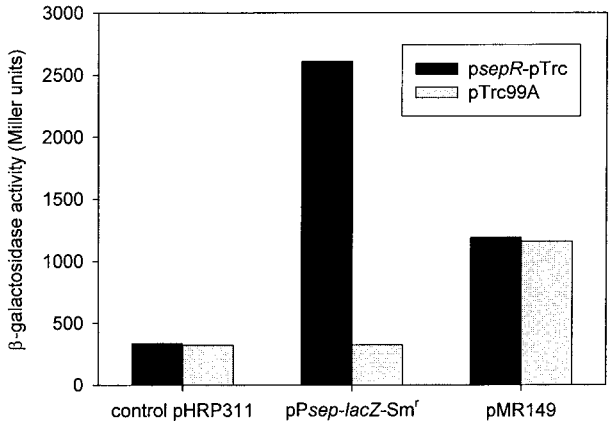

Fig. 4. A repressor role of SepR. β-Galactosidase activity was measured using transcriptional fusion constructs *sepABC-lacZ* (pP*sep-lacZ*-Sm^r) and *todX-lacZ* (pMR149), and the control plasmid pHRP311 in *E. coli* RFM443 strain. The plasmid containing the *sepR* gene (p*sepR*-p*Trc*) and control plasmid p*Trc*99A were co-expressed in the same strain.

doubled at 0.95 mM toluene, and reached a plateau of about 18 Miller units for toluene concentrations of 4.5 mM and above. The background activity without any toluene was low (<0.1 Miller units).

As the *tod* operon and the *sepABC* genes are both induced by toluene and TodS is required for *tod* gene induction, we investigated whether TodS signalling is required for *sepABC* induction by toluene. Strains *Pp*F1L2.1 and *Pp*F1(*todS*::Km^r)L2.1 were created by introducing the *sepABC-lacZ* fusion (pP*sep-lacZ*) into the chromosome of *Pp*F1 and *Pp*F1(*todS*::Km^r) respectively (Fig. 1). When the β-galactosidase activity was measured in both strains in the presence or absence of toluene (as a control), the same level of activity was observed in both, indicating that TodS is not required for *sepABC* induction by toluene.

Construction of biosensor strain PpF1G4

The tests with the *sep-lacZ* fusion showing solvent induction provided the incentive to further investigate the substrate specificity of SepR. To this end, we chose the *lux* reporter system because bioluminescence assays are rapid and easily quantifiable and several such whole-cell reporter systems have been designed for monitoring organic pollutants (King *et al.*, 1990; for a review: Keane *et al.*, 2002). The *luxCDABE* operon cloned from the terrestrial bacterium *Photorhabdus* (formerly *Xenorhabdus*; Boemare *et al.*, 1993) *luminescens* (Frackman *et al.*, 1990) was employed for the construction of the *sep-lux* biosensor. Use of the complete *lux* cassette permitted measurement of bioluminescence without the addition of

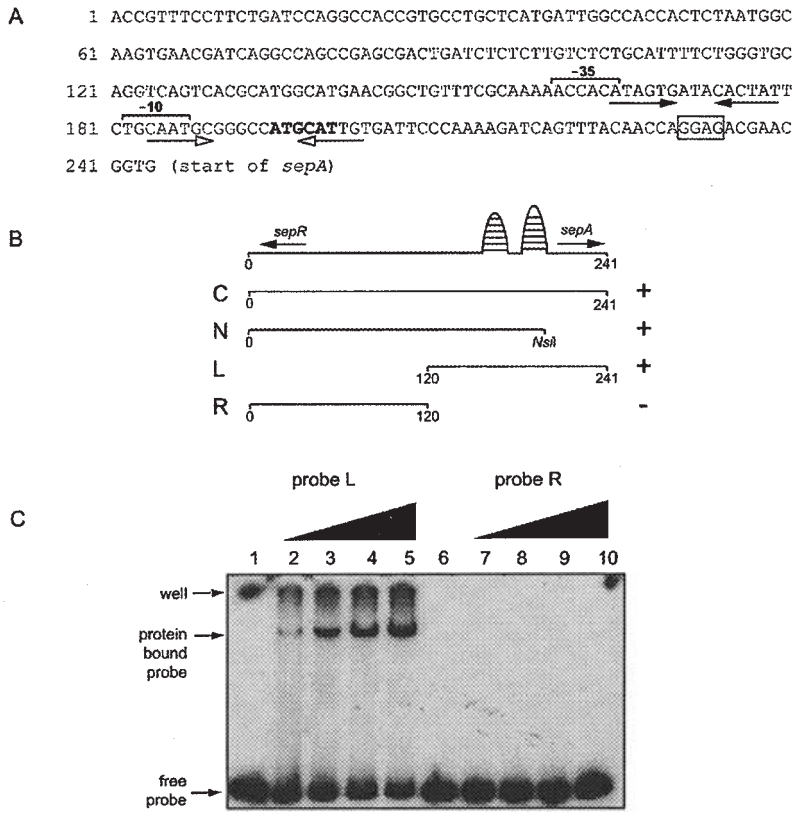

Fig. 5. A. Characteristics of the 241-bp *sepR-sepABC* intergenic sequence. The predicted Shine–Dalgarno sequence is boxed; the two pairs of converging arrows indicate possible hairpin structures each consisting of 6 bp inverted repeats; the *Nsi*I recognition sequence is boldfaced. Potential −10 and −35 promoter sequences are as indicated.
B. Fragments used in the mobility shift assays. Fragment C corresponds to the complete intergenic region (the positions of the potential hairpin structures are diagrammed above); fragment N ends at the *Nsi*I restriction site; fragments L and R represent the two halves of fragment C. The + and – signs indicate positive and negative bindings to MBP-SepR, respectively, as shown in the gel in C. The [^{32}P]-labelled probes (lanes 1–5, probe L; lanes 6–10, probe R) were incubated with varying amounts of purified MBP-SepR protein and run on TAE non-denaturing 4.5% polyacrylamide gel. The concentrations of MBP-SepR used were: lanes 1 and 6, no protein; lanes 2 and 7, 1 μg; lanes 3 and 8, 2 μg; lanes 4 and 9, 3 μg; lanes 5 and 10, 4 μg.

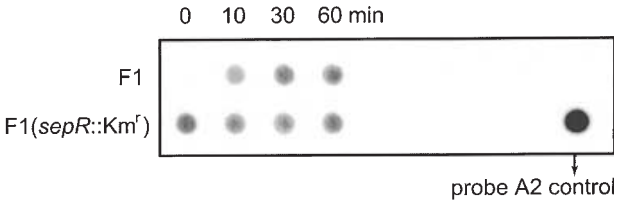

Fig. 6. Evidence of *sepA* repression and its de-repression. Dot-blot analysis of *sepA* RNA extracted from *Pp*F1 and the corresponding null mutant of *sepR, Pp*F1(*sepR*::Km^r), at 0, 10, 30 and 60 min after induction with toluene (2.17 mM). Purified RNA (3 μg) was used and hybridized with [^{32}P]-labelled probe A2 (Fig. 1), which corresponds to an internal fragment within *sepA*. Denatured DNA from unlabelled probe A2 was used as a positive control on the membrane.

the *n*-decanal substrate. One recombinant strain, designated *Pp*F1G4, was used for further investigation.

Results of the specific bioluminescent response of *Pp*F1G4 exposed to a variety of compounds are shown in Fig. 7. The test compounds are listed in order of their hydrophobicity, as expressed by their log *P*-values, where *P* is the octanol-water partition coefficient, a parameter that is widely used for correlating biological effects of organic substances (Inoue and Horikoshi, 1989). The log *P*-values for the compounds were either obtained from Howard and Meylan (1997) or calculated from fragmental constants with the software program KOWWIN (Meylan and Howard, 1995). The results indicated that SepR has a relatively broad effector specificity as it was activated by a wide range of aromatic compounds. The trend indicated a quasi-normal distribution, with peak bioluminescent responses occurring for compounds with log *P*-values in the range of about 1.5–3.7. With some exceptions, very hydrophobic (log $P > 4.0$) and very hydrophilic (log $P < 1.5$) compounds are not recognized as effectors. It is noteworthy that *o*-xylene induces the system equally well as the *m*- and *p*-substituted isomers. The effectors also include halogenated toluenes, phenylacetylene and TCE. As was found in the *sep-lacZ* assay, *p*-cymene and its acid derivative, *p*-cumate, as well as cumic alcohol did not cause a response. This is also the case with simple alco-

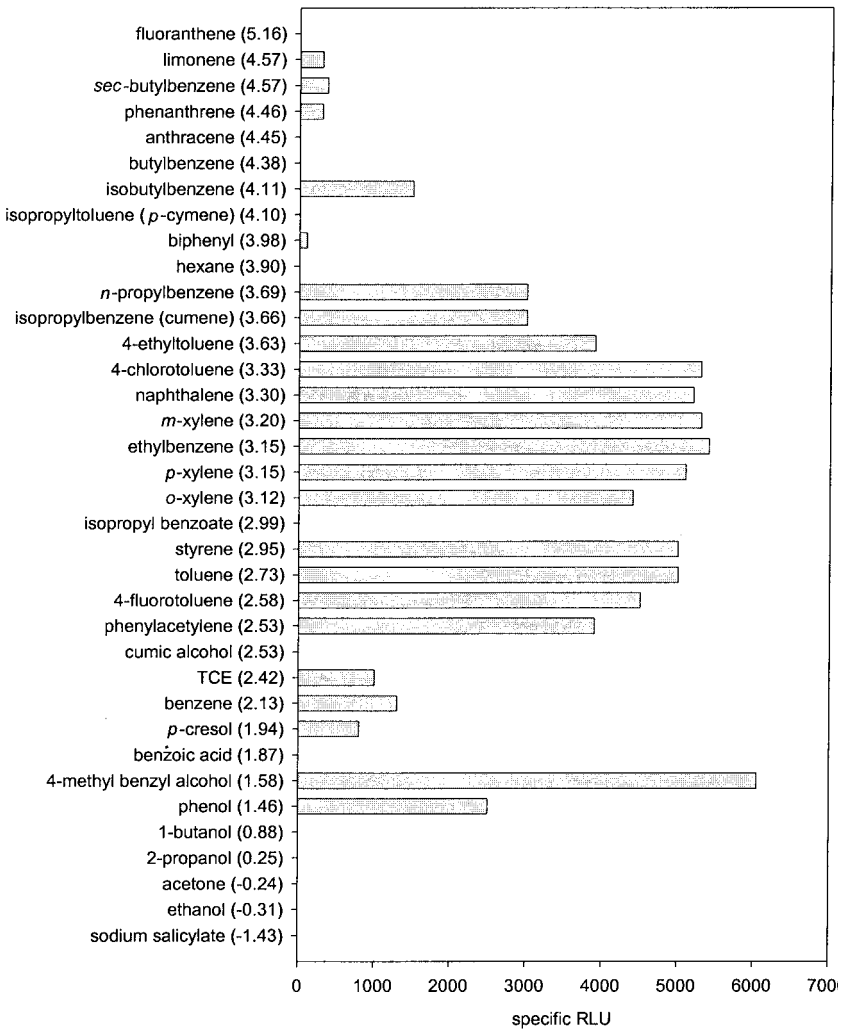

Fig. 7. Response of *Pp*F1G4 biosensor to a spectrum of organic compounds (log *P*-values are given in parentheses). Concentrations = 1 mM, except for sparingly soluble compounds, which were a 1:2 dilution of a saturated solution.

hols and acetone. Other non-effectors include benzoate acid and PAHs such as fluoranthene and anthracene, although phenanthrene had a slight response. It should be noted that a similar response to organic compounds was obtained with strain *Pp*F1APR1, which contains a pP*sep-lacZ*-Km^r fusion. This strain was also used to test gene expression in response to zinc chloride, cadmium chloride, and the antibiotics tetracycline and chloramphenicol. However, neither the antibiotics nor the heavy metals had any apparent effect (data not shown).

A comparison of two biosensors

As BTEX compounds are ubiquitous environmental pollutants, the *Pp*F1G4 response to these compounds was investigated further. It was determined that the intensity of the bioluminescent response of *Pp*F1G4 to BTEX compounds is concentration-dependent (Fig. 8). This is in good agreement with the response to toluene concentrations in the *sep-lacZ* system. At around 3 mM *m*-xylene and ethylbenzene, toxicity sets in causing the observed sharp decrease in light production.

We also tested the possible response of a number of ubiquitous multicomponent non-aqueous phase liquids (NAPLs), including gasoline, JP-4 jet fuel, diesel, coal tar creosote, and three varieties of crude oil (Brent Blend, Isthmus Maya and Menemota Venezuela). For these tests, bioluminescent assays were performed with both *Pp*F1G4 and strain TVA8 which is a *Pp*F1 derivative containing a modified mini-*Tn*5 chromosomal insertion of a *tod-lux* fusion (Table 1). As expected, all the NAPLs tested produced a bioluminescent response in both biosensors (Fig. 9). However, depending on the hydrocarbons, one system appears to work better than the other. Also, the

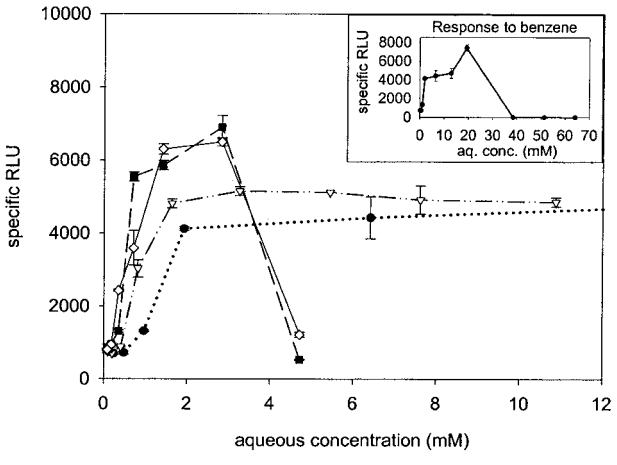

Fig. 8. Dosage response of *Pp*F1G4 to BTEX compounds. Error bars indicate standard deviations for triplicate determinations of bioluminescence. Legend key: ●, benzene; ▽, toluene; ■, ethylbenzene;◇, *m*-xylene. The inset shows the response to benzene and toxicity observed at higher concentrations.

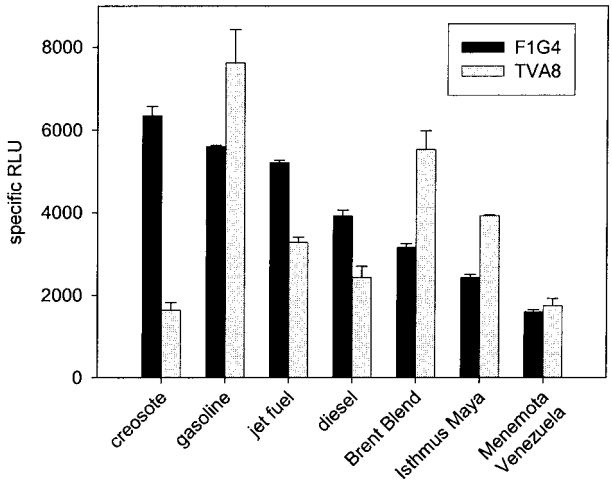

Fig. 9. A comparison of bioluminescent response of *Pp*F1G4 and TVA8 biosensors to multicomponent NAPLs. Brent Blend, Isthmus Maya and Menemota Venezuela are varieties of crude oil. Error bars indicate standard deviations for triplicate determinations of bioluminescence.

magnitude of the light response to a given NAPL cannot be readily correlated to its BTEX content. In gasoline and diesel, the BTEX content (weight per cent) is estimated to be 19.3 and 0.72, respectively, and for the three types of crude oils, it ranges from 0.6 to 2.5. As NAPLs are complex mixtures, the biosensors' light response may have been enhanced or suppressed by the presence of other unidentified components present within the NAPLs.

Solvent effect

It has been observed that, in the naphthalene reporter strain *P. fluorescens* HK44, which contains a *nahG-luxCDABE* fusion, non-inducing organic solvents produced a significant bioluminescent response (Heitzer *et al*., 1998). In that study, the analysis of mRNA levels confirmed that certain solvents did not induce *lux* gene expression, even though they triggered a bioluminescent response. This phenomenon has been termed the 'solvent effect'. It was postulated that exposure of HK44 to organic solvents resulted in membrane perturbation, causing increased fatty acid synthesis, which in turn elevated the aldehyde supply for the bioluminescence reaction. As the HK44 cells were found to be aldehyde-limited, the solvents affected the light response by increasing the aldehyde substrate level, which could then react with low basal levels of luciferase. Thus, the addition of *n*-decanal to cells had the effect of saturating the system with the aldehyde substrate, so that both induced and non-induced test cultures demonstrated increased light production. However, only test cultures containing compounds that induced gene expression yielded a significantly greater bioluminescent response compared to the control.

In order to verify that the light response of *Pp*F1G4 to test compounds was due to induction of the *sep* genes, and not to a 'solvent effect', the effect of adding *n*-decanal to *Pp*F1G4 cells exposed to 3 mM of toluene, *o*-xylene, TCE, and limonene was investigated. These compounds were chosen by virtue of the fact that the bioluminescent response they produced spanned the high and low of the measured unit (Fig. 7). Three different concentrations of *n*-decanal (2 mM, 1 mM and 0.5 mM) were tested by adding the appropriate volume of a 1% (v/v) aqueous solution of *n*-decanal to test solutions prior to the light measurement. A concentration of ~ 2 mM *n*-decanal has been used for similar bioluminescence assays (Sticher *et al*., 1997; Heitzer *et al*., 1998). The results showed that while at the two lower concentrations of *n*-decanal (0.5 mM and 1 mM), there was no significant increase in light production, at a concentration of 2 mM, there was actually an inhibition of light production in all cases. The fact that the addition of *n*-decanal did not boost bioluminescence in any of the test samples nor in the control suggests that *Pp*F1G4 cells were not aldehyde-limited. Thus, a 'solvent effect' may not be possible with this biosensor, and any light production can be correlated to luciferase levels and consequently, to gene induction. Further evidence that the bioluminescent response of *Pp*F1G4 is actually due to induction of the *sep* genes was provided by the fact that a *lacZ*-based fusion in *Pp*F1 (p*sep-lacZ*-Km^r) was induced by BTEX compounds and other solvents, as stated earlier. Moreover, it was shown that *sepA* mRNA levels increased upon exposure to toluene (Fig. 6).

Catabolite repression

Catabolite repression, also termed 'post-exponential induction' or 'exponential silencing' is an example of a situation where the expression of catabolic genes is influenced by the physiological and metabolic state of the cells (Cases and de Lorenzo, 2001). In this phenomenon, although bacteria grow rapidly on a nutrient-rich media, there is a lack of transcriptional activity, even in the presence of an inducing compound. However, once the growth rate of the bacteria subsides as they enter stationary phase, the promoter begins to respond to the effector. Figure 10 shows how the type of growth medium affects the bioluminescent response of *Pp*F1G4 and TVA8 under the same inducing condition of 4.5 mM toluene. The results indicated that the response of TVA8 culture is dramatically affected by a switch of nutrients from a minimal medium (M9 + glucose) to a relatively rich source (TB). In both LB and Terrific broth, which are richer than minimal medium (Sambrook *et al*., 1989), there was no response with TVA8. On the other hand, although the response was lower in the M9 medium compared to that of TVA8, *Pp*F1G4 is less affected by the nature of the nutrient media. In Terrific broth, a strong measureable bioluminescent response is evident for *Pp*F1G4. These preliminary results suggest that *Pp*F1G4, unlike TVA8, is a functional bioluminescent biosensor under nutrient-rich conditions.

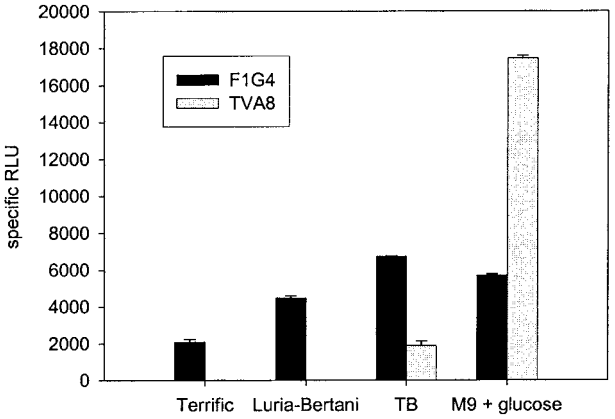

Fig. 10. Effect of growth medium on bioluminescent response of biosensors *Pp*F1G4 and TVA8 exposed to 4.5 mM toluene. Error bars represent standard deviations for triplicate determinations of bioluminescence. The compositions of the media are stated in *Experimental procedures*.

Presence of sep *gene homologues in the* P. putida *KT2440 genome and other aromatic hydrocarbon degraders*

The complete determination of the genome sequence of *P. putida* KT2440 (http://www.tigr.org/; GenBank accession number AE015451) rendered a unique opportunity to analyse for the presence of homologues of the *sep* encoding sequences. As a result of BLAST analysis using the TBLASTN algorithms, the query SepA, SepB and SepC protein sequences retrieved at least 11, 13 and 11 homologues respectively. In scoring these hits, only near full-length sequences of the respective proteins were considered and the sequence identity was found to be between 37 and 71% for SepA, 38–74% for SepB, and 38%-67% for SepC. On the other hand, SepR retrieved only two hits as well as giving a relatively low 40–42% sequence identity.

In terms of adjacency or proximity of the retrieved homologues, five appeared to be contiguous of the *sepABC* type and an equal number of the *sepAB* type. In one case, there is no apparent *sepB* homologue in between those of *sepA* and *sepC*. Taking the *sepR* homologue into consideration, there is only one instance where the *sepABC* and *sepR* homologues are in 'proximity' but this turned out to be some 9.714 kb away. Moreover, unlike the divergent arrangement of the *sepABC* and *sepR* genes in *Pp*F1, the said gene arrangement in KT2440 is a convergent one.

In earlier Southern hybridization experiments, we had probed for the presence of *sep* homologues in a number of *P. putida* strains and other degraders of environmental pollutants under stringent hybridization conditions. The molecular probes used were probe A1 for *sep A*, probe B for *sepB*, probe C for *sepC* (Fig. 1). Detection of *sepR* was done using the *Bam*HI insert fragment from pMBP-*sepR*. As a result, the following strains were probed positive for the presence of *sepABC* genes: *P. putida* strains G7 (naphthalene and salicylate), mt-2 (xylenes), PRS2000 (aromatic acids), Idaho (a solvent-tolerant strain), and *P. pseudoalcaligenes* strain KF707 (biphenyls and PCB). Among these five strains, only the Idaho strain probed positive with *sepR*. On the other hand, *Sphingomonas paucimobilis* EPA505 (fluoranthene), *Rhodococcus* sp. M5 (biphenyl and PCB) and *Rhodococcus* sp.ATCC 55309 (dibenzothiophene) were all negative. Other strains such as *Burkholderia* spp. LB400 (biphenyl and PCB), *P. pseudoalcaligenes* strain KF707 (biphenyl and PCB) and JS150 (toluene), *B. cepacia* G4 (toluene) and *S. yanoikuyae* B1 or Q1 (biphenyls and PCB) gave positive signals with *sepB* and/or *sepC* but none again probed positive with *sepR*.

Discussion

*Pp*F1 is an organism that continues to amaze in its genetic capacity. The *sep* system is the third solvent-responsive locus besides the prototypical *tod* pathway and the companion *cym/cmt* pathway. Although chemotaxis to aromatic hydrocarbons in *Pp*F1 is also an inducible phenomenon, its receptor or genetic locus is yet to be defined (Parales *et al*., 2000).

Among other organisms, *Pp*F1 has been shown to tolerate 'solvent shock' consisting of 1 and 10% (vol/wt) toluene in soil, although it is not as resistant in liquid media (Huertas *et al*., 1998; Huertas *et al*., 2000). The *sep* gene cluster described in our present study most likely provides the molecular basis for this strain characteristic. We confirmed the protective role of the solvent efflux system of *Pp*F1 in a solvent stressed environment. This finding is consistent with that reported for the Srp system of *P. putida* S12 (Kieboom *et al*., 1998a,b; for a review see Isken and de Bont, 1998) and the Ttg systems in *P. putida* DOT-T1E (Huertas *et al*., 1998; for a review see Ramos *et al*., 2002). After exposure to toluene shock, the survival of *Pp*F1 cells lacking the *sepB* or *sepC* gene, when pregrown in toluene, was greatly impaired. Assuming that the *sepABC* genes are maximally expressed under induced conditions, it is reasonable that lack of this genotype would be detrimental to the cells. When strains were not pregrown in toluene, only a de-repressed *sepR* mutant of *Pp*F1 showed an increased level of survival to toluene shock compared to the wild type. The presence of a threshold level of the SepABC proteins in the cells at the moment toluene is added seems to be necessary for the cells to survive a toluene shock. The mutant *Pp*F1(*sepR*::Km^r) was found to exhibit a shorter lag period compared to the wild type.

Pseudomonas putida DOT-T1E is the most studied solvent tolerant strain by way of three related efflux pumps, designated TtgABC (Ramos *et al*., 1998), TtgDEF (Mosqueda and Ramos, 2000) and TtgGHI (Rojas *et al*., 2001). This strain is also known to undergo *cis/trans* isomerization and vesicle formation to elaborate its tolerance strategy (Ramos *et al*., 2002). All three *ttg* systems have been shown to function in an operonic manner, and are divergently transcribed from the following established or putative regulators: TtgR is a TetR type of transcriptional regulator that negatively controls the expression from the *ttgABC* operon (Duque *et al*., 2001); TtgT is an IclR-type regulator that possibly controls the *ttgDEF* operon (unpublished data of Ramos *et al*., 2002); and TtgV (IclR-type) and TtgW (TetR-type) are two adjacently located regulators; the former negatively controls the *ttgGHI* system, but the role of the latter is, as of yet, unknown. In terms of a possible dual regulatory control, the *srpABC* system of *P. putida* S12 has been found to be negatively regulated by SrpS (IclR-type) and it was postulated that the adjacently located SrpR (TetR-type) may be necessary for an effective repression of the *srp* genes (Wery *et al*., 2001).

The *sep* system of *Pp*F1 is common with the *ttgDEF* and *srpABC* gene clusters in its inducibility by solvents (Kieboom *et al*., 1998b; Mosqueda and Ramos 2000). Yet there are subtle as well as gross differences. Besides aromatic compounds (toluene, benzene, styrene, *p*-xylene, ethylbenzene and propylbenzene), both aliphatic solvents and alcohols are capable of inducing the *srp* system which is not the case with *sep*. However, neither system is responsive to the tested antibiotics, heavy metals or the presence of sodium chloride. Interestingly, the TtgDEF system was found to be responsible for the efflux of styrene and toluene only. On the other hand, besides the two latter aromatics, the TtgABC and TtgGHI pumps are also responsible for pumping out xylenes, ethylbenzene and propylbenzene (Rojas *et al*., 2001). The TtgABC and TtgGHI pumps also remove antibiotics like chloramphenicol or tetracycline, but not TtgDEF (Rojas *et al*., 2001).

Three lines of evidence indicated that SepR acts as a repressor for the *sep* genes: (i) elevated mRNA levels observed in *Pp*F1(*sepR*::Km^r); (ii) a *lacZ* transcriptional fusion in which the activity in the de-repressed *sepR* mutant was increased (although only by 1.5-fold, this may be due to the fact that the *sepR* repressor gene is present in monocopy on the chromosome, whereas the *sepABC-lacZ*-Sm^r fusion was expressed from a plasmid, resulting

in some titration of the SepR repressor); and (iii) overexpression of SepR in *E. coli* completely abolished the expression of the *sepABC-lacZ*-Sm^r fusion. Moreover, a purified SepR overexpressed as a MBP fusion protein was able to bind specifically to the *sepR-sepABC* intergenic region proximal to *sepA*. Further experiments are required to define the SepR operator sequence. It is interesting to find the following sequence, CCATGCATTGTGATTC CCAAA, in the 3′ region of the potential −10 promoter element of *sepA.* The underlined nucleotides are identical to the consensus sequence (AAATGGAAATGATTC CACTA) suggested for an IclR-family DNA target (Zhang *et al*., 2002).

The specificity of SepR as seen through the *lux* reporter system in *Pp*F1G4 encompasses a broad range of compounds that includes trichloroethylene (TCE) and all three isomers of xylene, notably *o*-xylene. Although the *tod-lux* fusion in TVA8 produced a bioluminescent response when exposed to phenol, JP-4 jet fuel, benzene, toluene, ethylbenzene, *m*-xylene, and *p*-xylene, it was not responsive to *o*-xylene (Applegate *et al*., 1998). This was confirmed in our study (not shown). In fact, few organisms are known to degrade or grow on *o*-xylene as sole carbon source (Kim *et al*., 2002). On the other hand, the oxidation of TCE by toluene dioxygenase in *Pp*F1 and its induction in the *tod-lux* fusion in TVA8 have been well documented (Wackett and Gibson, 1988; Li and Wackett, 1992; Shingleton *et al*., 1998). Phenylacetylene has been explored as an alternative chromogenic substrate for toluene dioxygenase besides the conventional indole to indigo transformation (Keener *et al*., 1998; 2001). It is interesting that SepR responds to this alkyne compound as well. Among the PAHs tested, naphthalene elicits by far the strongest response. Although at a relatively low level, the *sep-lux* system also responds to biphenyl, phenanthrene, and limonene (a monocyclic monoterpene). Whereas the specificity of the *tod* and *sep* systems is by and large mutually inclusive, it appears that the specificity of the CymR repressor protein that regulates *p*-cymene (*p*-isopropyltoluene) degradation is exclusive. Besides *p*-cymene, its two metabolites, cumic alcohol and *p*-cumate, are non-effectors of SepR. In this regard, benzoic acid also does not produce a response. It is interesting that cumene (*p*-isopropylbenzene), which lacks a methyl group vis-à-vis *p*-cymene, is an effector molecule of SepR.

The exact molecular mechanisms by which SepR interacts with effectors are as yet unknown. However, a broad substrate specificity has been observed for other exclusion systems in bacteria, specifically proton motive force (PMF) dependent multidrug efflux pumps. For instance, the multidrug efflux regulatory proteins BmrR (in *Bacillus subtilus*), EmrR (in *E. coli*), and QacR (in *Staphylococcus aureus*) all have the ability to bind to structurally diverse compounds (Paulsen *et al*., 1996). Recently, the first crystal structure of an IclR member regulator was reported to consist of two domains: an N-terminal DNA-binding domain containing a winged helix–turn–helix motif, and a C-terminal regulatory domain involved in effector binding (Zhang *et al*., 2002). Notwithstanding structure determination, the specific function of this protein remains unknown. However, this information is potentially useful for an eventual SepR structure.

Compared to SepR, we know less of the Sep structural proteins. SepA, SepB and SepC proteins are predicted to function as periplasmic efflux protein, inner membrane efflux protein and outer membrane efflux protein, respectively. Otherwise these proteins can be referred to as the membrane fusion protein, cytoplasmic membrane transporter of the RND family (translocase), and outer membrane factor, respectively (Paulsen *et al*., 1997). By analogy with the better characterized MexA-MexB-OprM efflux pump in *P. aeruginosa* (for a review see Poole, 2001), SepA is predicted to provide the connection between the function of SepC, a potential lipoprotein in the outer membrane and SepB, a large 'xenobiotic-exporting' component in the inner membrane. Like many of its homologues, SepB is characterized by its internal tandem repeated sequence (sequence of the first half resembles the second half) and the protein is predicted to cross the membrane 12 times. At this time, it may be possible to model SepC after the structure of the *E. coli* outer membrane protein, TolC (Koronakis *et al*., 2000), as has been done with TtgC (Ramos *et al*., 2002). However, TolC is an unacylated protein. As a whole, it is essential to elucidate the structure and membrane topology of the individual pump subunits to gain an understanding of how this pump extrudes solvents or xenobiotics.

Although this is an essential step, complexity arises as to the multiplicity of the various efflux systems. We know the presence of at least three Ttg systems in strain DOT-T1E (Ramos *et al*., 2002), as is the case with the multidrug efflux pumps of *P. aeruginosa* (Poole, 2001). Analysis of the *P. putida* KT2440 genome sequence revealed the presence of at least 11 groups of *sepABC* homologues. This is in keeping with the independent analysis of the KT2440 genome carried out at the Institute of Genomic Research (TIGR; K. Nelson, pers. comm.). Referred to as the RND/MFP/OMF gene clusters, at least one has been shown to partake in toluene resistance, and some were found to be adjacent to the BenF/PhaF family of porins.

In contrast, the distribution of *sepR* homologues is comparatively low. This was borne out by DNA-DNA hybridization experiments where a positive signal of the *Pp*F1 *sepR* homologue was detected only in *P. putida* Idaho, a strain capable of growth in *p*-xylene, toluene and other alkylbenzenes at concentrations of 5–50% v/v in the cul-

ture medium (Cruden *et al*., 1992). It is interesting to note that the presence of *sepR* homologues in *P. putida* mt-2 was not apparent by the said hybridization experiment whereas the sequence of KT2440 gave two such sequences (KT2440 is a derivative of strain mt-2 cured of the pWW0 plasmid). This is likely due to a low level of sequence identity. It goes without saying that DNA sequence comparison is by far the most stringent form of hybridization and method for finding homologues. Insofar as the configuration of the *sepABC* and *sepR* genes described in our present study, clearly strain difference is evident among the various *putida* species.

The presence of a phage-like integrase sequence among bacterial degradative pathways has so far rarely surfaced (van der Meer *et al*., 2001 and references therein). One of the few was the so-called *clc* element (105-kb) of *Pseudomonas* sp. B13 that carries genes for chlorocatechol degradation (Ravatn *et al*., 1998a). This integrase (Int-B13) however, is unusually large in that it consists of 657 amino acids, the first 420 amino acids of which are most similar to the integrases of the phage P4 family. Interestingly, it has been shown that the *clc* element could be transferred to *Pp*F1 resulting in a transconjugant capable of degrading both toluene and chlorocatechol, and as a bonus, able to degrade chlorobenzenes (Ravatn *et al*., 1998b). Integration of the *clc* element in *Pp*F1 was found to occur at the 3′-ends of two glycine tRNA genes (Ravatn *et al*., 1998a). The presence of *int-F1* adjacent to *sep* and the upstream *tod* and the c*ym/cmt* pathways is suggestive of a catabolic transposon. If not defective, it is probably of low frequency as there is no reported transfer of the *Pp*F1 catabolic capabilities to other strains. Instead, the converse is true, perhaps by virtue of the relative ease in transferring a plasmid into another strain. Nonetheless, evidence of gene disruption and the presence of repeated DNA elements that may be involved in an insertion event have been reported within the *Pp*F1 degradative gene clusters: a defective *todR* upstream of the *tod* operon (Wang *et al*., 1995), and the presence of GC-rich repeated sequences upstream and downstream of the *cym/cmt* gene clusters (Eaton, 1997).

To date, biosensors developed for the detection of organic compounds have all been based on fusions with promoters from specific catabolic pathways. These include biosensors for naphthalene (King *et al*., 1990), toluate (de Lorenzo *et al*., 1993a), phenols (Shingler and Moore, 1994), BTEX compounds (Burlage *et al*., 1994; Applegate *et al*., 1997; 1998; Ikariyama *et al*., 1997; Willardson *et al*., 1998), isopropylbenzene and related compounds (Selifonova and Eaton, 1996), middle-chain alkanes (Sticher *et al*., 1997), polychlorinated biphenyls (Layton *et al*., 1998), as well as 2,4-D and 2,4-dichlorophenol (Hay *et al*., 2000). For recent reviews, see Daunert *et al*. (2000) and Keane *et al*. (2002). *Pp*F1G4 is one of the few chromosomally based biosensors and its versatility is thus far illustrated by being a full-fledged sensor for BTEX isomers besides exhibiting a broad specificity spectrum, and the ability to produce a reliable response in both nutrient-rich and minimal media. The latter aspect is important as any environmental application is challenged with an undefined nutrient source. Improvements to *Pp*F1G4 are expected to be made once we learn more about the structure of SepR and the global control or mechanism in the functioning of the *sep* promoter. Finally, useful as solvent-tolerant bacteria can be in biocatalysis and biotransformations of toxic and water-insoluble compounds into value-added chemicals (de Bont, 1998), it is fitting to say that the *sep* and related efflux systems are pumped up to go interesting places.

Experimental procedures

Organisms and culture conditions

Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely cultured at 37°C, whereas *Pp*F1 derived strains were grown at 30°C. Media components were purchased from Difco. Throughout this study, bacterial cells were grown in LB (10 g bacto tryptone, 5 g bacto yeast extract, 5 g NaCl per litre solution) or M9 media (Miller, 1992) supplemented with glucose (0.2%) and an appropriate trace metal solution (Stanier *et al*., 1966). Solid media contained 1.5% agar. When needed, medium was supplemented with antibiotics at the following concentrations: ampicillin, 100 μg ml^{-1}; gentamycin, 20 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}. Cultures were shaken on an orbital shaker at 250 r.p.m. For the catabolic repression tests, the other media used were TB (10 g bacto tryptone, 5 g NaCl per litre) and Terrific Broth (12 g bacto tryptone, 24 g bacto yeast extract, 4 ml glycerol per litre) (Sambrook *et al*., 1989).

Chemicals

The organic compounds or solvents that were used in the β-galactosidase and bioluminescence assays were purchased from Sigma-Aldrich and were of reagent grade purity or better. The regular unleaded gasoline (octane number 87) and diesel were obtained from an Esso gas station in Montreal, Quebec. The JP-4 jet fuel was procured from the Dorval International Airport in Montreal. The coal tar creosote was obtained from Kopper Industries, Carbon Materials and Chemicals Division (Pittsburg, PA). The following three crude oils were obtained from the Petro Canada Refinery in Montreal: Brent Blend originating from the North Sea, Isthmus Maya, a blend of Isthmus and Maya crude oils from Mexico, and Menemota from Venezuela. BTEX analysis for gasoline and diesel was performed on a Perkin-Elmer Sigma 2000 gas chromatograph, 'DB-petro100′ capillary column of dimensions 100 m × 0.25 mm × 0.25 μm, through headspace analysis. Typical compositions for Brent Blend, Isthmus, and Maya crude oils were obtained from an online database for the

properties of crude oils and oil products, maintained by the Environmental Technology Centre, a division of Environment Canada (Environmental Technology Centre, Environment Canada, 2001).

DNA techniques

Plasmids used in this study, as well as their detailed construction, are listed in Table 1. The plasmid pPF9SX, containing the sequence that encompasses the *todS–todT* genes, was obtained by performing a *Sac*I-*Xho*I digestion of genomic DNA from strain *Pp*F1 *(todS::*Km^r) and by generating a library in plasmid pBluescriptKS(–), digested with the same enzymes. The resulting plasmid pPF9SX was obtained after kanamycin resistance (Km^r) selection of the corresponding clones transformed in *E. coli*. This plasmid was then used as a probe to obtain the subsequent overlapping clone pGEM-3.8PB. Cloning procedures, including digestions with restriction enzymes, electrophoresis, Southern hybridizations, and transformations were carried out using standard methods (Sambrook *et al*., 1989; Ausubel *et al*., 1990). *E. coli* DH5α was routinely used for recombinant plasmid maintenance and isolation, unless otherwise specified. Enzymes used in cloning procedures were purchased from Pharmacia or New England Biolabs. Chromosomal DNA was isolated using a Genomic-tip System (Qiagen), plasmid DNA was purified with a QIAprep Spin Miniprep kit (Qiagen), and DNA fragments from agarose gels were purified using a QiaexII gel extraction system (Qiagen). Nucleotide sequencing reactions were performed with purified double strand plasmid DNA using ABI PRISM® dRhodamine Terminator Cycle Sequencing Kit, as recommended by the supplier, and the products were detected using an automated DNA sequencer (model 377, Applied Biosystems). Nucleotide sequences were analysed by the BLAST program, available from the National Center for Biotechnology Information (NCBI) server (Altschul *et al*., 1990) and the PC/Gene package (Intellgenetics). Labelling of DNA probes for southern and RNA dot-blot analysis was performed using a Random Primed DNA Labelling kit (Roche Molecular Biochemicals) with [α-^{32}P]-dCTP. Polymerase chain reaction was performed on a Perkin-Elmer DNA Thermal Cycler 480, using Taq DNA polymerase.

Expression of sep genes with T7 polymerase

Escherichia coli strain K38 harbouring the T7 polymerase gene on plasmid pGP1-2 was transformed with pT7-5 or pT7-6 derived plasmids carrying the individual s*ep* genes (Table 1). Proteins were labelled with [^{35}S]-methionine (Amersham) and separated in sodium dodecyl sulphate (SDS)-10% polyacrylamide gel as described previously (Wang *et al*., 1995).

Construction of strains

To construct null mutants of *sepR, sepB,* and *sepC*, a Km resistance cassette from pUC4K was introduced in unique restriction sites within the genes of interest, cloned on pUC13-*oriT* derived plasmids. The resulting plasmids were then conjugated into *Pp*F1 from *E. coli* S17-1. Km^r transconjugants were first selected on M9 glucose plates, allowing counterselection against *E. coli*. Transconjugants resulting from double cross-over recombinations were obtained as Km^r and ampicillin sensitive (Ap^s) mutants. As *Pp*F1 is already resistant to 100 μg ml^{-1} of ampicillin, transconjugants resulting from integration of pUC13 into the *Pp*F1 chromosome were ruled out by screening with 750 μg ml^{-1} of ampicillin. To confirm that the wild type genes had been replaced by the Km^r 'knocked-out 'allele, selected transconjugants were verified by polymerase chain reaction (PCR) amplification of the genomic DNA and by Southern hybridization.

For the construction of the *sep-lux* biosensor, the pGLS11 plasmid was used as a source of the *luxCDABE* gene cassette from *Photorhabdus luminescens* (Frackman *et al*., 1990). The five gene *lux* cassette permitted measurement of bioluminescence without the addition of an aldehyde substrate. $CaCl_2$ treated *E. coli* DH5α cells were transformed with the ligation mixture containing plasmid pBB5.23-*oriT-lux* (Table 1) and plated on LB plates containing 100 μg ml^{-1} ampicillin. Plates were inspected in the dark for the production of light. Plasmid DNA was isolated from positive clones and analysed with restriction endonucleases to confirm the presence of the *sep-lux* construct. This recombinant DNA was then introduced into $CaCl_2$ treated *E. coli* S17-1 cells and transferred into *Pp*F1 by conjugation. Transconjugants were plated on minimum M9 glucose medium with 750 μg ml^{-1} ampicillin in order to counterselect for the recipient against the donor *E. coli* S17-1, which cannot grow on minimal media alone.

Because the delivery plasmid cannot be maintained in *Pp*F1, we assumed that it became integrated into the bacterial chromosome after the cells were grown in liquid minimum M9 glucose medium without selective pressure, to ensure loss of the plasmid. After several generations of growth in order to ensure construct stability, the culture was plated on minimum M9 glucose medium containing 750 μg ml^{-1} ampicillin, to select for colonies where the plasmid DNA had been integrated into the chromosome. The viable colonies were grown overnight with and without the presence of toluene vapour, and then tested for light production. One clone in particular, designated 'F1G4', was chosen because it had low background light levels in the absence of an inducer, and was the brightest when exposed to toluene. As the growth of the biosensor strain *Pp*F1G4 in the presence of toluene as sole carbon source was similar to that of the parent *Pp*F1 *(*data not shown), it was assumed that the insertion of the *lux* genes imposed no apparent burden on the cell metabolism.

A similar approach was used to construct *Pp*F1 strain derivatives with a *sepABC-lacZ* fusion inserted in their chromosomes. *Pp*F1APR1 was obtained by conjugation with *E. coli* S17-1, containing the pP*sep-lacZ*-Km^r plasmid. This plasmid has a *lacZ*-Km^r cassette excised from pKOK6.1 and inserted in the *sepA* gene of pBB5.23-*oriT* (Fig. 1). The transconjugants resulting from single cross-over were selected as Km^r and Ap^r clones, after many passages without selective pressure, in order to assure proper stable insertion in the chromosome. Strains *Pp*F1L2.1 and *Pp*F1(*todS*::Km^r)L2.1 were constructed in a similar way, but using *E. coli* S17-1, containing plasmid pP*sep-lacZ*, as the donor in the conjugation. In this plasmid a *lacZ* cassette is inserted into the *sepA* gene (Fig. 1), thus allowing only an ampicillin resistance (Ap^r) selection for clones presenting a single cross-over.

Growth of PpF1 derivatives on toluene and survival in response to toluene shock

Survival of *Pp*F1 strain derivatives in response to toluene shock was determined in LB media. When cells were preexposed to toluene via the gas phase, toluene was introduced into the glass bulb of a central vessel placed in the culture flask, in such a way as to avoid direct contact of the solvent with the liquid media. Cells were pregrown with or without the presence of toluene until the cultures reached an A_{600} of 1.0. Toluene was then added at a concentration of 28.5 mM and the number of viable cells was determined right before and after toluene addition over time.

Overexpression and purification of SepR

SepR was overproduced as an MBP fusion protein in *E. coli* UT5600 cells harbouring the plasmid pMBP-*sepR* (Table 1), using the pMAL system (NEB). Primers used in PCR amplification of *sepR* for its cloning in pMal-c2X were: solreg-5′ (ATGGATCCATGAGCGATTCGGAAGAAAG) and solreg-3′(ATGGATCCTCTAATCAACCCGCAAACTC). The addition of *Bam*HI sites at the 5′ and 3′ ends allowed cloning into the corresponding sites of the vector. These measures were taken so that the ATG translational start site of *sepR* would be in the same reading frame as the MBP protein. Expression of the fusion protein was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for two hours. The protein was purified using the amylose resin as affinity ligand, as recommended by the manufacturer. A modification to the original protocol was the use of protease inhibitor (Complete, EDTA-free, Roche Diagnostics) during all the purification steps preceding the binding to the resin. Protein concentration was determined with the Bradford reagent (Bio-Rad protein assay), using bovine serum albumin (BSA) as a standard.

Gel mobility shift assays

To perform the gel shift assays, DNA fragments originating from the intergenic region of *sepR-sepABC* were synthesized by PCR, using plasmid pGEM-3.8PB as a template. The following oligonucleotides were used to generate the different DNA probes described in Fig. 7: probe C, opsep-5′ (TCTCACCGTTCGTCTCCTGG) and opsep-3′ (TTCTGATC CAGGCCACCGTG); probe L, opsep-5′ and opL-3′ (TCACG CATGGCATGAACGGC); probe R, opR-5′ (CGTGACTGAC CTGCACCCAG) and opsep-3′. Probe N was obtained by digesting probe C with *Nsi*I. The corresponding fragments were isolated from a 1.6% agarose gel and then labelled with [γ-^{32}P]-ATP with T4 polynucleotide kinase (Ausubel *et al*., 1990), and purified on a CentriSep column (Princeton Separations). Binding between labelled DNA (0.1 ng) and varying amounts of purified MBP-SepR protein was carried out for 30 min at room temperature in 20 μl of binding buffer (20 mM Tris-Cl pH 7.4, 2 mM $MgCl_2$, 2 mM EDTA, 10 mM KCl, 0.3 mM DTT, 300 μg ml^{-1} BSA, 50 μg ml^{-1} poly(dI-dC)poly(dI-dC), glycerol 2.5%(v/v)). Half of the mixture was run on a 4.5% polyacrylamide gel (acrylamide/bisacrylamide: 30/0.8) made in TAE buffer. Gels were dried at 80°C under vacuum and subjected to the autoradiography.

β–galactosidase activity measurements

The β-galactosidase activity of the various *lacZ* transcriptional fusions used throughout this work was determined according to Miller (1992). Cells were permeabilized with chloroform and sodium dodecyl sulphate (0.002%) prior to β-galactosidase measurements.

Determination of the activity of the plasmid-encoded *sep-lacZ* fusion (p*sep-lacZ*-Sm^r) in *E. coli* RFM443 strain derivatives was performed as follows: cells were grown in LB glucose (0.4%). As the strains carried a second plasmid (either p*Trc*99A or p*sepR*-p*Trc*), antibiotics for the marker of each plasmid were added to the media. When the cells reached an A_{600} of 0.5, IPTG was added to a final concentration of 1 mM. Cells were incubated further for 140 min before permeabilization of the cells. Activity of the control *lacZ* plasmids (pMR149 or pHRP311) was measured in a similar way.

*Pp*F1 strain derivatives carrying chromosomal *sepABC-lacZ* fusions (*Pp*F1APR1 and *Pp*F1L2.1) were grown to an A600 of 0.5 in LB, at which point toluene or other inducers were added, and then cells were grown for an additional 2 h at 30°C prior to β-galactosidase activity measurements. The same approach was used when *Pp*F1 strain derivatives had been mobilized with plasmids, except that no inducers were used, and the appropriate antibiotics were added to the media.

RNA isolation and analysis

*Pp*F1 derived strains grown in M9 glucose were induced with liquid toluene (2.17 mM) when they reached an A_{600} of 0.6. A volume of 3 ml of cell culture was sampled at 10-minute intervals for 30 min, centrifuged for 30 s in 1.5 ml microtubes, and quickly frozen at −80°C, until RNA was extracted. Total RNA was isolated using an RNeasy extraction Kit (Qiagen), according to the manufacturer's instructions, except for the following modifications: lysosyme final concentration in TE buffer was increased to 12 mg ml^{-1} and the volume of lysis buffer was doubled. Samples were on-column digested with Rnase Free Dnase (Qiagen) prior to elution from the column. The integrity of purified RNA was checked by rapid electrophoresis in 1% agarose TBE gel (data not shown). RNA was mixed with two volumes of denaturing buffer, consisting of 9.25% formaldehyde (w/v), 75% (v/v) formamide, and heated 10 min at 60°C before electrophoresis. For dot-blot analysis, 3 μg of purified RNA was immobilized on a positively charged nylon membrane (Roche Molecular Biochemicals), using a Bio-Dot apparatus (Bio-Rad Laboratories). RNA blotting, as well as its denaturation prior to its application to the membrane, were done according to Sambrook *et al*. (1989). After UV crosslinking, the membrane was hybridized with a *sepA* ^{32}P-randomly labelled probe (probe A2, Table 1) using standard protocol. Washes were performed at 55°C and the final high stringency wash was done in 0.2 × SSC, 0.1% SDS.

Bioluminescence assays

In preliminary experiments, it was determined that the strongest and most consistent light response occurred when the cells were induced at an A_{600} of 0.5 and incubated for a period of 2 h. Thus, these conditions were employed for all further

experiments. Minimum M9 glucose medium was used for all bioluminescence assays, except for the experiments that evaluated the catabolic repression of biosensors *Pp*F1G4 and TVA8 in rich growth media, where Terrific, LB, and TB broths were used. A fresh overnight culture was used to inoculate flasks containing the growth medium, and cells were grown with shaking at 250 r.p.m. at 30°C to an A_{600} of 0.5, then centrifuged and resuspended in 1/25 of the original volume. The concentrated cell suspension was diluted 25 times by adding 200 μl to 4.8 ml of growth medium with a known concentration of analyte, thus attaining the original A_{600} for the cells. For the sparingly soluble test compounds used in the screening experiments, dilutions were prepared from near saturated or saturated solutions in minimum M9 medium to attain the desired concentration of the analyte (either 1 mM or a 1:2 dilution of a saturated solution). For the NAPL tests, 4 μl of either gasoline, JP-4 jet fuel, diesel, coal tar creosote, or the three varieties of crude oil were added to 4.8 ml of minimum M9 glucose medium prior to addition of the cells. The contents of the test vials were well mixed on a vortex, and incubated at 30°C in a shaker with agitation at 250 r.p.m. for 2 h. A sample from each test vial was diluted to an A_{600} of 0.3, in order to reduce the light quenching effects related to high cell densities. Light was measured in triplicate, using opaque 96-well plates, in a Dynex MLX Microtiter Plate Luminometer. The exact value of A_{600} after dilution was measured and recorded for each sample, in order to express the light signal in terms of specific RLU (i.e. RLU/A_{600}).

Distribution of sepABC and sepR genes in other bacterial isolates

Southern transfer of DNA was done on a positively charged nylon-based membrane (GeneScreen Plus from Dupont). We followed the aqueous hybridization buffer protocol from the supplier using [^{32}P]-labelled DNA probes. The probes used for the detection of the individual genes were probe A1 for Sep A, probe B for Sep B, probe C for SepC (Fig. 1). Detection of SepR was done using the *Bam*HI insert fragment from pMBP-*sepR* as a probe. Prehybridization and hybridization were done at 65°C, and then samples were washed at 60°C in 2 × SSC (Sambrook *et al*., 1989), 1% SDS.

Nucleotide sequence accession number

The 9319 bp DNA sequence encompassing the *sep* and *int-F1* genes has been deposited in the GenBank database under an updated accession number U72354.

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