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Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*

(Mini-Tn5; *Pseudomonas*; TOL pathway; NAH pathway; XylS; XylR; bioremediation; broad host range)

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SUMMARY

Four recombinant mini-Tn5 transposons are described which contain outward-facing *Pm*, *Pu* or *Psal* promoters from the catabolic plasmids TOL and NAH of *Pseudomonas putida*, along with their cognate wild-type regulatory genes (*xylS*, *xylR*, *nahR*) or mutant varieties (*xylS2*). Transcription from such promoters is activated when the host bacteria encounters certain aromatic compounds, such as alkyl- and halobenzoates (XylS, XylS2), alkyl- and halotoluenes (XylR) or salicylates (NahR). These transposons enable the generation of conditional phenotypes dependent on the presence of specific effectors, as well as the engineering of strains expressing heterologous genes that are regulated by aromatic inducers. A mini-Tn5 *xylS/Pm::luxAB*, was used to construct *Pseudomonas* strains emitting light upon exposure to concentrations of *m*-toluate as low as 5–10 μ M. The broad-host-range transposition system of Tn5 and the stability of the inserted genes due to the loss of the transposase-encoding gene during delivery of the mobile element make these transposons particularly well suited for the construction of stable strains exhibiting halo/alkyl aromatic-regulated conditional phenotypes in the absence of antibiotic selection, as is required for some uncontained bioremediation and biomonitoring applications.

INTRODUCTION

Genetically engineered bacteria destined for environmental applications should exhibit high levels of ecological predictability (Colwell, 1991; de Lorenzo, 1992; de Lorenzo and Timmis, 1992). Central aspects of such pre-

dictability are the stable maintenance of the engineered phenotype and an adequate level of expression of that phenotype under the conditions prevailing in the location where the bacteria are expected to perform. We recently reported the development of mini-Tn5 and mini-Tn10 transposon vectors carrying non-antibiotic resistance-

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Abbreviations: A, absorbance (1 cm); aa, amino acid; Ap, ampicillin; β Gal, β -galactosidase; *bla*, gene encoding β -lactamase; bp, base pair(s); kb, kilobase(s) or 1000 bp; Km, kanamycin; MCS, multiple cloning site; NAH, catabolic pathway for naphthalene biodegradation; NahR, regulator protein of the NAH pathway; nt, nucleotide; *P.*, *Pseudomonas*; ppm, parts per million (μ g/ml); Pip, piperacillin; *Pm*,

promoter of *meta*-TOL operon; *Psal*, promoter of the *meta*-NAH operon; *Pu*, promoter of the *upper*-TOL operon; ^R, resistant/resistance; Rif, rifampicin; SDS, sodium dodecyl sulfate; ^S, sensitive/sensitivity; Sm, streptomycin; Sp, spectinomycin; Tn, transposon; *tnp**, transposase-encoding gene of the IS50R of Tn5 devoid of *NotI* sites in its nt sequence; TOL, catabolic pathway for toluene/*m*-xylene/*p*-xylene biodegradation; Tp, trimethoprim; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; XylS, regulator protein of the *meta*-TOL pathway; XylR, regulator protein of the *upper*-TOL pathway; ::, novel junction (fusion or insertion); (), denotes prophage.

selection determinants for the stable insertion of foreign genes in the chromosome of Gram⁻ bacteria (Herrero et al., 1990; de Lorenzo et al., 1990; 1993). In the present study we have combined the properties of the mini-Tn5 elements with the ability of native and mutant activator proteins of the regulatory circuits of plasmids TOL (toluene degradation) and NAH (naphthalene degradation) of *Pseudomonas putida* to permit activation of transcription from their cognate catabolic promoters to a variety of halo- and alkyl-aromatics, including several xenobiotic compounds which are environmental pollutants (Harayama and Timmis, 1989). The utility of these expression systems to engineer phenotypes responsive to specific aromatic effectors was demonstrated by inserting a specialized transposon carrying a *Pm::luxAB* fusion into the chromosome of *P. putida*. When exposed to traces of XylS effectors as low as 1 ppm (7 μ M), the resulting strains emitted enough light to be quickly and quantitatively detected with a standard *luxblot* assay.

RESULTS AND DISCUSSION

(a) Rationale for the design and utilization of mini-Tn5 elements carrying outward-facing catabolic promoters

The mini-Tn5s described earlier (Herrero et al., 1990; de Lorenzo et al., 1990; 1993) which are used as the basis for the constructions reported here are fully artificial transposons which utilize the mechanism of transposition of Tn5 and produce random insertions into the chromosome of a variety of Gram⁻ bacteria of any nt sequence bracketted by the 19-bp I and O termini of Tn5. They all utilize the same Tn suicide delivery system, the core of which is the pUT plasmid (Fig. 1). The concept of the system facilitates the construction of a variety of genetic elements within the limits of the mobile unit which eventually becomes inserted in a target chromosome. An important feature of mini-Tn5 elements is that inserts are very stable due to the loss of the transposase gene (*tnp**) during transposition.

We have exploited the modular organization of the mini-Tn5 transposons to construct new mobile elements which permit the generation in different Gram⁻ bacteria of conditional mutations and the expression of cloned heterologous genes which are dependent on alkyl- and haloaromatic effector molecules. This involved the introduction into the mini-transposons of four expression systems based on promoters of two well characterized catabolic operons of the TOL and NAH plasmids of *Pseudomonas*. *Pm* is the promoter of the *meta*-cleavage operon of the TOL plasmid of *P. putida*. Transcription is initiated from *Pm* when its cognate regulatory protein XylS is activated by substrates (like benzoate and tolu-

ates) of the *meta*-cleavage pathway and several others halo- and alkyl-substituted structural analogues (Ramos et al., 1986; 1990). As shown in Fig. 1, we have utilized in one case *Pm* in combination with the wt *xylS* gene (mini-Tn5 *xylS/Pm*) and in another *Pm* in combination with the mutant *xylS2* gene (mini-Tn5 *xylS2/Pm*), which has a Arg⁴⁵→Thr substitution in its aa sequence (Ramos et al., 1990), broadening the range of aromatic inducers which activate the protein. In the mini-Tn5 *xylR/Pu* we utilized the *Pu* promoter and its cognate regulator XylR which controls transcription of the upper catabolic operon of the TOL plasmid in response to the presence of not only substrates of the upper route like toluene and xylenes but also of a variety of mono- and disubstituted methyl-, ethyl- and chlorotoluenes and their derived benzyl alcohols and benzaldehydes (Abril et al., 1989). Transcriptional activity of the *Pu* promoter requires not only activated XylR protein but also the σ^{54} -containing form of the RNA polymerase and integration host factor (de Lorenzo et al., 1991). The fourth expression system employs the salicylate-responsive promoter of catabolic plasmid NAH7 (Schell and Poser, 1989; Yen, 1991). Structural genes for *nahG* and regulator *nahR* of NAH pathway are divergently transcribed in a fashion in which NahR activates the *Psal* promoter in front of *nahG* in response to the presence of salicylate (Schell and Poser, 1989). For the construction of mini-Tn5 *nahR/Psal*, we trimmed off all non-essential sequences of that region to introduce a *nahR-Psal* expression cassette in mini-Tn5 *nahR/Psal*.

(b) Engineering of chromosomally based heterologous expression systems regulated by aromatic inducers

Organization of the mini-Tn5 derivatives shown in Fig. 1 allows cloning of foreign DNA inserts into the single *NotI* site located downstream from each catabolic promoter and just upstream from the I end of the transposon and their subsequent transposition into the chromosome of target bacteria where they will become subject to regulated transcription in monocopy dosage. Cloning of heterologous genes within the transposon is facilitated by the use of plasmid vectors p18Not and pUC18Not, which provide an easy means of flanking DNA fragments with *NotI* sites (Herrero et al., 1990).

To evaluate the transposons for this purpose and also to have comparative estimates of the relative strength of each promoter in *P. putida* and *E. coli*, we cloned a promoterless *trp::lacZ* reporter gene from pUJ8 (Table I) as a *NotI* fragment in the four *NotI* sites of each of the transposon expression vectors. The resulting hybrid transposons were then introduced into the chromosome of *P. putida* as explained in de Lorenzo et al. (1990), and the strains thereby generated were assayed for β Gal pro-

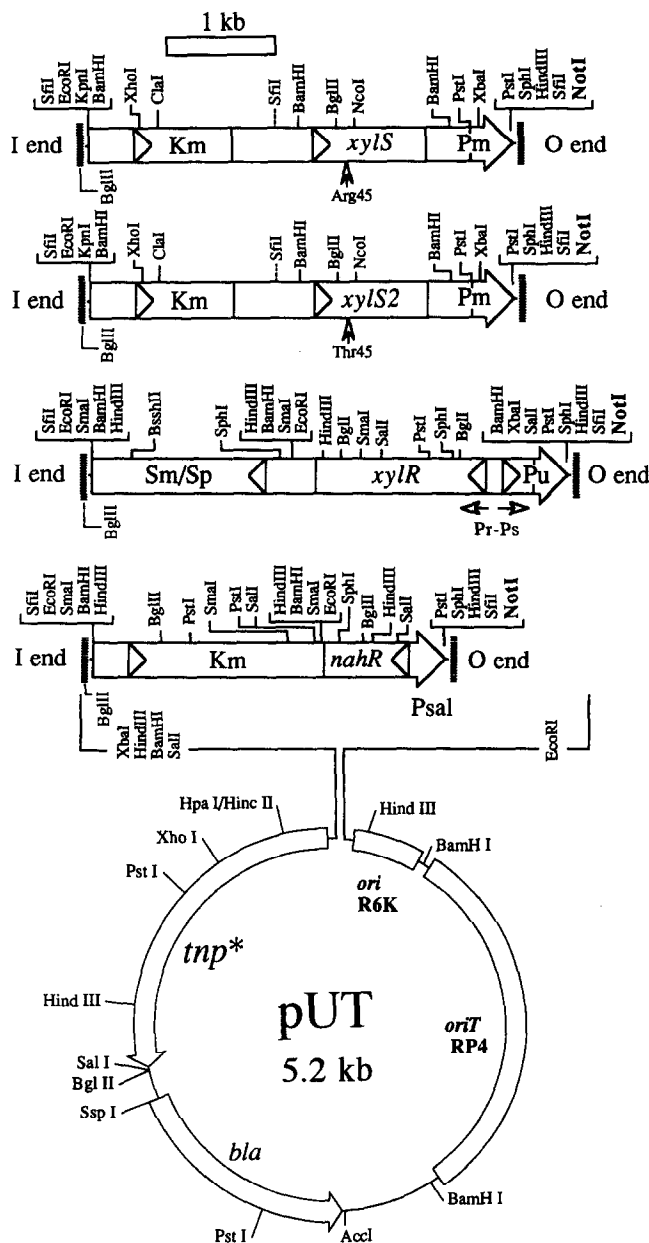


Fig. 1. Organization of mini-transposons containing outward-facing catabolic promoters. The upper part of the figure shows the new mini-Tn5 elements, which are distinct for each of the constructions discussed, whereas the lower part displays the universal mini-Tn5 delivery plasmid pUT (Herrero et al., 1990), shared by all of them. Note the *NotI* site downstream from each catabolic promoter, which is unique in all delivery plasmids, and the presence of the O and I ends of Tn5 which afford transposition of the DNA bracketted by them (Berg, 1989). **Construction of mini-Tn5 *xylS*/Pm and mini-Tn5 *xylS2*/Pm:** The *Km^R* gene of Tn903 was cloned as a 1.7-kb *BamHI* fragment into the pUC18 *Sfi* vector in the orientation in which the resistance gene is transcribed towards the *PstI* site of the MCS. The *ClaI-PstI* insert of the resulting construction was then replaced by a *ClaI-PstI* 3.6-kb fragment of pNM185 (Mermod et al., 1986a). This reconstructed the *Km^R* gene and introduced into the pUC18 *Sfi* derivative a complete *xylS* sequence which is transcribed from the *Km^R* gene promoter. A 0.4-kb *PstI* fragment containing the TOL plasmid *Pm* promoter (Mermod et al., 1984) was subsequently introduced into the single *PstI* site, resulting in the assembly of a 4.4-kb restriction fragment arranged as *SfiI-Km^R-xylS/Pm-SfiI*. This fragment was excised and cloned into the corresponding *SfiI* sites of the pUT backbone (Herrero et al., 1990), giving rise to delivery plasmid

duction in the presence of specific effectors of the catabolic promoters (Fig. 2). In all cases, induction in response to aromatic inducers was clearly evident. Under the conditions employed, the induction levels of β Gal in *P. putida* varied from 5- to 500-fold and covered a wide range of strengths and basal levels. Mini-transposons carrying wt regulatory genes *xylS*, *xylR* and *nahR* afforded induced levels of β Gal relatively independent of the site of insertion and within the range of the examples shown in Fig. 2. On the contrary, β Gal levels of chromosomal mini-Tn5 *xylS2*/Pm::*lacZ* insertions varied significantly

pCNB1 (or pUT/mini-Tn5 *xylS*/Pm; Table 1). Construction of pCNB2 (or pUT/mini-Tn5 *xylS2*/Pm) was carried out in a similar fashion except that pERD2 (Ramos et al., 1988) instead of pNM185 was used as the source of the mutant *xylS2* regulatory gene. The arrows below *xylS* and *xylS2* in the figure indicate the location of the single aa change responsible for the broader specificity of XylS2 (Ramos et al., 1990). **Construction of mini-Tn5 *xylR*/Pu:** pEZ4 is a *Pu-lacZ* fusion plasmid constructed by cloning a 298-bp *SmaI-HaeIII* fragment from pED3306 (Mermod et al., 1986b) into the unique *SmaI* site of pMLB1034 (Silhavy et al., 1984). This construction has single *EcoRI* and *SmaI* sites upstream from the *Pu* sequence. The 2.4-kb *HpaI* fragment of pTK19 (Table 1) containing the structural *xylR* gene with its native promoter, *Pr*, and a portion of the divergent and XylR-controlled *xylS* promoter, *Ps* (Inouye et al., 1987), was then inserted into the unique *SmaI* site. Ligation mixtures were transformed into *E. coli* CC118 strain (Table 1) and plated on M9/glucose-casamino acids/XGal medium, and well-grown *Ap^R* colonies were subsequently exposed to *m*-xylene vapours. Those which turned intense blue were further examined for the presence of the *xylR*-containing insert. Out of the two possible orientations, we selected the one in which the *Ps* promoter was placed in tandem with the *Pu* promoter of the *Pu-lacZ* fusion. This construction had a 2.7-kb segment with the structure *EcoRI-xylR-Ps-Pu-BamHI* which was excised as an *EcoRI-BamHI* fragment and transferred into the corresponding sites of pUC18Sfi. A *Sm/Sp^R* interposon element (Fellay et al., 1987) was then inserted into the unique *EcoRI* site, resulting in a cassette with the structure *SfiI-Sm^R-xylR-Ps/Pu-SfiI*. This cassette was then introduced into the pUT backbone to obtain the delivery plasmid derivative pCNB3 (or pUT/mini-Tn5 *xylR*/Pu). **Construction of mini-Tn5 *nahR*/Psal:** pMS15 (Schell and Sukordhaman, 1989) is a pUC8 derivative into which a *HindIII-PstI* of approximately 1.6 kb containing the whole *nahR* gene and a portion of the *nahG* gene (Schell and Poser, 1989; Schell and Sukordhaman, 1989) has been inserted. A *fl* phage origin of replication was then inserted as a 450-bp *EcoRI* fragment (Heitman et al., 1989) into the unique *EcoRI* site of pMS15 to produce pMS15ori. Single-stranded, uracil-containing pMS15ori DNA was then obtained to introduce by site-directed mutagenesis (Kunkel et al., 1987) two new restriction sites within the plasmid flanking the minimal sequence containing *nahR* and *Psal*: an *EcoRI* site was created immediately downstream from the terminal stop codon of the *nahR* structural sequence (Schell and Sukordhaman, 1989), and a *PstI* site was created between the *nahG* transcription start point and the first ATG codon of the structural gene. The introduction of these sites allowed the excision of a *nahR/Psal* cassette as a 1.1-kb *EcoRI-PstI* fragment in which transcription from *Psal* is oriented towards the *PstI* site. This fragment was cloned between the corresponding sites of pUC18Sfi, and an Ω -*Km^R* element (Fellay et al., 1987) was added at its single *EcoRI* site. The resulting plasmid contains a 3.4-kb fragment including the following elements: *SfiI-Km^R-nahR/Psal-SfiI*, which was excised and cloned in a pUT-derived plasmid (Herrero et al., 1990) to generate the delivery plasmid pCNB4 (or pUT/mini-Tn5 *nahR*/Psal).

TABLE 1
Bacteria and plasmids

Species and strain	Relevant genotype/properties ^a	Reference/origin
<i>E. coli</i> CC118	$\Delta(\text{ara-leu})$, <i>araD</i> , ΔlacX74 , <i>galE</i> , <i>galK</i> , <i>phoA20</i> , <i>thi-1</i> , <i>rpsE</i> , <i>rpoB</i> , <i>argE</i> (am), <i>recA1</i>	Herrero et al. (1990)
<i>E. coli</i> CC118(λpir)	<i>E. coli</i> CC118, λpir lysogen; recipient of pCNB derivatives (see plasmids below)	Herrero et al. (1990)
<i>E. coli</i> S17-1(λpir)	λpir , <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ⁻ M ⁺ , RP4:2-Tc:Mu:Km Tn7Tp ^R Sm ^R ; mobilising strain for pCNB derivatives	de Lorenzo et al. (1993)
<i>P. putida</i> KT2442	<i>hsdR</i> , Rif ^R prototrophic	Lab stock
<i>P. putida</i> SF001	KT2442 with a chromosomal mini-Tn5 <i>xylS/Pm::luxAB</i> insertion	This work (Fig. 3)
Plasmids	Genotype/phenotype/characteristics ^b	Reference
p18 Not	Ap ^R ; identical to pUC18 but with <i>NotI/EcoRI/SalI/HindIII/NotI</i> as MCS	Herrero et al. (1990)
pUC18 Sfi	Ap ^R ; identical to pUC18 but with <i>SfiI</i> /MCS of pUC18/ <i>SfiI</i> as new MCS	Herrero et al. (1990)
pUC18 Not	Ap ^R ; identical to pUC18 but with <i>NotI</i> /MCS of pUC18/ <i>NotI</i> as new MCS	Herrero et al. (1990)
pGP704	Ap ^R , <i>oriR6K</i> , <i>oriTRP4</i> , MCS of M13tg131	Miller and Mekalanos (1988)
pNM185	Km ^R , <i>xylS/Pm</i> -based broad-host-range expression vector, source of <i>xylS</i> gene	Mermod et al. (1986a)
pMLB1034	Ap ^R , <i>lacZ</i> promoter probe plasmid	Silhavy et al. (1984)
pERD2	Km ^R , <i>xylS2/Pm</i> -based broad-host-range expression vector, source of <i>xylS2</i> gene	Ramos et al. (1988)
pEZ4	Ap ^R , <i>Pu::lacZ</i> fusion plasmid	M. Metzke
pUJ8	Ap ^R , <i>trp::lacZ</i> promoter probe plasmid	de Lorenzo et al. (1990)
pED3306	Ap ^R , source of <i>Pu</i> promoter sequences	Mermod et al. (1986b)
pTK19	Km ^R , <i>xylR</i> cloned as 2.4-kb <i>HpaI</i> fragment in pKT231, source of <i>xylR</i> gene	T. Köhler
pMS15	Ap ^R , <i>nahR/Psal</i> sequences clones as a 1.6-kb <i>HindIII-PstI</i> insert in pUC8	Schell and Sukordhaman (1989)
pMS15ori	Ap ^R , pMS15 added with a <i>f1 ori</i> as a 450-bp <i>EcoRI</i> fragment	This work
pUT	Ap ^R ; <i>tnp*</i> gene of Tn5-IS50 R inserted in <i>SalI</i> site of pGP704	Herrero et al. (1990)
pCNB1	Ap ^R Km ^R , <i>ori R6K</i> , <i>mobRP4</i> , pUT/mini-Tn5 <i>xylS/Pm</i>	This work
pCNB1- <i>lacZ</i>	Ap ^R Km ^R , pCNB1 with <i>trp::lacZ</i> reporter downstream of <i>Pm</i>	This work
pCNB1- <i>luxAB</i>	Ap ^R Km ^R , pCNB1 with <i>luxAB</i> reporter downstream of <i>Pm</i>	This work
pCNB2	Ap ^R Km ^R , <i>ori R6K</i> , <i>mobRP4</i> , pUT/mini-Tn5 <i>xylS2/Pm</i>	This work
pCNB2- <i>lacZ</i>	Ap ^R Km ^R , pCNB2 with <i>trp::lacZ</i> reporter downstream of <i>Pm</i>	This work
pCNB3	Ap ^R Sm/Sp ^R , <i>ori R6K</i> , <i>mobRP4</i> , pUT/mini-Tn5 <i>xylR/Pu</i>	This work
pCNB3- <i>lacZ</i>	Ap ^R Sm/Sp ^R , pCNB3 with <i>trp::lacZ</i> reporter downstream of <i>Pu</i>	This work
pCNB4	Ap ^R Km ^R , <i>ori R6K</i> , <i>mobRP4</i> , pUT/mini-Tn5 <i>nahR/Psal</i>	This work
pCNB4- <i>lacZ</i>	Ap ^R Km ^R , pCNB2 with <i>trp::lacZ</i> reporter downstream of <i>Psal</i>	This work

^a λpir is a λ phage carrying the *pir* gene, the product of which (the π protein) is essential for the replication of R6K plasmid and its derivatives, such as pUT (Herrero et al., 1990).

^bAll mini-Tn5 derivatives described in this work utilize a suicide delivery system based on the properties of the pUT plasmid (Herrero et al., 1990). Plasmid pUT is a derivative of pGP704 (Miller and Mekalanos, 1988) to which a Tn5 transposase *tnp** has been added. The Tnp product promotes the transposition of any DNA segment flanked by the 19-bp termini of Tn5 when the segment is located in *cis* with respect to *tnp**. Replication of pUT occurs only in specialized λpir lysogens, like *E. coli* S17-1 (λpir), and *E. coli* CC118 (λpir). Plasmid pUT and its derivatives can be transferred by conjugation into a variety of Gram⁻ bacteria through RP4-mediated mobilization, thus allowing the generation of random chromosomal insertions of the DNA cloned within the boundaries of the mini-transposons. Insertional mutagenesis of a target strain with the mini-Tn5 elements is carried out by filter-mating as described in detail in de Lorenzo et al. (1990), using *E. coli* S17-1 (λpir) as the donor strain. Authentic transposition is distinguished from cointegrate formation on the basis of the resistance of exconjugants to the β -lactam antibiotic piperacillin (Pip), which is specified by the *bla* gene of the delivery vector (Fig. 1). Operational insertion frequencies were in the range of 10^{-5} – 10^{-6} when *P. putida* KT2442 was used as the target strain.

between different exconjugants, probably due to the sensitivity of the *XylS2/Pm* system to local changes in DNA structure.

In our hands, the most practical construction was that derived from *xylS/Pm*, since it had a relatively low basal level of expression, which was quickly triggered to 10 000–14 000 βGal units upon addition of the inducer *m*-toluate. Regarding induction ratios, the four expression systems seemed to be regulated better in the *Pseudomonas* host than in *E. coli* (Fig. 2). The induction pattern of

mini-Tn5 *xylR/Pu* was particularly interesting (Fig. 2). The level of expression of the *Pu*-driven *trp::lacZ* gene present in the transposon was indistinguishable from the background in the absence of inducer, whereas it reached about 5000 units of βGal after overnight exposure to vapours of the effector *m*-xylene. Transcriptional activity of *Pu* is subjected to various controls which include not only *XylR* in combination with the σ^{54} -containing RNA polymerase, but also integration host factor (de Lorenzo et al., 1991) and growth rate regulation (Hugovieux et al.,

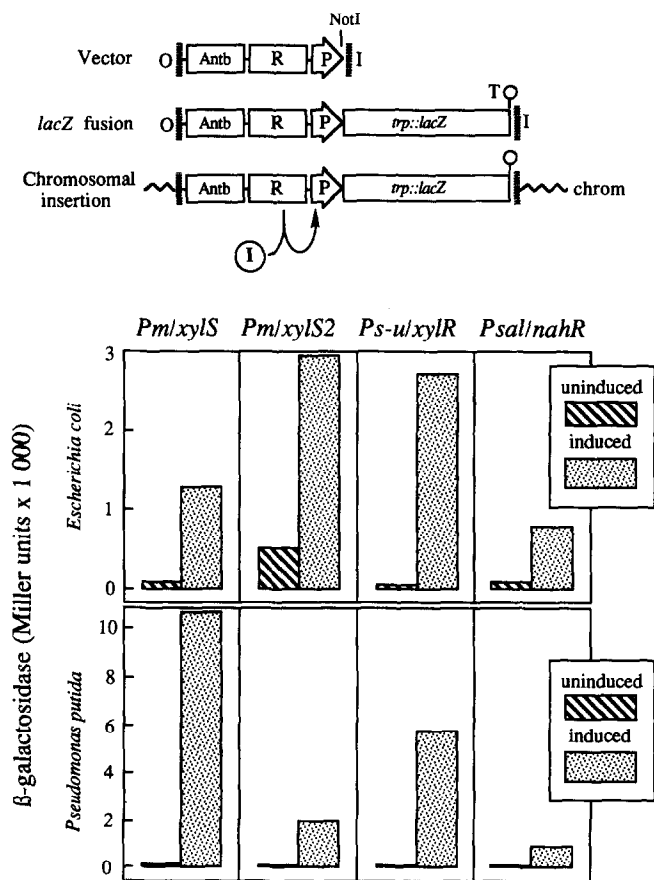


Fig. 2. Assessment of promoter strength in different transposon-based heterologous expression systems. The upper part of the figure indicates the way insertions were arranged in subsequent steps of their construction. The *trp::lacZ* reporter gene followed by a T7 terminator (T) of pUJ8 (Table I) was inserted as a *NotI* fragment into each of the four delivery vectors pCNB1, pCNB2, pCNB3 and pCNB4 (Fig. 1, Table I) which carry different antibiotic-resistance markers (Antb) and promoter/regulator pairs (P/R), and the resulting elements were then transposed into the chromosome of either *E. coli* CC118 or *P. putida* KT2442 as described (de Lorenzo et al., 1990; see legend to Table I). The levels of β Gal induction upon the addition of inducer to one example of each of the resulting exconjugants are shown. In the case of strains containing mini-Tn5 *xylS/Pm::lacZ*, mini-Tn5 *xylS2/Pm::lacZ*, and mini-Tn5 *nahR/PsaI::lacZ*, cells were grown in LB medium at 30°C with aeration until $A_{600nm} = 0.4$, after which they were supplemented with 2 mM of inducer *m*-toluate, *p*-methoxybenzoate and salicylate, respectively. Incubation was continued for 3 more h and the β Gal levels measured (Miller, 1972). In the case of mini-Tn5 *xylR/Pu::lacZ*, cells grown in LB medium as before were induced by overnight exposure to vapours of *m*-xylene. Note the different ranges of β Gal units between *E. coli* and *Pseudomonas* hosts.

1990). The result of this is the absence of significant transcription in uninduced cells and low or no activity of the *Pu* promoter during the exponential growth of induced cells. This property can be exploited for cloned genes whose products are deleterious for growing cells: appropriate conditions can be selected which prevent promoter functioning during growth followed by activation once it has ceased, thus uncoupling the bulk of biomass production from specific gene expression. Furthermore, since

the expression system can be activated upon exposure to vapours of inducer rather than by direct addition to the medium, activation of a desired gene may be arranged in a non-disruptive manner.

(c) Aromatic-responsive light emission in *Pseudomonas putida* inserted with mini-Tn5 *xylS/Pm::luxAB*

As an application, we have utilized one of the transposons described above to examine whether activation of the *Pm* promoter by a variety of benzoates could be exploited to construct a sensing scheme which would translate the aromatic-dependent activity of *Pm* into a signal easily detectable by physical means. For this purpose, we constructed mini-Tn5 *xylS/Pm::luxAB*, as described in the legend to Fig. 3, and inserted it into the chromosome of *P. putida* KT 2442. The resulting strain, *P. putida* SF001 (Table I) was subsequently grown in the presence of different *m*-toluate concentrations and subjected to a *luxdot* assay. Fig. 3 shows that light emission mediated by the activity of the *luxAB* genes is readily detected even at inducer concentrations as low as 5–10 μ M, i.e., in the range of 1 ppm. This result further expands the utility of *luxAB* as a reporter for monitoring aromatic compounds when coupled to specific catabolic promoters (Burlage et al., 1990; Weger et al., 1991).

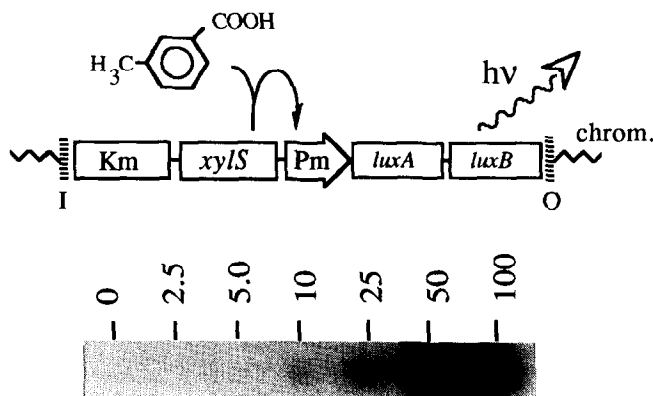


Fig. 3. Aromatic-responsive light emission by *Pseudomonas putida* SF001. The *Pseudomonas* strain used in this experiment (Table I) carries a chromosomal insertion of the transposon schematically represented above. To construct mini-Tn5 *xylS/Pm::luxAB*, a 3.3-kb *NotI* fragment containing promoterless *luxAB* genes from *Vibrio harveyi* (de Lorenzo et al., 1990) was inserted at the unique *NotI* site of pCNB1. This transposon ends upon host cells a phenotype of *luxAB*-mediated light emission in response to exposure to a number of benzoate derivatives (Ramos et al., 1990). For the *luxdot* assay (Peabody et al., 1989) shown in the lower part of the figure, a culture in LB medium of *P. putida* SF001 (Table I) was grown at 30°C to $A_{600nm} = 0.4$, after which it was split into several aliquots which were supplemented with *m*-toluate at the concentrations (μ M) indicated in the figure and further incubated overnight. Then 10- μ l samples of the cultures were adsorbed onto a nitrocellulose paper, exposed to traces of decanal and then sandwiched to a x-ray film for 10 min. The resulting autoradiograph is shown.

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REFERENCES

- Abril, M.A., Michan, C., Timmis, K.N. and Ramos, J.L.: Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J. Bacteriol.* 171 (1989) 6782–6790.
- Berg, D.: Transposon Tn5. In: Berg, D. and Howe, M. (Eds.), *Mobile DNA*. American Society for Microbiology, Washington, DC, 1989, pp. 185–210.
- Berg, D.: Conditional mutations in procaryotes. In: Drlica, K. and Riley, M. (Eds.), *The Bacterial Chromosome*. American Society for Microbiology, Washington, DC, 1990, pp.15–26.
- Burlage, R., Sayler, G. and Larimer, F.: Monitoring of naphthalene catabolism by bioluminescence with *nah-lux* transcriptional fusions. *J. Bacteriol.* 172 (1990) 4749–4757.
- Colwell, R.: Risk assessment in environmental biotechnology. *Curr. Opin. Biotechnol.* 2 (1991) 470–471.
- de Lorenzo, V.: Genetic engineering strategies for environmental applications. *Curr. Opin. Biotechnol.* 3 (1992) 227–231.
- de Lorenzo, V. and Timmis, K.N.: Specialized host-vector systems for the engineering of *Pseudomonas* strains destined for environmental release. In: Galli, E., Silver, S. and Witholt, B. (Eds.), *Pseudomonas: Molecular Biology and Biotechnology*. American Society for Microbiology, Washington, DC, 1992, pp. 415–428.
- de Lorenzo, V., Herrero, M., Jacobzik, U. and Timmis, K.N.: Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J. Bacteriol.* 172 (1990) 6568–6572.
- de Lorenzo, V., Herrero, M., Metzke, M. and Timmis, K.N.: An upstream XylR- and IHF-induced nucleoprotein complex regulates the σ^{54} -dependent *Pu* promoter of TOL plasmid. *EMBO J.* 10 (1991) 1159–1167.
- de Lorenzo, V., Eltis, L., Kessler, B. and Timmis, K.N.: Analysis of *Pseudomonas* gene products using *lacI^q/Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene* 123 (1993) 17–24.
- de Weger, L., Dunbar, P., Mahafee, P., Lugtenberg, B. and Sayler, G.: Use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. *Appl. Environ. Microbiol.* 57 (1991) 3641–3644.
- Fellay, R., Frey, J. and Krisch, H.: Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. *Gene* 52 (1987) 147–154.
- Harayama, S. and Timmis, K.N.: Catabolism of aromatic hydrocarbons by *Pseudomonas*. In: Hopwood, A. and Chater, K.F. (Eds.), *Genetics of Bacterial Diversity*. Academic Press, London, 1989, pp. 151–174.
- Heitman, J., Treisman, J., Davis, N. and Russel, M.: Cassettes of the *f1* intergenic region. *Nucleic Acids Res.* 17 (1989) 4413.
- Herrero, M., de Lorenzo, V. and Timmis, K.N.: Transposon vectors containing non-antibiotic selection markers for cloning and stable chromosomal insertion of foreign DNA in Gram-negative bacteria. *J. Bacteriol.* 172 (1990) 6557–6567.
- Hugovieux, N., Köhler, T., Rekik, M. and Harayama, S.: Growth phase dependent expression of the *Pseudomonas putida* TOL plasmid pWW0 catabolic genes. *J. Bacteriol.* 172 (1990) 6651–6660.
- Inouye, S., Nakazawa, A. and Nakazawa, T.: Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. *Proc. Natl. Acad. Sci. USA* 84 (1987) 5182–5186.
- Kunkel, T., Roberts, J. and Zakour, R.: Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154 (1987) 367–382.
- Maniatis, T., Fritsch, E.F. and Sambrook, J.: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
- Mermod, N., Lehrbach, P., Reineke, W. and Timmis, K.N.: Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of coordinately and positively regulated overlapping promoters. *EMBO J.* 3 (1984) 2461–2466.
- Mermod, N., Ramos, J.L., Lehrbach, P. and Timmis, K.N.: Vector for regulated expression of cloned genes in a wide range of Gram-negative bacteria. *J. Bacteriol.* 167 (1986a) 447–454.
- Mermod, N., Harayama, S. and Timmis, K.N.: New route to bacterial production of indigo. *Bio/Technology* 4 (1986b) 321–324.
- Miller, J.H.: *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972.
- Miller, V.L. and Mekalanos, J.J.: A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* 170 (1988) 2575–2583.
- Peabody, D.S., Andrews, C.L., Escudero, K.W., Devine, J.H., Baldwin, T.O. and Bear, D.G.: A plasmid vector and quantitative techniques for the study of transcription termination in *Escherichia coli* using bacterial luciferase. *Gene* 75 (1989) 289–296.
- Ramos, J.L., Stolz, A., Reineke, W. and Timmis, K.N.: Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. *Proc. Natl. Acad. Sci. USA* 83 (1986) 8467–8471.
- Ramos, J.L., Carrero, M.G. and Timmis, K.N.: Broad host range expression vectors containing manipulated meta-cleavage pathway regulatory elements of the TOL plasmid. *FEBS Lett.* 226 (1988) 241–246.
- Ramos, J.L., Michan, C., Rojo, F., Dwyer, D. and Timmis, K.N.: Signal-regulator interactions. Genetic analysis of the effector binding site of XylS, the benzoate-activated positive regulator of *Pseudomonas* TOL plasmid meta-cleavage pathway operon. *J. Mol. Biol.* 211 (1990) 373–382.
- Schell, M. and Poser, E.: Demonstration, characterization and mutational analysis of NahR protein binding to *nah* and *sal* promoters. *J. Bacteriol.* 171 (1989) 837–846.
- Schell, M. and Sukordhman, M.: Evidence that the transcription activator encoded by the *Pseudomonas putida nahR* gene is evolutionarily related to the transcription activators encoded by the *Rhizobium nodD* genes. *J. Bacteriol.* 171 (1989) 1952–1959.
- Silhavy, T.J., Berman, M.L. and Enquist, L.W.: *Experiments with Gene Fusions*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984.
- Yen, K.: Construction of cloning cartridges for development of expression vectors in Gram-negative bacteria. *J. Bacteriol.* 173 (1991) 5328–5335.