MiniReview

Mini-transposons in microbial ecology and environmental biotechnology

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Abstract

Mini-transposon is the generic name given to the members of a collection of genetic assets derived from transposons Tn*10* and Tn*5*, in which the naturally occurring functional segments of DNA have been rearranged artificially to originate shorter mobile elements. In the most widespread design (that known as the pUT system), any heterologous DNA segment can be conveniently cloned within the boundaries of a mini-Tn*5* vector and finally inserted into the chromosome of target Gram-negative bacteria after a few simple genetic manipulations. The large variety of antibiotic, non-antibiotic and excisable selection markers available has been combined at ease with DNA fragments encoding one or more phenotypes of interest for ecological or biotechnological applications. These include the tagging of specific strains in a community with selectable and/or optical marker genes, the production of stable gene fusions for monitoring transcriptional regulation in single cells, the metabolic engineering of strains destined for bioremediation, the non-disruptive monitoring of gene transfer and the assembly of gene containment and strain containment circuits for genetically manipulated microorganisms.   © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction: mini-transposons in perspective

The development of the gene cloning technology by Cohen and Boyer in the early 1970s constituted a revolution for all of the biological sciences, seeded new industries based on these sciences, and ushered in the era of biotechnology. Though the mainstream of effort in the late 1970s lay in the development of contained bioreactor-based biotechnological applications, during the 1980s, interest in developing recombinant plants and microorganisms for uncontained environmental applications in agriculture, medicine (live vaccines), waste disposal etc. grew rapidly. This in turn fuelled scientific and public debate on possible ecological risks of such applications and stimulated the extension of research in microbial ecology.

The use of recombinant organisms for environmental applications differs from that for contained applications in important respects. Instead of being propagated as a monoculture in an optimised, controlled environment with nutrients in excess, the recombinant organism is introduced into a community

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of diverse organisms where it must establish itself, interact with other members of the community in unknown ways, and face a multitude of poorly controllable external factors, some of which place it under considerable stress. Some environmental situations, such as those encountered in bioremediation, are patently hostile for the recombinant organism. Thus, whereas contained applications are mainly based on a few well characterised microorganisms, such as *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and some cell lines which perform well in bioreactors, open applications are based on a more diverse range of organisms able to survive and perform in natural communities in the environment, such as *Pseudomonas*, *Rhizobium*, *Salmonella*, etc. Efforts in the early 1980s focused on the development of new plasmid vectors based on the broad host range RSF1010 and RP4/RK2 replicons. However, these vectors suffered as well from the disadvantages generally common to plasmids. The specific characteristics of open biotechnological applications clearly necessitated the development of novel genetic tools exhibiting new properties. These included stability without selection, minimal physiological burden, small size, non-antibiotic selection markers and minimal lateral transfer of cloned genes to indigenous organisms.

Our first attempts to construct recombinant bacteria for environmental applications without using plasmid vectors involved the use of natural transposons. Specifically, genes for the biosynthesis of the type-specific lipopolysaccharide O-antigen of *Shigella dysenteriae* serotype 1 (Shiga's bacillus) a protective antigen against Shiga dysentery, were cloned in the mercury resistance transposon Tn*501*, which was then transposed to the chromosomes of *aroA* mutant attenuated salmonellae live vaccines [1]. The selective marker used for the various genetic manipulations was resistant to mercury salts. This generated stable, monocopy recombinants that were antibiotic sensitive. Despite their obvious advantages over plasmid vectors for gene cloning, natural transposons [2] have disadvantages: they are large and hence unwieldy for genetic manipulations; they have termini composed of repeated sequences which tend to promote DNA rearrangements; their transposases which mediate transposition also mediate transposition immunity, which prevents multiple cloning in

the same strain; and very few encode markers other than antibiotic resistances which can be used for selection purposes.

We therefore decided in 1987 to design from first principles novel mini-transposon cloning vectors which would have the advantages of natural transposons, but lack their disadvantages [3]. These efforts relied heavily on the extensive progress that had been made on the structure and function of transposons, primarily of Tn*5* and Tn*10*, by the groups of D. Berg, N. Kleckner, and others [2].

We started by assessing non-antibiotic resistance markers, such as heavy metal ions (mercury, arsenite, chromate, nickel) and herbicides, which might serve well for selection of genetic events. From this first survey, we selected 3 new markers: (1) the broad-specificity *mer* system of plasmid pDU1358, endowing resistance to mercuric salts and organomercurials; (2) the *ars* genes of plasmid R773, encoding a pump for tolerance to arsenite; and (3) resistance to the herbicide phosphinotricin tripeptide (PTT or Bialaphos), encoded by the *bar* gene of *Streptomyces hygroscopicus* [4]. At the same time, we became aware of the observations made by N. Keckner that the transposase of Tn*10* could work very efficiently in *cis*, but outside the bounds of the terminal Tn*10* ends [5]. On this basis, we considered assembling the non-resistance markers next to one or more restriction sites for cloning of heterologous DNA fragments and flanking the whole assemblage with the termini of the IS sequences of Tn*10*, followed by the Tn*10* transposase in *cis*. Such an array of elements can be delivered in *E. coli*, for instance, with a lambda phage system (such as was already published with various Tn*10*-derivatives [5]), but not in other bacteria. On this issue, we took some inspiration from the elegant suicide delivery system for Tn*5* developed in Pühler's Laboratory in 1983 for the analysis of soil Pseudomonads [6]. It consisted in the assembly of both Tn*5* and the origin of transfer of plasmid RP4 (*oriT*) in a pBR322-type plasmid which cannot replicate in the target bacterium. The resulting construct can be mobilised to many Gram-negative bacteria with an RP4-based transfer system (see below), but it cannot replicate in them, thereby leading to a suicide delivery scheme. However, we were interested in inserting the mini-transposon in *Shigella*, in which pBR322 derivatives can replicate,

so this approach could not be automatically applied. Our first attempts tried to assemble the whole array of DNA segments (Tn*10* ends flanking non-antibiotic resistance genes adjacent to cloning sites, IS*10* transposase and RP4*oriT*) in a thermosensitive replicon, but the outcome was very poor. The ultimate breakthrough came from the work by Miller and Mekalanos [7] on the use of an universal suicide delivery system based in the mechanism of replication of plasmid R6K (see below). By assembling these elements in a single replicon, we were able to come up with the first series of Tn*10*-based mini-transposon vectors known as the pLOF series [4]. Soon after, we noticed that a much smaller version of them could be constructed based on Tn*5*, with the advantage that the mechanism of Tn*5* transposition seems to have one of the broadest ranges known [2,8]. Although mini-Tn*10* vectors have indeed been used to fulfil their initial role in for designing vaccine strains [9,10], their utilisation as general genetic tools has been far less frequent that the counterparts based on Tn*5*. Since this review is intended for applications in Microbial Ecology, we will focus exclusively on mini-transposons derived from Tn*5*. Alternative, Tn*7*-based systems for integration of DNA segments into the chromosome of strains destined for the environment have been developed also (see [3] for a review).

2. Organisation and functioning of mini-Tn*5* transposon vectors

Tn*5* is a composite transposon, i.e. its mobility is determined by two insertion sequences (IS*50*L and IS*50*R) flanking the DNA region encoding the kanamycin/neomycin/resistance genes [2]. Interestingly, as in Tn*10*, the transposase determined by IS*50*R (the product of the *tnp* gene) still functions when the gene is artificially placed outside of the mobile unit, though preferably placed in *cis* to the cognate terminal sequences. As shown in Fig. 1, this allows the construction of recombinant transposons in which only those elements essential for transposition (i.e. IS terminal sequences and transposase gene) have been retained and arranged such that the transposase gene is adjacent to, but outside of, the mobile DNA segment [4,8,11]. Due to the loss of the *tnp* gene after
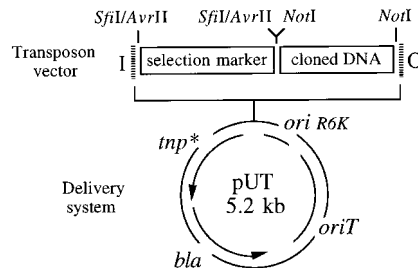

Fig. 1. Organization of Tn*5*-based transposon vectors and their suicide delivery plasmid. The scheme represents the features common to all constructions, the whole being generally named as the pUT system. The transposase gene of Tn*5* devoid of *Not*I sites is present nearby but outside the mobile element (transposon vector) which includes sites for unique and very unusual restriction enzymes *Sfi*I/*Avr*II and *Not*I. This sequence is flanked by the two 19 bp I and O termini of Tn*5*. The *Sfi*I/*Avr*II site is then used for the insertion of a variety of antibiotic and non-antibiotic selection markers whereas the *Not*I site is used for cloning heterologous DNA fragments encoding desired phenotypes. These constructs are maintained in host strains that produce the R6K-specified π protein. For mobilization, pUT plasmids are transformed into an *E. coli* strain with a chromosomally integrated RP4 that provides conjugal transfer functions which act on the *oriT* sequence present in the plasmid.

insertion, mini-transposons are stably inherited and are not prone to cause DNA rearrangements or other forms of genetic instability. Inserted cells do not become immune to further rounds of transposition, thus allowing the organism to be re-inserted with the same system, provided that subsequent transposons contain distinct selection markers. A critical feature of the mini-transposons is the system to deliver them into the target strain, based on the narrow-host range plasmid R6K (Fig. 1). Plasmids having the R6K origin of replication require the R6K-specified replication protein π and can be maintained only in host strains producing this protein. Mini-transposon delivery plasmids have the R6K origin of replication as well as the origin of transfer (*oriT*) of RK2. Delivery plasmids are thus maintained stably in λ*pir* lysogens or in *E. coli* strains with the *pir* gene recombined in their chromosome [12], and can be mobilised into target *Pseudomonas* cells through RP4 transfer functions. The combination of all these genetic assets was the production of a complete system for construction and suicide delivery of hybrid mini-Tn*5*s which have become known as the pUT system. A very detailed description of the practical use of mini-Tn*5*s can be found in [11].
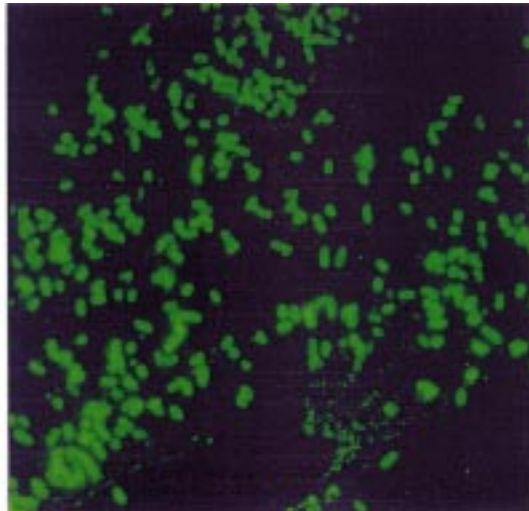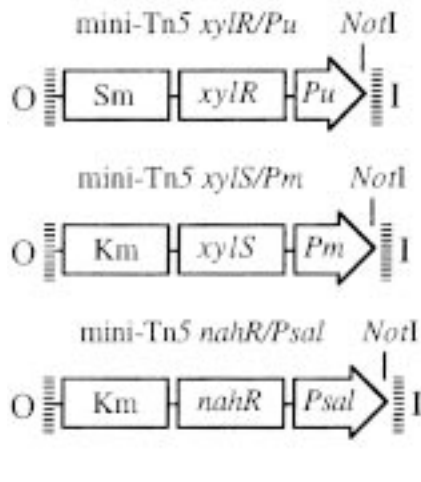
Fig. 2. Expression cassettes based on catabolic promoters of *Pseudomonas putida*. The organization of mini-Tn5 *xylR/Pu*, mini-Tn5 *xylS/Pm* and mini-Tn5 *nahR/Psal* is sketched. The first two contain, respectively, the *Pu* and *Pm* catabolic promoters of the TOL plasmid of *P. putida* pointing towards a *Not*I site, along with their cognate regulatory genes *xylR* or *xylS*. Expression of heterologous genes cloned at the *Not*I site of the transposon is induced upon exposure of the cells inserted with the hybrid construction to TOL inducers. The overall organization of the mini-Tn5 *nahR/Psal* element, bearing regulatory elements from the naphthalene degradation pathway NAH7, is identical to that of the counterparts of the TOL pathway, and it permits engineering expression of heterologous genes in response to salicylate. The photograph to the right is an epifluorescence image of *P. putida* cells carrying transposon mini-Tn5 *xylS/Pm* inserted in its *Not*I site with the gene for the antigenic surface reporter system based on the LamB protein and explained in the text [32]. Cells shown were induced with *m*-toluate for expression of the reporter antigen, which is detected with a fluorescent monoclonal antibody. Non-induced cells give a fully dark field.

3. Chromosomal integration of heterologous DNA segments

As discussed above, although plasmids have been the favoured tools for gene cloning and expression projects, they do present a number of difficulties for maintaining a recombinant phenotype under working environmental conditions. In strains destined for environmental or agronomical release, it is, therefore, of essence that the desired traits are encoded by the chromosome, thus ensuring the stability and predictability of the engineered phenotype. Mini-transposons provide a straightforward tool to clone and insert foreign genes stably into the chromosomes of various Gram-negative bacteria such as Pseudomonads and their relatives. To this end, it was necessary to equip the mini-transposons with cloning sites. In keeping with our philosophy to restrict their size to a minimum, and in contrast to the then current wisdom of providing as many cloning sites as possible in cloning vectors ('clone site banks'), we

introduced only two sites, those of the rare cutters *Not*I and *Sfi*I. The latter, which in our constructs is also an *Avr*II site, has consistently been used for insertion of the selection marker (Fig. 1). Thus, the original mini-transposons [4,8] only had a single *Not*I site for cloning DNA fragments. Hybrid transposons bearing the DNA segment of interest can be easily assembled with the help of the specialised cloning vectors, pUC18*Not* or pUC18*Sfi* [4], which flank with *Not*I or *Sfi*I/*Avr*II sites, respectively, any restriction fragment cloned at their polylinker. This modular nature of the mini-transposons facilitates the construction of mobile elements à la carte, in which not only new selective markers can be introduced (Sm, Km, Cm, Tc), but also DNA segments of various origins, promoterless reporter genes and a whole variety of expression devices can be engineered (see below). As mentioned above, multiple insertions in the same strain are only limited by the availability of distinct selection markers. On this basis, mini-transposons are, to this day, the most powerful genetic

assets for any strain-construction project that involves the combination of otherwise separate traits encoded by cloned DNA segments [13,14].

4. Novel assets assembled in mini-transposons

4.1. Expression cassettes and optical markers

Since the series of mini-transposon vectors published in the early 1990s, a number of significant improvements have been incorporated in the basic scheme of the pUT system. Some versions now available incorporate more restriction sites within the transposon vector [15] that avoid (at least in some cases) the need of pre-cloning the insert of interest in pUC18*Not* or pUC18*Sfi*. In other cases, very rare restriction sites have been entered into the mini-transposon in order to facilitate the physical mapping of the insert with pulse-field gel electrophoresis [16,17]. Also, a collection of *Sfi*I expression cassettes have been devised to drive expression of genes cloned as promoterless *Not*I inserts. These include not only the typical isopropyl β-D-thiogalactopyranosyde (IPTG)-responsive *lac*-based promoters [18,19], but also regulated expression cassettes recruited from biodegradative operons of *Pseudomonas* [20]. In this way (Fig. 2), expression of a gene of interest can be directed through the accidental or deliberate presence in the medium of benzoate, salicylate, toluene and many other aromatics [20–22]. In a further design (Fig. 3), we combined a benzoate-responsive catabolic promoter in one transposon from the TOL plasmid of *P. putida* mt2, driving expression of the T7 RNA polymerase gene and then expression of the desired gene in a second transposon under the control of a T7 promoter [23]. This permitted the engineering of expression cascades to amplify the response to a given chemical signal.

Other lines of improvement have dealt with the combination of mini Tn*5* vectors with novel markers and reporter genes which can be monitored through optical means, such as light emission or fluorescence. These markers are particularly useful for non-disruptive monitoring of genetically modified microorganisms (GMOs) in the environment and even for quantifying specific gene transfer, expression or metabolic status of genes at the level of single cell in a complex
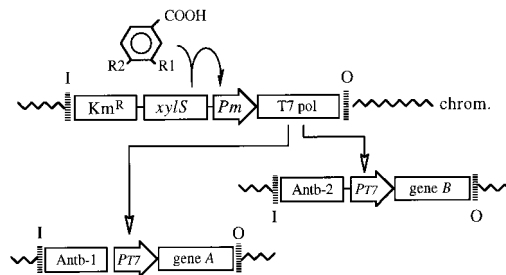

Fig. 3. Assembly of regulatory cascades with mini-transposons combined with T7 polymerase and T7 promoters. The figure summarizes the utilization of mini-Tn*5* *xylS*/*Pm*::*T7pol* for constructing strains which carry disparate heterologous genes whose expression is simultaneously triggered by addition of benzoates [23]. The mobile element schematically represented at the top of the figure and inserted into the chromosome of the target strain synthesizes T7pol in response to inducers of the *xylS*/*Pm* system, e.g. benzoate derivatives. Once produced, T7pol activates transcription from T7 promoters (*P*$_{T7}$) of different genes (*gene A, gene B*) carried by mini-transposons which have been inserted with their different resistance markers (Antb-1, Antb-2, etc.).

community. The favourite marker to this end is the GFP, the jellyfish green fluorescent protein [24–28]. Various laboratories have published mini-Tn*5* variants [29,30] which, themselves, bear GFP mutants with different properties [31]. Other mini-Tn*5* versions endow the cells with the surface presentation of a very distinct epitope which can be recognised with cognate monoclonal antibodies [32]. These, in turn, can be coupled to either fluorescein for epifluorescence (Fig. 2) or for enrichment of GMOs with immunomagnetic beads (Fig. 4). Some applications of these markers and reporters are discussed below.

4.2. Constructing quasi-natural recombinant strains

One of the major recent developments in mini-transposons tailored for environmental applications has taken place in the nature of the selectable markers assembled in them. The non-antibiotic selection markers (e.g. herbicides or heavy metal resistances), initially proposed by Herrero et al. [4], do present some problems in practical use. Resistance to herbicides, such as Bialaphos, is flawed by the high level of tolerance and/or spontaneous mutation rate observed in Gram-negative bacteria. Resistance to mercuric salts (encoded by the *mer* genes) is also
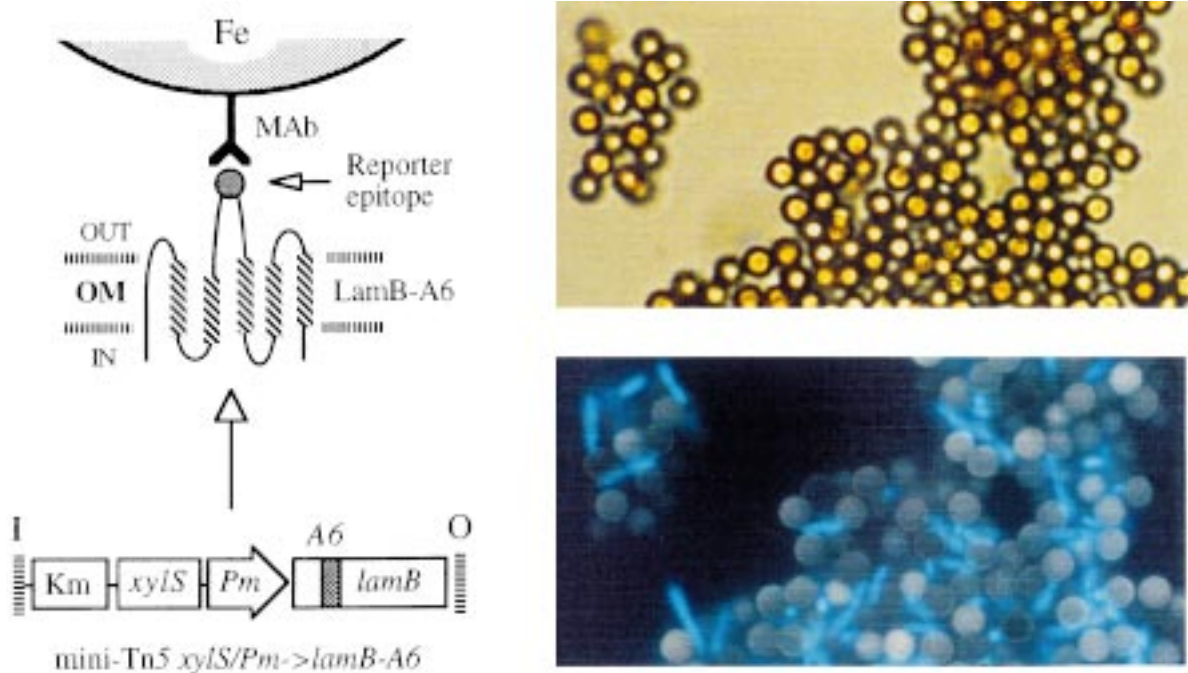
Fig. 4. Immunomagnetic separation of GMOs labeled with an antigenic surface reporter. Construction of a hybrid LamB-A6 protein as a surface reporter of promoter activity. The LamB protein of *E. coli* contains 12 outer membrane (OM) spanning domains (symbolically represented in the figure) connected by various polypeptide loops facing the periplasm (IN) or the external medium (OUT). Amino acid position 153 is located in one permissive loop which allows exposure of heterologous peptides (like the TGEV A6 reporter epitope) on the cell envelope. This antigen can react in situ with a specific monoclonal antibody (MAb) bound directly or indirectly to immunomagnetic beads. The scheme below shows the organization of the chromosomal insertion of mini-Tn5 *xylS/Pm→lamB-A6* [32] carrying relevant regulatory elements (not to scale) engineered in a transposon vector bounded by the I and O termini of Tn5. The top photograph is a phase-contrast microscopy image of magnetic beads coated with anti-A6 MAb and mixed with a culture of a *P. putida* strain inserted with mini-Tn5 *xylS/Pm→lamB-A6*, induced with *m*-toluate. The bottom photograph is the epifluorescence image of the same sample stained with diamidine phenylindole dihydrochloride (DAPI). Note the cells attached to the beads. (Photographs by A. Cebolla.)

Fig. 5. Novel non-antibiotic selection markers. The different DNA segments encoding the various *Sfi*I/*Avr*II cassettes are shown on top. The use of the resistance to potassium tellurite encoded by the *kilAtelAB* cluster as selection marker is discussed in [35]. Colonies expressing the resistance have a distinct colour (A). For production of the DNA fragment shown encoding resistance to glyphosate (the monoisopropylamine salt of *N*-phosphonomethyl glycine) the *aroA* gene of *Salmonella typhimurium* (encoding 5-enolpyruvyl shikimate 3-phosphate synthase) was amplified as a 1.3-kb *Bam*HI–*Sal*I segment mutated in vitro to generate a gene variant (*aroA**) resistant to the herbicide [34]. This fragment was then added with a short *Eco*RI–*Bam*HI segment bearing the promoter of the β-lactamase (*Pbla*) and finally assembled in pUC18*Sfi* to flank the segment with *Sfi*I/*Avr*II sites (resulting in plasmid pJMSA12). Selection for tolerance to glyphosate is made in minimal medium with 8 mg ml^{-1} of the herbicide. Various mini-Tn5s are available [12,33] which employ growth on lactose as the selection marker. The segments shown in the figure include a promoterless *lacZY* segment originated as a 5.1-kb *Eco*RI–*Nru*I from plasmid pRS551 [65] which was flanked by *Sfi*I/*Avr*II sites by cloning in *Eco*RI/*Hin*cII-digested pUC18*Sfi*. The delivery plasmid for the corresponding transposon, bearing a single *Not*I site for cloning heterologous fragments, is called pJMSA2 (pUT/mini-Tn5 *lacZY*). A different version of the same transposon bears a *xylR::lacZY* fusion, in which the hybrid marker gene is driven by the constitutive *Pr* promoter of the TOL plasmid. In this case, the delivery plasmid is called pJMSA1 (pUT/mini-Tn5 *xylR::lacZY*). Selection for growth on lactose is effected in minimal medium with 0.2% of this carbohydrate as the only carbon source and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosyde (Xgal, B). The segments below show the organization of excisable markers carrying elements of the multimer resolution system (*mrs*) of RP4 as explained in [36]. The genes and functions determined by the different elements are specified in each case: *npt*, kanamycin resistance gene of Tn5; *xylE*, gene encoding catechol 2,3-dioxygenase; *res*, resolution sites, targets of the site-specific resolvase ParA. (C) shows the phenotypic monitoring of ParA transient expression. A *P. putida* strain carrying a chromosomal insertion of mini-Tn5 *res-npt-xylE-res* was mated with *E. coli* CC118λ*pir* bearing the *parA+* plasmid pJMSB8 and a helper. The mating was then plated on minimal citrate medium for counterselection of the donor and, after incubation, sprayed with catechol for revealing the maintenance or loss of the *xylE* gene. About 15% of the colonies had become Km^s and C2,3O$^-$ as shown.

hampered by the very narrow and varying window of concentrations of the agent in which selection is effective. Finally, tolerance to arsenite may not be easy to select due to the potential interference with the phosphate in the medium. Some alternatives have been developed to overcome these problems.

These include the use of *lacZY* as both a reporter gene, a visual tag and a selectable marker, since many soil bacteria expressing these genes are able to grow on lactose as the only carbon source [12,33], as shown in Fig. 5. We have also employed resistance to glyphosate, encoded by an *aroA* gene
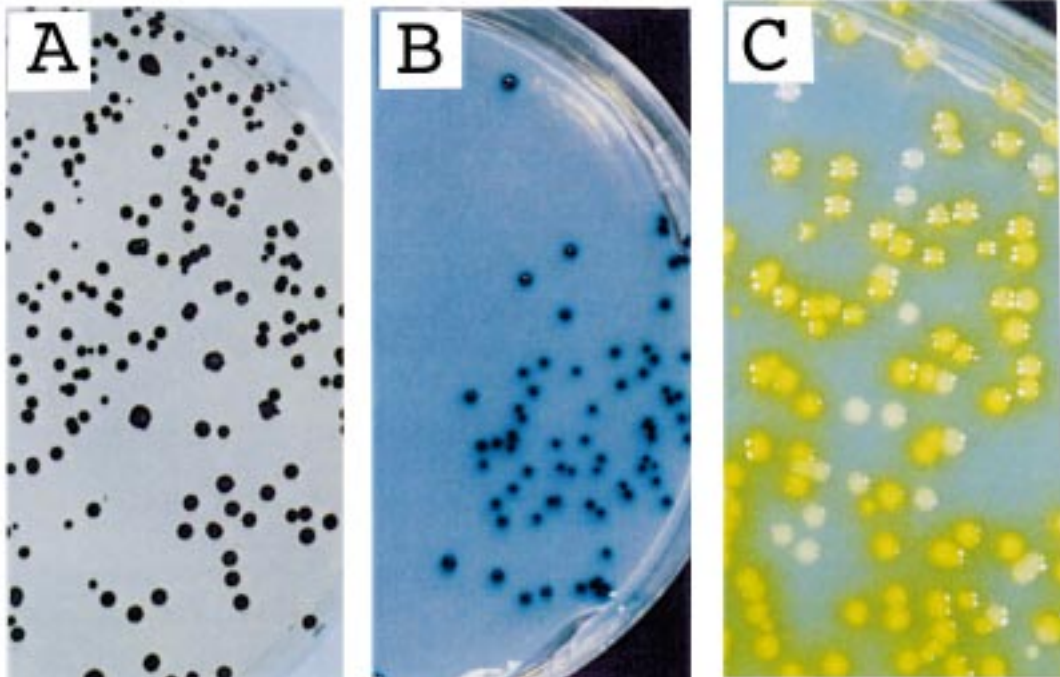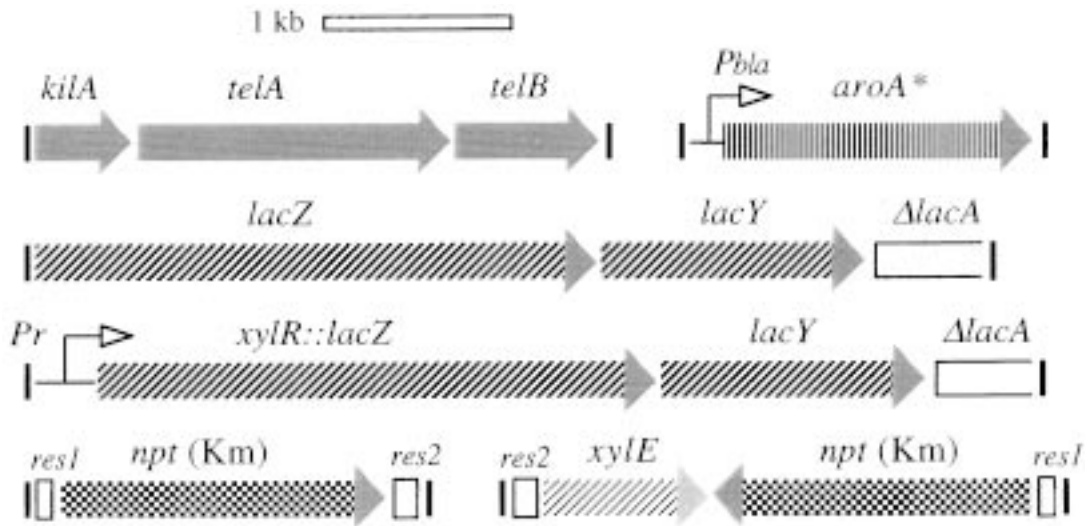
mutant of *Salmonella* encoding a variant of 5-enol-pyruvyl shikimate 3-phosphate synthase resistant to the herbicide [34]. Finally, we have used, with considerable success, the resistance to tellurite salts encoded cryptically by plasmid RK2, since again, it provides a clear-cut selection [35] besides a notorious visual labelling (Fig. 5).

These alternative markers do remain in the target strain after the transposition event without any real utility after selection. The next challenge was, therefore, developing a genetic system to remove such markers, antibiotic or not, after they fulfilled their role of accompanying the DNA segments encoding the phenotypes of interest and allow selection of their insertion in the chromosome of the desired strains. To this end, we resorted to a well-known site-specific recombination system encoded by the broad host range RP4 plasmid. Previous work on its multimer resolution system (*mrs*) which participates in the process of plasmid replication, had shown that DNA segments flanked by tandem *res* sites and cloned in a multicopy vector could be precisely deleted in vitro and in vivo by the product of the *parA* gene. On this basis, this *mrs* system was exploited to develop a general method that permits the precise excision of antibiotic resistance markers present in mini-transposon vectors after the transposition event has taken place [36]. This is based on site-specific recombination between two directly repeated 140 bp resolution (*res*) sequences of RP4 effected by a resolvase encoded by the *parA* gene and borne by a conditional replication plasmid. This strategy permits the stable inheritance of heterologous DNA segments virtually devoid of the sequences used initially to select their insertion. The mechanism of multimer resolution (which works over 60 kb in RP4), makes this strategy generally applicable to DNA fragments of virtually any size, even large chromosomal portions (Fig. 6). Although various applications of the ParA-*res* system can be easily envisioned, it seems to be particularly adequate to generate chromosomal insertions of heterologous DNA segments eventually devoid of any selection marker, as is generally required for metabolic engineering of strains destined for environmental release [3,13,37]. This procedure has been used successfully to construct specialised *Pseudomonas* strains able to grow on toluene through a hybrid biodegradation
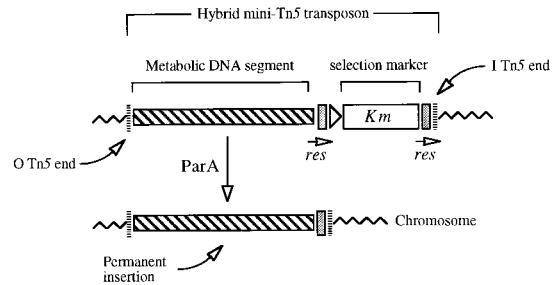

Fig. 6. Metabolic engineering of *Pseudomonas* with mini-transposon vectors bearing excisable antibiotic resistances. In this scheme, the selection marker is placed within the transposon vector flanked by tandem *res* sites of the multimer resolution system of plasmid RP4. Chromosomal insertion of a DNA segment (encoding a metabolic function of interest) cloned in the mini-transposon can be selected through the marker present in the vector (Km in the figure). Subsequent transient expression of the cognate resolvase ParA gene (e.g. from an unstable plasmid) causes the selection marker gene to be specifically deleted, thus leaving behind only the DNA segment of interest stably inserted in the chromosome [36,38].

pathway which involved formation of a benzoate intermediate and an *ortho*-cleavage of the corresponding catechol (Fig. 6) [38]. Due to the loss of recombinant markers during the construction process, these genetically engineered strains are hardly different from their non-recombinant counterparts. We propose the term *quasi-natural* for recombinant strains constructed with such a method, since they have been originated with genetic methods which resemble the natural recombination processes which lead to the creation of novel phenotypes in Nature [39].

4.3. Remaining drawbacks

A general problem of mini-transposons is that their insertion involves necessarily the disruption of a chromosomal sequence. Environmentally important functions can thus be affected. In this respect, all strains generated by the transposon insertion approach are mutants, although it is at least possible to screen against growth-defective lesions in the laboratory. One more practical drawback originates from the very pedigree of the vectors. The basic pUT plasmids were constructed in the late 1980s, when the polymerase chain reaction (PCR) and the now standard automated sequencing procedures were a rarity.

They were assembled by cutting and pasting a large number of DNA segments from many origins and thus they carry an excessively high number of restriction sites. These complicate considerably the orientation of cloned DNA segments and the mapping of the chromosomal insertions. These problems, which were unimportant at the time of the earlier applications, have become limiting for some more recent applications, in particular the sequencing of the DNA regions flanking the site of the insertions. A second problem which may deserve further improvement in the future is the suboptimal performance of the suicide delivery system in some target strains which do not behave well as recipients of RP4-mediated conjugal transfer [11]. In most cases, this problem cannot be solved by electroporating the suicide donor replicon into the strain of interest, and therefore, other tightly controlled conditional replication systems have to be developed. Another general issue is the lack of equivalent mini-transposons for manipulations in Gram-positive bacteria. Although the mobility mechanism system of these transposons may work in genera, such as in *Bacillus*, *Listeria* or *Pneumoccocus* we are unaware of any successful attempt to use the standard mini-Tn*10*s or mini-Tn*5*s in these microorganisms.

5. Applications of mini-transposons in microbial ecology and biotechnology

5.1. Tagging with marker genes

The use of markers and bioreporters tailored for applications in the field [40] is in itself a separate aspect of Environmental Microbiology which has been the subject of a recent excellent review [41]. There is no real border between the two concepts and, depending on the specific construct, the same gene or genes can be used alternatively or simultaneously as markers or reporters. The antibiotic and non-antibiotic resistance genes initially engineered in mini-transposons can be used as simple markers, to differentiate a certain strain within a community. Instead of encoding selection determinants, other markers encode enzymes which act on chromogenic substrates, such as β-galactosidase, β-glucoronidase, alkaline phosphatase and catechol 2,3-dioxygenase.

The corresponding genes have been assembled in mini-transposons for an easy delivery into the strains of interest [8,42] and used extensively in projects involving the monitoring of a certain strain or a plasmid in the environment [41,43,44]. One interesting development of these traditional markers is the use of the β-glucosidase gene of *Pyrococcus furiosus* or *celB* [45]. This enzyme has basically the same activity on chromogenic substrates as β-galactosidase, but it is extremely heat-resistant. This allows the suppression of virtually all background activity in an environmental sample and the detection and measurement of this gene as either marker or reporter. For other applications, the utility of GFP as a luminescent tag became very evident soon after its publication in the literature, to the point of nearly displacing other optical markers such as *lux* [41] or *luc* [46,47]. A third type of marker/reporter relies on the physical properties of the gene product, the best example being the ice nucleation gene of *Pseudomonas syringae* encoded by the gene called alternatively *ice* or *ina* [48]. Although the notions behind the use of this gene as a reporter are extremely elegant, ice nucleation is not yet a widespread marker system because of difficulties in interpreting the data resulting from its utilisation. As mentioned above, other types of physical markers are those termed surface reporters [32]. These are based on the expression of a distinct epitope, for which a very specific monoclonal antibody exists, on the outermost envelope of the bacteria of interest. The protein best suited to this end is the LamB protein of *E. coli*, the receptor for the phage lambda. This outer membrane protein bears an outward facing, very permissive site around position 153 of the amino acid sequence. Genetic insertion of the A6 epitope of a coronavirus in such a site and its expression in *Pseudomonas putida* gave rise to cells which can not only be traced through immunofluorescence in the complex medium of an activated sludge [32], but also efficiently recovered from a bacterial mixture with the use of immunomagnetic beads (Fig. 4).

5.2. Gene fusions: monitoring transcriptional regulation in the environment

The information obtained on promoter activity in the Laboratory may have little to do with the actual

performance of the same promoters in an open, i.e. natural environment, where the number of variables increases and the final outcome of transcriptional activity may be determined by signals very different to those identified in vitro [21]. In addition, bacteria frequently enter a non-culturable state, which leaves useless most reporter systems whose assay requires the growth of the cells under study. A frequent application of mini-transposons has been the assembly of specialised reporter units to tackle this issue. Insertions of mini-Tn5s bearing promoterless reporter genes have been used to generate transcriptional fusions for identifying promoters responsive to environmental signals [49]. Alternatively, promoters already known have been assembled in front of adequate reporters in a mini-transposon and the whole unit placed into the chromosome for regulation studies [50,51]. Although the use of β-galactosidase for monitoring promoter activity has not yet been outmatched, a whole variety of genes encoding enzymatic, optical, immunological and physical markers (see below) have also been combined in the pUTs for studying specific promoters in response to external chemical or physical inputs.

Some of these new constructs have been applied to examine in situ the regulation of catabolic promoters of biodegradative pathways of *Pseudomonas*, e.g. those encoded by the TOL (toluene degradation) plasmid. Although the regulation of these promoters has been studied in the laboratory in great detail, it has not been possible so far to determine their performance in complex natural environments. This is an important issue, since understanding how promoters are regulated in situ may also be of essence if the regulated functions are to be used in bioremediation of polluted sites [21]. Various recently published articles have dealt with this issue through the construction of *Pseudomonas* strains inserted in their chromosome with monocopy fusions of the TOL promoters to optical (GFP) or immunological markers for in situ monitoring of gene expression in biofilms [50–52] or in activated sludge [25,32].

5.3. Metabolic engineering

Adaptation of bacteria to novel carbon sources is frequently accompanied by the acquisition or loss of single or multiple DNA segments which determine the corresponding metabolic functions. The sequence of molecular events which leads to the assembly of metabolic pathways for the degradation of xenobiotic compounds seems to have resulted from joining DNA segments recruited from pre-existing pathways [36,39]. The series of transposon vectors derived from Tn5 which permits the insertion and stable inheritance of heterologous DNA elements into the chromosome of target bacteria resemble to some extent the existing mechanisms of acquisition or loss of adaptation-related phenotypes. This is particularly true when the transposon vectors used permits, as discussed above, the addition of heterologous DNA segments devoid of any other phenotypic marker to the genome of the target bacterium. We have employed transposon-vectors with excisable selection markers [36] for the insertion of the reconstructed and fully functional *upper* TOL pathway into the chromosome of *P. putida*. The resulting strains convert toluene into benzoate and further metabolise this compound through the housekeeping *ortho*-ring cleavage pathway of the catechol intermediate [38]. Due to the loss of recombinant markers during the construction process, these GMOs are hardly different from their non-recombinant counterparts. That the *tnp* gene (encoding the Tn5 transposase) is not inherited in the resulting strain makes it extremely unlikely that the insertions can be moved again. In this respect, the new DNA segment(s) added to the genome of the recipient strain can be even more stable and predictable than many catabolic genes of natural *Pseudomonas* isolates, the DNA sequences of which used to be systematically subjected to rearrangements [53]. The use of mini-transposons is, therefore, optimal in metabolic engineering projects that require a high predictability of the biocatalyst. Examples of this kind include the construction of surfactant-producing *Pseudomonas* strains inserted with a gene cassette from *Rhodococcus* for desulfurisation of dibenzothiophene [54] as well as the production of root-colonizing, polychlorobiphenyl (PCB)-degrader recombinant *Pseudomonas* for rhizo-remediation of soil polluted with these chloroaromatic compounds [55].

5.4. In situ monitoring of gene transfer

Mini-transposon vectors have not only permitted

the construction of strains with biodegradative phenotypes, but also the design of bacteria used as live probes to detect and quantify in a non-disruptive manner otherwise intractable molecular events in the environment. One of the most elegant schemes in this respect has been the assembly of host-dependent conditional expression vectors which allow detection of DNA transfer in a complex community at the level of single cells. In one case [29], expression of a reporter gene (either visual or selectable on antibiotic plates) is achieved only if it moves from the donor into another specialised recipient which provides the functions required for expression of such a reporter. In a second instance, the marker gene is fully repressed in the donor strain, but becomes expressed to high levels as soon as it enters a different host [56]. The use of these elements in combination with optical reporters (e.g. GFP) and confocal laser microscopy is, at the moment, the most powerful methodology to examine bacterial behaviour and lifestyle in complex microbial consortia [50–52,57].

5.5. Engineering gene and strain-containment circuits

The notion of biological containment of GMOs was coined in the late 1980s to describe various genetic circuits which would make recombinant bacteria commit suicide when they are no longer needed in a given biotechnological application or when they escape accidentally a predetermined location [37,58,59]. Although various model systems engineered with plasmids proved to be effective in some cases [59], the full applicability of such circuits could not be realised before their combination with transposon vectors. Two types of containment systems have been developed depending on whether the GMO is required to die after fulfilling a certain function (such as biodegradation of an environmental pollutant) or whether inhibition of the lateral transfer of the recombinant genes is desired. In a well-developed instance, *Pseudomonas* strains degrading 4-ethyl benzoate are programmed to activate a killing function after this substrate has been consumed from the environment [60,61]. In another genetic design, recombinant genes are associated with a killing gene which is counteracted by an antidote gene in *trans* in the GMO [62,63]. However, the antidote gene is lost after transfer of the genes of interest into a new recipient, thus causing its death. This last approach has been instrumental in constructing fully contained *Pseudomonas* strains for biodegradation of PCBs in which horizontal transfer of the recombinant genes is well below any of the currently available methods for detection [64].

6. Conclusions

In spite of the various drawbacks intrinsic to the original design of the mini-Tn*10* and mini-Tn*5* transposon vectors, these molecular tools have not yet been outmatched as a sort of *genetic Swiss Army knife* for engineering Gram-negative bacteria destined for environmental release. Their main virtue is their ease for insertion of one or more segments of heterologous DNA in the chromosome of the strain of interest. If placed in a non-essential site of the genome, these inserted DNA segments remain stably inherited and produce no burden to the carrier strain. This allows a large number of combinations between markers, reporter genes, degradative pathways and toxic functions which are amenable to precise monitoring with a collection of increasingly sophisticated optical methods. At the same time, GMOs engineered with mini-transposons can be destined for environmental release as biocatalysts in bioremediation or as biological control agents in agriculture with a degree of predictability and biological containment which can hardly be found in natural bacterial isolates [37,58,61]. In fact, by exploiting natural mechanisms of insertion and excision of DNA fragments, these genetic tools diffuse the boundary between natural and recombinant bacteria and may provide a more rational basis to the still ongoing discussion on the use of GMOs in Biotechnology.

References

[1] Fält, I., Mills, D., Schweda, E., Timmis, K.N. and Lindberg, A.A. (1996) Construction of a recombinant *aroA* salmonellae stably producing the *Shigella dysenteriae* serotype 1 O-antigen and structural characterization of the *Salmonella/Shigella* hybrid LPS. Microb. Pathogen. 20, 11–30.

[2] Berg, D.E. and Mowe, M.M. (1989) Mobile DNA. American Society for Microbiology, Washington, DC.

[3] de Lorenzo, V. (1992) Genetic engineering strategies for environmental applications. Curr. Opin. Biotechnol. 3, 227–231.

[4] Herrero, M., de Lorenzo, V. and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. J. Bacteriol. 172, 6557–6567.

[5] Way, J.C., Davis, M.A., Morisato, D., Roberts, D.E. and Keckner, N. (1984) New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene 32, 369–379.

[6] Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. BioTechnology 1, 784–791.

[7] Miller, V. and Mekalanos, J. (1988) 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, 2575–2583.

[8] de Lorenzo, Herrero, M. and Timmis, K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative bacteria. J. Bacteriol. 172, 6568–6572.

[9] Tzchaschel, B., Klee, S., de Lorenzo, V., Timmis, K.N. and Guzmán, C. (1996) Towards a vaccine candidate against *Shigella dysenteriae 1* : expression of the Shiga toxin B subunit in an attenuated *Shigella flexneri aroD* carrier strain. Microb. Pathogen. 21, 277–288.

[10] Tzchaschel, B., Guzmán, C., Timmis, K.N. and de Lorenzo, V., 1996. An *Escherichia coli* hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like Toxin IIe B subunit by *Salmonella typhimurium aroA.* Nature Biotechnol. 14, 765–769.

[11] de Lorenzo, V. and Timmis, K. (1994) Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5 and Tn10-derived mini-transposons. Methods Enzymol. 235, 386–405.

[12] Hansen, L.H., Sorensen, S.J. and Jensen, L.B. (1997) Chromosomal insertion of the entire *Escherichia coli* lactose operon into two strains of *Pseudomonas* using a modified mini Tn5 delivery system. Gene 186, 167–173.

[13] Ramos, J.L., Díaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C. and Timmis, K.N. (1994) Behavior of bacteria designed for biodegradation. BioTechnology 12, 1349–1356.

[14] Sánchez-Romero, J.M. and de Lorenzo, V. (1998) Genetic engineering of non-pathogenic *Pseudomonas* strains as biocatalysts for industrial and environmental processes. In: Manual of Industrial Biotechnology, 2nd edn. (Davies, J. and Cohen, G., Eds.). American Association of Microbiology Press, Washington, DC, in press.

[15] Alexeyev, M., Shokolenko, I. and Croughan, T. (1995) New mini-Tn5 derivatives for insertion mutagenesis and genetic engineering in Gram-negative bacteria. Can. J. Microbiol. 41, 1053–1055.

[16] Wong, K. and McClelland, M. (1992) Dissection of the *Salmonella typhimurium* genome by use of a Tn5 derivative carrying rare restriction sites. J. Bacteriol. 174, 3807–3811.

[17] Hiller, B., Frey, B. and Schumann, W. (1994) Tn5 Map, a transposon for the rapid mapping of restriction sites in plasmids. FEMS Microbiol. Lett. 115, 151–156.

[18] de Lorenzo, V., Eltis, L., Kessler, B. and Timmis, K. (1993) Analysis of gene products of *Pseudomonas* with *lacIq/Ptrp-lac* plasmids and transposons conferring conditional phenotypes. Gene 123, 17–24.

[19] Pérez-Martín, J. and de Lorenzo, V. (1996) VTR expression cassettes for engineering conditional phenotypes in *Pseudomonas*: activity of the *Pu* promoter of the TOL plasmid under limiting concentrations of the XylR activator protein. Gene 172, 81–86.

[20] de Lorenzo, V., Fernández, S., Herrero, M. and Timmis, K. (1993) Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. Gene 130, 41–46.

[21] de Lorenzo, V. (1994) Designing microbial systems for gene expression in the field. Trends Biotechnol. 12, 365–371.

[22] de Lorenzo, V. (1994) Genetic strategies to engineer expression systems responsive to relevant environmental signals. In: Molecular Ecology of Rhizosphere Microorganisms (O'Gara, F., Dowling, D. and Boesten, B., Eds.), pp. 91–101. VCH, Weinheim.

[23] Herrero, M., de Lorenzo., V., Ensley, B. and Timmis, K.N. (1993) A T7 RNA polymerase-based system for the construction of *Pseudomonas* strains with phenotypes dependent on TOL-meta pathway effectors. Gene 134, 103–106.

[24] Eberl, L., Givskov, M., Poulsen, L.K. and Molin, S. (1997) Use of bioluminescence for monitoring the viability of individual *Pseudomonas putida* cells. FEMS Microbiol. Lett. 149, 133–140.

[25] Eberl, L., Schulze, R., Ammendola, A., Geisenberger, O., Erhart, R., Sternberg, C., Molin, S. and Amman, R. (1997) Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. FEMS Microbiol. Lett. 149, 77–83.

[26] Eberl, L., Ammendola, A., Geisenberger, O., Schulze, R., Givskov, M., Sternberg, C., Molin, S. and Schleifer, K.H. (1998) Use of green fluorescent protein for online, single cell detec-

tion of bacteria introduced into activated sludge microcosms. Biofilm 3, 1.

[27] Sternberg, C., Eberl, L., Poulsen, L.P. and Molin, S. (1997) Detection of bioluminescence from individual bacterial cells: a comparison of two different low-light imaging systems. J. Biolumin. Chemolumin. 12, 7–13.

[28] Burlage, R.S., Yang, Z.K. and Mehlhorn, T. (1996) A transposon for green fluorescent protein transcriptional fusions: application for bacterial transport experiments. Gene 173, 53–58.

[29] Christensen, B.B., Sternberg, C. and Molin, S. (1996) Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (Gfp) from *Aequora victoria* as a marker. Gene 173, 59–65.

[30] Suarez, A., Guttler, A., Stratz, M., Staendner, L.H., Timmis, K.N. and Guzman C. (1997) Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. Gene 196, 69–74.

[31] Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjørn, S.P., Givskov, M. and Molin, S. (1998) New unstable variants of green fluorescent protein (GFP) for studies of transient gene expression in bacteria. Appl. Environ. Microbiol., 64, 2240–2246.

[32] Cebolla, A., Guzmán, C. and de Lorenzo. V. (1996) Nondisruptive detection of activity of catabolic promoters of *Pseudomonas* with an antigenic surface reporter system. Appl. Environ. Microbiol. 62, 214–220.

[33] Fedi, S., Brazil, D., Dowling, D. and O'Gara, F. (1996) Construction of a modified mini-Tn5 *lacZY* non-antibiotic marker cassette: ecological evaluation of a *lacZY* marked *Pseudomonas* strain in the sugarbeet rhizosphere. FEMS Microbiol. Lett. 135, 251–257.

[34] Stalker, D.M., Hiatt, W.R. and Comai, L. (1985) A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. J. Biol. Chem. 260, 4724–4728.

[35] Sánchez-Romero, J.M., Díaz, R. and de Lorenzo, V. (1998) Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas*, Appl. Env. Microbiol., in press.

[36] Kristensen, C.S., Eberl, L., Sanchez-Romero, A., Givskov, M., Molin, S. and de Lorenzo, V. (1995) Site-specific deletions of chromosomally located segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. 177, 52–58.

[37] Ramos, C., Diaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, J.L. and Timmis, K.N. (1994) The behavior of bacteria designed for biodegradation. BioTechnology 12, 1349–1356.

[38] Panke, S., Sánchez-Romero, J.M. and de Lorenzo, V. (1998) Engineering *quasi*-natural *Pseudomonas putida* strains for metabolism of toluene through an *ortho*-cleavage degradation pathway. Appl. Environ. Microbiol. 64, 748–751.

[39] Hallet, B. and Sherrat, D.J. (1997) Transposition and site-specific recombination: adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. FEMS Microbiol. Rev. 21, 157–178.

[40] Jansson, J.K. (1995) Tracking genetically engineered microorganisms in nature. Curr. Opin. Biotechnol. 6, 275–283.

[41] Jansson, J.K. and De Bruijn, F.J. (1998) Biomarkers and bioreporters to track microbes and monitor their gene expression. In: Manual of Industrial Biotechnology, 2nd edn. (Davies, J. and Cohen, G., Eds.). American Association of Microbiology Press, Washington, DC, in press.

[42] Wilson, K., Sessitsch, A., Corbo, J., Giller, K., Akkermans, A. and Jefferson, R.A. (1995) β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology 141, 1691–1705.

[43] Top, E.M., Van Daele, P., De Saeyer, N. and Forney, L.J. (1998) Enhancement of 2,4 dichlorophenoxyacetic acid (2,4 D) degradation in soil by dissemination of catabolic plasmids. Antonie van Leeuwenhoek 73, 87–94.

[44] Top, E.M., Malstseva, O. and Forney, L.J. (1996) Capture of a catabolic plasmid that encodes 2,4-dichlorophenoxyaceticacid-α-ketoglutaric acid dioxygenase (TfdA) by genetic complementation. Appl. Environ. Microbiol. 62, 1470–1476.

[45] Sessitsch, A., Wilson, K.J., Akkermans, A. and De Vos, W.M. (1996) Simultaneous detection of different *Rhizobium* strains marked with either the *Escherichia coli gusA* gene or the *Pyrococcus furiosus celB* gene. Appl. Environ. Microbiol. 62, 4191–4194.

[46] Cebolla, A., Ruiz-Berraquero, F. and Palomares, A. (1993) Stable tagging of *Rhizobium meliloti* with the firefly luciferase gene for environmental monitoring. Appl. Environ. Microbiol. 59, 2511–2519.

[47] Cebolla, A., Vázquez, M.E. and Palomares, A. (1995) Expression vectors for the use of eukaryotic luciferases as bacterial markers with different colors of luminescence. Appl. Environ. Microbiol. 61, 660–668.

[48] Lindow, S.E., Lahue, E., Govindarajan, A.G., Panopoulos, N.J. and Gies, D. (1989) Localization of ice nucleation activity and the *iceC* gene product in *Pseudomonas syringae* and *Escherichia coli*. Mol. Plant-Microbe Interact. 2, 262–272.

[49] Kim, Y., Watrud, L.S. and Matin, A. (1995) A carbon starvation survival gene of *Pseudomonas putida* is regulated by σ^{54}. J. Bacteriol. 177, 1850–1859.

[50] Poulsen, L.K., Dalton, H.D., Angles, M.L., Marshall, K.C., Molin, S. and Goodman, A.E. (1997) Simultaneous determination of gene expression and bacterial identity in single cells in defined mixtures of pure cultures. Appl. Environ. Microbiol. 63, 3698–3702.

[51] Møller, S., Sternberg, C., Andersen, J.B., Christensen, B.B. and Molin, S. (1998) In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. Appl. Environ. Microbiol. 64, 721–732.

[52] Christensen, B.B., Sternberg, C., Andersen, J.B., Eberl, L., Møller, S., Givskov, M. and Molin, S. (1998) Establishment of new genetic traits in a microbial biofilm community. Appl. Environ. Microbiol. 64, 2247–2255.

[53] van der Meer, J.R., de Vos, W.M., Harayama, S. and Zehnder, A.J.B. (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56, 677–694.

[54] Gallardo, M.E., Fernández, A., de Lorenzo, V., García, J.L.

and Díaz, E. (1997) Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. J. Bacteriol. 179, 7156–7160.

[55] Brazil, D., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D. and O'Gara, F. (1995) Construction of a rhizosphere Pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. Appl. Environ. Microbiol. 61, 1946–1952.

[56] Jaenecke, S., de Lorenzo, V., Timmis, K.N. and Díaz, E. (1996) A stringently-controlled expression system to analyse lateral gene transfer between bacteria. Mol. Microbiol. 21, 293–300.

[57] Normander, B., Christensen, B.B., Molin, S. and Kroer, N. (1998) The effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of bush bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 64, 1902–1909.

[58] Ramos, J.L., Andersson, P., Jensen, L.B., Ramos, C., Ronchel, M.C., Diaz, E.D., Timmis, K.N. and Molin, S. (1995) Suicide microbes on the loose. BioTechnology 13, 35–37.

[59] Molin, S., Klemm, P., Poulsen, L.K., Biehl, H., Gerdes K. and Andersson, P. (1987) Conditional suicide system for containment of bacteria and plasmids. BioTechnology 5, 1315–1318.

[60] Jensen, L.B., Ramos, J.L., Kaneva, Z. and Molin, S. (1993) A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli gef* gene. Appl. Env. Microbiol. 59, 3713–3717.

[61] Ronchel, M.C., Ramos, C., Jensen, L.B., Molin, S. and Ramos, J.L. (1995) Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. Appl. Environ. Microbiol. 61, 2990–2994.

[62] Díaz, E., Munthali, M., de Lorenzo, V. and Timmis, K.N. (1994) Universal barrier to lateral spread of specific genes among microorganisms. Mol. Microbiol. 13, 855–861.

[63] Munthali, M.T., Timmis, K.N. and Diaz, E. (1996) Use of colicin E3 for biological containment of microorganisms. Appl. Env. Microbiol. 62, 1805–1807.

[64] Munthali, M.T., Timmis, K.N. and Diaz, E. (1996) Restricting the dispersal of recombinant DNA: design of a contained biological catalyst. Nature Biotechnol. 14, 189–191.

[65] Simons, R.W., Houman, F. and Kleckner, N. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene 53, 85–96.