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A new approach for containment of microorganisms: Dual control of streptavidin expression by antisense RNA and the T7 transcription system

(heterologous gene expression/bioremediation/*Pseudomonas putida*)

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ABSTRACT **The use of microorganisms in the open environment would be of less concern if they were endowed with programmed self-destruction mechanisms. Here, we propose a new genetic design to increase the effectiveness of cell suicide systems. It ensures very tight control of the derepression of cell death by the combination of the bacteriophage T7 RNA polymerase-lysozyme system and an inducible synthesis of antisense RNA and the *Escherichia coli* LacI repressor. Functionality of this regulatory concept was tested by applying it to containment of Gram-negative bacteria, based on the conditional expression of the lethal *Streptomyces avidinii* streptavidin gene. Toxicity of streptavidin is derived from its exceptionally high binding affinity for an essential prosthetic group, D-biotin. The entire construct was designed to allow the soil bacterium *Pseudomonas putida* to survive only in the presence of aromatic hydrocarbons and their derivatives which it can degrade. Under favorable growth conditions, clones escaping killing appeared at frequencies of only 10^{-7}–10^{-8} per cell per generation. The general requirement for biotin through the living world should make streptavidin-based conditional lethal designs applicable to a broad range of containment strategies.**

Although genetically engineered microorganisms (GEMs) offer great benefits in environmental applications, it is difficult to predict their behavior in natural ecosystems or how recombinant DNA can spread among indigenous bacterial populations. Potential risk associated with deliberate or unintentional release of GEMs into the open environment can be minimized by the use of debilitated strains. An alternative, and perhaps more appropriate, approach is the introduction of conditional or stochastic maintenance functions into GEMs (1–3). In such a case, the viability of GEMs depends on the expression of an essential gene or on the repression of a lethal gene controlled by a regulatory promoter responding to changes in the chemical or physical constitution of the environment, or by a promoter undergoing recombinational switches. However, the effectiveness of suicide systems is limited by relatively high frequency of their mutational inactivation, resulting in positive selection of uncontained clones. Utilizing several independent killing functions and tightening the regulation of their expression are potential solutions to this problem.

In this report, we address the need both for new killing genes and improved strategies to control their expression. Described here is a potentially universal conditional lethal system based on the tightly regulated derepression of the streptavidin gene (*stv*) (4) from the actinobacterium *Streptomyces avidinii*. It targets the metabolism of one-carbon units at the oxidation level of carbon dioxide by depleting an essential prosthetic group, D-biotin (vitamin H); this should complement cell suicide systems for which direct targets are cell membranes and walls, or nucleic acids. Any incompletely repressed expression of the *stv* gene was eliminated at the level of its transcription, targeting directly the RNA polymerase, as well as at the level of its translation by antisense mRNA. This novel regulatory strategy for containment of GEMs is apparently responsible for the excellent performance of the whole design.

The entire system was tested in the Gram-negative soil rod *Pseudomonas putida* potentially useful for bioremediation of areas polluted with aromatic hydrocarbon-based organic solvents and petroleum. A containment system for pseudomonads is of particular importance because their nutritional versatility for low molecular weight organic compounds and fast growth rates allow them to rapidly colonize a wide range of habitats and predominate in soil or water microflora. Coupling this system to regulatory elements derived from the *P. putida* TOL catabolic plasmid (5) conditioned survival of bacteria only in the presence of an aromatic carboxylic acid that they can degrade.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. Subcloning was performed by using an *Escherichia coli* K-12 strain XL1-Blue MRF′ (*lacI*^{q} and Tet^r on F′) (Stratagene). Suicide constructs were tested in *P. putida* mt-2 strain KT2440 {*hsdR1*} (6). DNA used was a replicative form of a bacteriophage M13 clone, mGP1–2 (7), and plasmids pKK223–3 (Amp^r) (8) (Pharmacia), pCC102 (Kan^r) (9), pGEM-*luc* (Amp^r) (Promega), pLysE (Cml^r) (7), pRO1614 (Amp^r, Tet^r) (10), pTSA-13 (Amp^r) (11), pUC19 (Amp^r), and pVLT33 (Kan^r) (12).

Bacteria were grown aerobically in Luria–Bertani (LB) medium at 30°C, unless otherwise stated. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml (*E. coli*) or 800 μg/ml (*P. putida*; optimized for this particular isolate); kanamycin, 25 μg/ml (*E. coli*) or 75 μg/ml (*P. putida*); chloramphenicol, 50 μg/ml (*E. coli*); and tetracycline, 10 μg/ml (*E. coli*). Isopropyl β-D-thiogalactopyranoside (IPTG) and *m*-methylbenzoate (3MB) were used at concentrations of 1 mM and 0.2 mM, respectively.

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Abbreviations: GEM, genetically engineered microorganism; IPTG, isopropyl β-D-thiogalactopyranoside; 3MB, *m*-methylbenzoate; LB, Luria–Bertani.

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Recombinant DNA Techniques. DNA manipulations were carried out by standard procedures (13). *P. putida* was transformed by a RbCl method (14) or by electroporation (Gene Pulser apparatus; Bio-Rad).

Construction of Each Element of the System. The rationale of the genetic design is given in *Results*. The *stv* gene, the P_{tac} promoter, and the bacteriophage T7 transcription system (i.e., the *ϕ10* promoter, and the RNA polymerase and lysozyme genes), were placed on two compatible plasmids, pCC102 (an RSF1010 derivative bearing the *xylS2* gene and the P_m::*lacI* fusion) and pRO1614 (pMB1 and pRO1600 replicon), as shown in Fig. 2*A*. Fragments of pUC18 and pGEM-*luc* plasmids, bearing convenient cloning sites, were inserted into pCC102, downstream of the *lacI* gene, to facilitate subcloning of the *ϕ10*::*stv* fusion and subsequent DNA manipulations. The *stv* gene used here encodes a core streptavidin consisting of amino acid residues 16–133 of the mature streptavidin (11). This protein has a higher binding stoichiometry to biotinylated macromolecules than natural core streptavidin. A 1.3-kb fragment of the *luc* gene, derived from pGEM-*luc*, was inserted into pRO1614 as a spacer to reduce expression of the T7 lysozyme gene. This also makes the construct comparable to the corresponding region of pRO-*llp*, in which the lysozyme gene is separated from the P_{tet} promoter by the 1-kb *lacI* gene (*lacI* inserted between the *Eco*RI and *Bam*HI sites of the Tet^r locus).

For construction of pCC-*s*04 carrying the *lac* operator (O_{lac}) immediately upstream of *ϕ10*::*stv*, a *Bgl*II–*Hin*dIII fragment of pTSA-13 carrying *ϕ10*::*stv* was first inserted into the *Sma*I–*Hin*dIII site of pKK223-3. Then, a *Bam*HI fragment carrying O_{lac}*ϕ10*::*stv* was cloned into the *Bam*HI site of pVLT33. Finally, a *Kpn*I–*Sal*I fragment of the resulting plasmid was placed in the *Kpn*I–*Sal*I site of pCC-*s*05.

Fluctuation Tests. Frequency of the appearance of clones resistant to the induction of cell death was estimated following the Luria–Delbrück approach as described (15).

RESULTS

Design of a Conditionally Lethal Construct. The killing function in our system is based on the almost irreversible binding ($K_d \approx 10^{-15}$ M) of D-biotin by streptavidin (16), a tetrameric protein produced by *S. avidinii*. Cell death results from depletion of free biotin and direct inhibition of biotin-dependent carboxylases, decarboxylases, and transcarboxylases (17). Inactivation of these enzymes blocks the first committed step of fatty acid biosynthesis and affects gluconeogenesis, amino acid metabolism, replenishment of the Krebs cycle, and substrate uptake by some anaerobes.

A simple strategy for using streptavidin as a suicide factor would be to place its gene directly under the control of a promoter negatively regulated by a repressor protein synthesized in response to an environmental signal. The most serious drawback of such a design is incomplete repression (leakiness) of regulatory promoters. To achieve tighter control of the induction of a lethal phenotype, an additional regulatory circuits, involving a heterologous RNA polymerase and its inhibitor, were generated by coupling expression of the *stv* gene to the bacteriophage T7 transcription system (7) (Fig. 1). In this case, the *stv* gene was transcribed from the T7 gene *10* promoter (*ϕ10*) by T7 RNA polymerase. The T7 gene *1*, encoding RNA polymerase, was fused to the *E. coli* hybrid *trp-lac* (*tac*) promoter, negatively regulated by the LacI repressor. The *E. coli* LacI-O_{lac} system has been shown to be active in a broad range of microorganisms, including *P. putida* (18) and yeasts (19). The leakiness of the P_{tac} promoter was compensated by an inhibitor of T7 RNA polymerase, T7 lysozyme (20). The lysozyme, in addition to its muramidase activity, binds to T7 RNA polymerase and blocks its transcription activity. The T7 gene *3.5*, encoding lysozyme, was constitutively transcribed from the P_{tet} promoter. The *lacI* gene was transcribed from the P_m promoter induced by XylS protein-aromatic carboxylic acid complexes. Both the P_m promoter and *xylS* regulatory gene (used *xylS2* allele encodes $XylS^{thr45}$ with altered effector specificity and increased affinity for benzoates) are derived from the *P. putida* TOL plasmid where they control expression of genes clustered in the *meta*-cleavage pathway operon (5). Streptavidin should be synthesized upon inactivation of LacI with IPTG or by interception of LacI synthesis in response to depletion of a benzoic acid effector of the XylS protein, such as 3MB. Positive effectors of the XylS protein can be taken up by *P. putida* or produced within the cell by oxidation of toluene, *m*- and *p*-xylenes, and their derivatives, through the TOL-encoded upper catabolic pathway (3MB from *m*-xylene) (5). Note that the bacteriophage T7 system, in the configuration described above, by itself causes stress to the cell when induced. Transcription from the strong *ϕ10* and P_{tac} promoters, and overexpression of T7 RNA polymerase, for example, engage a large pool of ribonucleotides, and subsequently amino acyl tRNAs, and ribosomes.

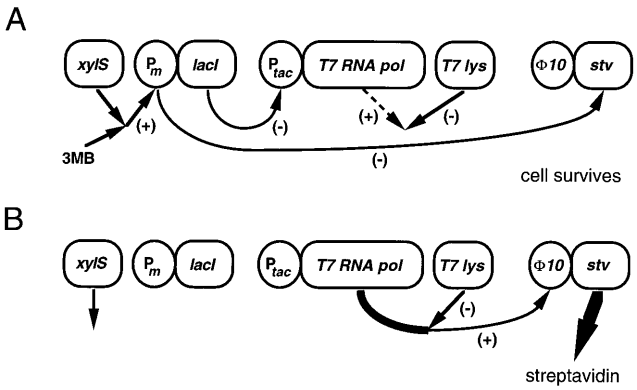

FIG. 1. Scheme of a tightly regulated biological containment system to control survival of bacteria by availability of 3MB or other hydrocarbon effectors of the XylS protein. (*A*) Survival. (*B*) Induction of a lethal phenotype.

An additional level of regulation of *stv* gene expression was achieved by placing the *ϕ10*::*stv* fusion immediately downstream of the *lacI* gene, but in the opposite orientation (Figs. 1 and 2*B*). This should further decrease uninduced expression of the *stv* gene by generating an antisense RNA (21) complementary to the *stv* transcript. On the other hand, RNA synthesized from *ϕ10* promoter upon induction of suicide should similarly block the remaining *lacI* mRNA and improve the kinetics of bacterial culture decay.

The T7 lysozyme and T7 RNA polymerase genes are also oppositely oriented. However, in this case, the level of accumulating countertranscript is likely to be lower because of the distance between P_{tet} and P_{tac} promoters (≈6 kb).

Induction of the Lethal Phenotype. The entire suicide construct was prepared as a pCC-*s*05 (*xylS2*, *lacI*, and *stv* genes)/pRO-*ilp* (T7 RNA polymerase and lysozyme genes) broad host-range two-plasmid system (Fig. 2). Copy numbers of pCC-*s*05 and pRO-*ilp* in *P. putida* were estimated to be 1–3 and 10–20, respectively. Transfer of *P. putida* (pCC-*s*05, pRO-*ilp*) from a 3MB-containing medium to a 3MB-free medium resulted in inhibition of the culture growth within 3 h at 30°C (Fig. 3*A*). Replacement of the *stv* gene with a truncated gene encoding only the N-terminal half of the protein or the use of pCC102 plasmid lacking the *ϕ10*::*stv* fusion, instead of pCC-*s*05, caused almost no response of *P. putida* to the absence of 3MB inducer (data not shown).

The efficiency of host cell killing by intracellularly synthesized streptavidin was quantitated by counting viable cells before and after the removal of 3MB (Fig. 3*B*). Bacterial samples were periodically collected and incubated for a week

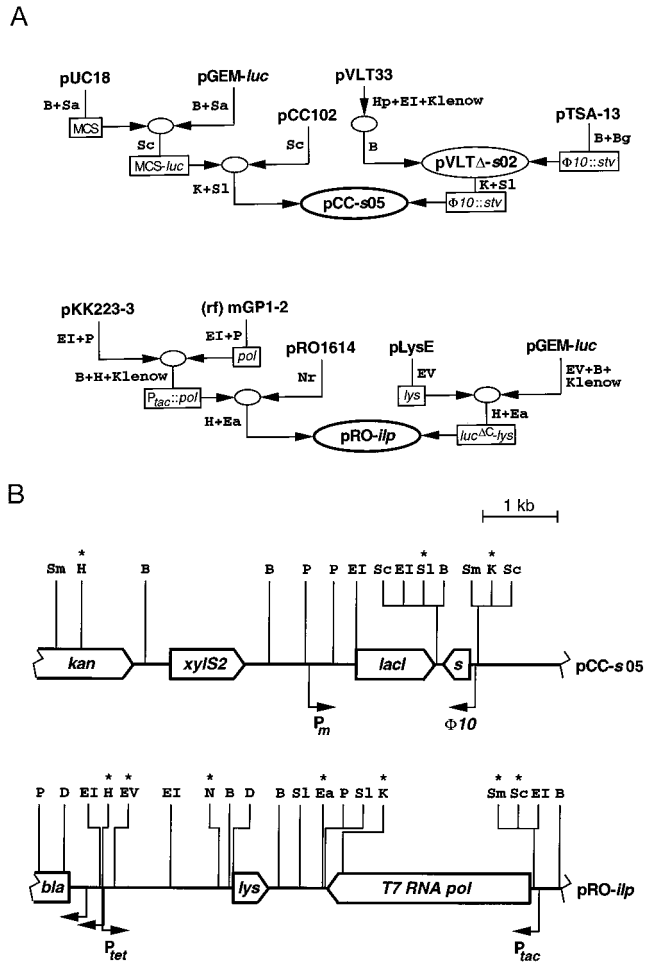

FIG. 2. Construction of plasmids pCC-*s*05 and pRO-*ilp* (*A*), and physical maps of their killing and regulatory elements (*B*). *stv* or *s*, Streptavidin gene; *T7 RNA pol* or *pol*, T7 RNA polymerase gene (T7 gene *1*); *lys*, T7 lysozyme gene (T7 gene *3.5*); *kan*, kanamycin-resistance gene; $luc^{\Delta C}$, a truncated *luc* gene encoding an N-terminal fragment of luciferase; MCS, a fragment of pUC18 containing part of the multiple cloning site. B, *Bam*HI; Bg, *Bgl*I; D, *Dra*I; Ea, *Eag*I; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nhe*I; Nr, *Nru*I; P, *Pst*I; Sa, *Sca*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I. Unique cleavage sites are marked with asterisks.

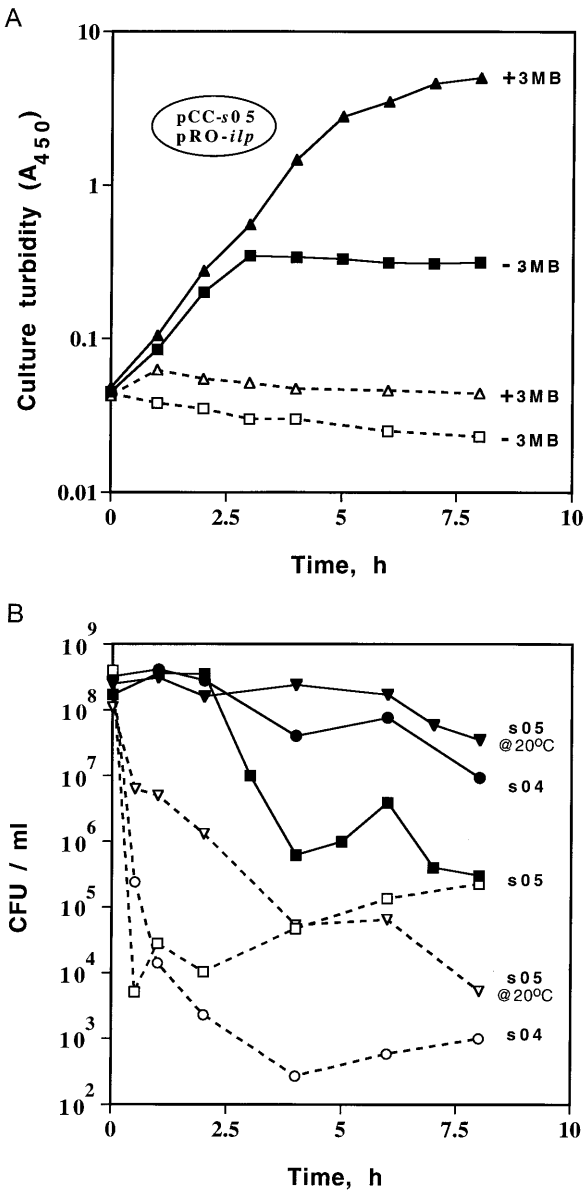

FIG. 3. Kinetics of the induced suicide of *P. putida* cultures. Bacteria were grown to a mid-exponential growth phase in LB medium containing 3MB, ampicillin, and kanamycin. After washing with LB, cells were diluted 100-fold with LB supplemented with antibiotics with or without 3MB (*A*), or resuspended in the same medium without 3MB (*B*), and further incubated. Broken lines with open symbols refer to the additional presence of IPTG in LB. Plasmid combinations: s04, pCC-*s*04/pRO-*ilp*; s05, pCC-*s*05/pRO-*ilp*. CFU, colony forming unit. Each data point is the mean of two or three independent experiments.

on agar plates supplemented with 3MB, 50 μg/ml biotin, and appropriate antibiotics. At 4–8 h after induction of the *stv* gene expression by the removal of 3MB, up to 99.9% of the *P. putida* cells did not renew growth even after prolonged incubation in the presence of biotin. Longer incubation times were needed to contain the culture at lower temperatures. For example, 8–9 h were required to reduce the bacterial population by 90% at 20°C. As expected, removal of 3MB, together with the addition of IPTG, induced faster and more efficient cell death.

Replacement of pCC-*s*05 with pCC-*s*04 containing the O_{lac} (with P_{tac}) 41 bp upstream of *ϕ10*, at the edge of the DNA sequence covered by a promoter-bound T7 RNA polymerase, resulted in remarkably slower killing upon removal of 3MB. However, in the additional presence of IPTG it resulted in killing of as much as 6 orders of magnitude of the initial bacterial population in 4 h. This represents one of the most rapid and efficient eliminations of a bacterial culture reported. Derepression of P_{tac} preceding *ϕ10* on pCC-*s*04 by removal of 3MB did not induce a lethal level of the *stv* gene expression by bacterial RNA polymerase, presumably because of the presence of a putative transcription terminator/attenuator immediately downstream of *ϕ10* and residual antisense expression in the absence of 3MB. The need for the T7 lysozyme also in *P. putida* with the O_{lac}*ϕ10* fusion on pCC-*s*04 was verified by removing the lysozyme gene from pRO-*ilp* (cutting the plasmid with *Sph*I and religating). Although the resulting *P. putida* (pCC-*s*04, pRO-*ip*) was virtually contained upon removal of 3MB, the cell population grew very slowly, and the accumulation of mutants was significantly higher.

Rates of escape of *P. putida* from killing were estimated by fluctuation tests. Table 1 compares bacteria carrying plasmid constructs (*i*) producing different uninduced basal levels of active T7 RNA polymerase—i.e., with and without T7 lysozyme (pCC-*s*04/pRO-*ilp* or pCC-*s*04/pRO-*ip*), (*ii*) with or without direct LacI-O_{lac}-dependent accessibility of the *ϕ10* promoter for RNA polymerase (pCC-*s*05/pRO-*ilp* or pCC-*s*04/pRO-*ilp*), and (*iii*) with or without countertranscript protection against basal expression of the *stv* gene (pCC-*s*05/

Table 1. Survival of *P. putida*, carrying different constructs for protection against uninduced expression of the *stv* gene, upon induction of suicide

Plasmid combination	Surroundings of the *stv* gene	Survival per cell per generation
pVLTΔ-*s*02, pRO-*llp*	$T \overset{kan}{\leftarrow} T \overset{stv}{\leftarrow} \phi 10$	10^{-4}–10^{-5}
pCC-*s*05, pRO-*ilp*	$P_m \overset{lacI}{\rightarrow} \overset{stv}{\leftarrow} \phi 10$	10^{-6}–10^{-7}
pCC-*s*04, pRO-*ilp*	$P_m \overset{lacI}{\rightarrow} \overset{stv}{\leftarrow} \phi 10, O_{lac}$	10^{-7}–10^{-8}
pCC-*s*04, pRO-*ip*	$P_m \overset{lacI}{\rightarrow} \overset{stv}{\leftarrow} \phi 10, O_{lac}$	10^{-5}–10^{-6}

Each arrow indicates the direction of transcription. T, bacterial transcription terminator; O_{lac}, *lac* operator.

pRO-*ilp* and pVLTΔ-*s*02/pRO-*llp*). The construction of pVLTΔ-*s*02 is shown in Fig. 2*A*. pRO-*llp* differs from pRO-*ilp* by having the pCC102-derived *lacI* gene instead of a 1.3-kb *luc* DNA spacer. The level of accumulation of killing-resistant clones in *P. putida* with the pCC-*s*05/pRO-*ilp* plasmid combination was about two orders of magnitude lower than in the construct without antisense expression, although pCC- and pVLT-based hybrid plasmids (both RSF1010 derivatives) are not completely isogenic. For the pCC-*s*04/pRO-*ilp* combination, which has the O_{lac} also next to $\phi 10$, killing-resistant mutants appeared with the lowest frequency. The absence of T7 lysozyme within a cell increased the level of mutation by two orders of magnitude.

Restriction nuclease cleavage analysis of plasmids from 20 killing-resistant clones showed, in most cases, changes in DNA digestion patterns involving the *stv* and T7 RNA polymerase gene regions (data not shown).

DISCUSSION

The conditional lethal system presented here consists of the streptavidin gene as a key suicide element. The biotin-binding ability of streptavidin in *P. putida* is notable because it requires not only proper peptide folding but also formation of the correct tetrameric structure. So far, there are only several genes that have been proven to be bactericidal upon expression in the members of genus *Pseudomonas.* These are genes encoding cell membrane destabilizing peptides Hok (*E. coli* plasmid R1) (22) and Gef (*E. coli*) (23), lysis genes of bacteriophages λ and φX174 (24), and the *colE3* gene encoding an RNase (*E. coli*) (25). It is also worth mentioning that the T7 lysozyme maintains its T7 RNA polymerase inhibition ability in a heterologous *P. putida* system.

The rate of inactivation, a basic determinant of the efficiency of suicide designs, in our *stv*-based system is 10^{-7}–10^{-8} per cell per generation. This value is lower than those already reported for constructs based on single toxic functions (10^{-2}–10^{-6}), even in *E. coli*, which is easier to contain (9, 23, 26, 27). It apparently reflects the very low basal level of streptavidin in cells. Despite the fact that the coupled T7 transcription/LacI-O_{lac} system is less leaky than the *lac* system alone (Table 1), an additional explanation of the better protection of cells against uninduced expression of the *stv* gene is synthesis of the antisense RNA originating from the P_m promoter. The lack of such protection, especially without a transcription terminator (e.g., LacI-O_{lac} complex) upstream of $\phi 10$ as in pVLTΔ-*s*02, resulted in a remarkably higher mutation frequency of 10^{-4}–10^{-5} per cell per generation. The LacI-O_{lac} complex formed upstream of the $\phi 10$ in pCC-*s*04 apparently interferes also with binding of bacteriophage RNA polymerase to the $\phi 10$ promoter (slower induction of the *stv* gene expression upon removal of 3MB) and further decreases the frequency of appearance of killing-resistant clones by reducing uninduced transcription of the *stv* gene. Preliminary data on the killing of *E. coli* by IPTG-induced expression of the *stv* gene suggest that, as expected, this system should be effective also in enteric bacteria.

A temperature shift from 30°C to 20°C alters the kinetics of the culture decay. This effect was more pronounced in a bacterial population depleted of 3MB in the absence of IPTG, indicating a contribution of both weaker interaction of T7 RNA polymerase with $\phi 10$ and the longer lifetime of the LacI repressor and/or its mRNA within a cell. Continuing decline of the culture after the addition of IPTG indicates a lower rate of mutational inactivation of the construct at 20°C than at 30°C.

The suicide design was tested under rich nutrient conditions—i.e., in LB medium containing some biotin. The growth conditions were favorable for cell survival, since killing occurs by depletion of biotin. In fact, slightly higher levels of IPTG-induced cell death were observed in minimal M9 medium (not shown). Under mostly starving conditions in, for instance, soil or seawater, this system may perform even more effectively. A *Sma*I fragment of pCC-*s*05 containing *xylS2*, *lacI*, and *stv* genes (Fig. 2*B*) has been already stably integrated into *P. putida* chromosome via mini-Tn*5*-mediated transposition (data not shown). Other known killing genes will be placed under control of $\phi 10$ promoter and integrated into the chromosome of *P. putida* {*hsdR1 stv lacI xylS2* Kan^r} to create multiple back up systems following incorporation of the T7 RNA polymerase-lysozyme regulatory module. Alternatively, stability of pRO-*ilp* upon induction of suicide in the absence of selection for plasmid maintenance can be achieved, for instance, by inserting into it the *parB* (*hok*/*sok*) locus of R1.

To summarize, additional regulatory circuits arranged to reduce the basal level of the expression of killing gene can remarkably increase the effectiveness of cell suicide machinery. Because of the general demand of biotin as a carboxyl carrier in the living world, the *stv* gene can serve as a universal cassette for programmed cell death. The *stv*-based system in combination with, for example, biotinylated solid supports and biotinylated fluorescent probes could also allow very sensitive monitoring of the presence of recombinant microorganisms.

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