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A Substrate-Dependent Biological Containment System for *Pseudomonas putida* Based on the *Escherichia coli gef* Gene

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A model substrate-dependent suicide system to biologically contain *Pseudomonas putida* KT2440 is reported. The system consists of two elements. One element carries a fusion between a synthetic *lac* promoter ($P_{A1-04/03}$) and the *gef* gene, which encodes a killing function. This element is contained within a transposaseless mini-Tn*5* transposon so that it can be integrated at random locations on the *Pseudomonas* chromosome. The second element, harbored by plasmid pCC102, is designed to control the first and bears a fusion between the promoter of the *P. putida* TOL plasmid-encoded *meta*-cleavage pathway operon (Pm) and the *lacI* gene, encoding the Lac repressor, plus *xylS2*, coding for a positive regulator of Pm. In liquid culture under optimal growth conditions and in sterile and nonsterile soil microcosms, *P. putida* KT2440(pWWO) bearing the containment system behaves as designed. In the presence of a XylS effector, such as *m*-methylbenzoate, the LacI protein is synthesized, preventing the expression of the killing function. In the absence of effectors, expression of the $P_{A1-04/03}$::*gef* cassette is no longer prevented and a high rate of cell killing is observed. Fluctuation test analyses revealed that mutants resistant to cell killing arise at a frequency of around 10^{-5} to 10^{-6} per cell per generation. Mutations are linked to the killing element rather than to the regulatory one. In bacteria bearing two copies of the killing cassette, the rate of appearance of mutants resistant to killing decreased to as low as 10^{-8} per cell per generation.

A large number of xenobiotics have been introduced into the environment in recent years. Bioremediation is an area of increasing interest for decontamination of polluted areas; such treatments involve, among other strategies, the release of large numbers of wild-type or recombinant microorganisms to decontaminate polluted soils, water, and sediments.

The TOL plasmid pWWO of *Pseudomonas putida* codes for a *meta*-cleavage pathway for the catabolism of benzoate and certain alkylbenzoates to Krebs cycle intermediates (2). Transcription of the *meta* operon is initiated at the Pm promoter and is positively regulated by the *xylS* gene product in the presence of effectors, e.g., *m*-methylbenzoate (22). The TOL *meta*-cleavage pathway has been used as a model system for the expansion of the range of aromatic carboxylic acids which a bacterium can degrade. The recombinant TOL plasmid pWWO-EB62 allows a host bacterium to grow on *p*-ethylbenzoate. It was generated by introducing two successive mutations in TOL plasmid pWWO, namely, a point mutation in the *xylS* gene regulator, that enables the mutant regulator XylS2 (Arg-45 → Thr) to recognize *p*-ethylbenzoate as an effector, in contrast to wild-type XylS; and another mutation in the *xylE* gene that renders a catechol 2,3-dioxygenase resistant to inactivation by *p*-ethylcatechol (23). On the other hand, the TOL *xylS* and *xylXYZ* genes have been used to expand the range of haloaromatics degraded by *Pseudomonas* sp. strain B13 (6, 12, 24, 25).

To minimize the risk of spreading of genetically modified microorganisms and/or recombinant DNA, active biological containment system based on the use of genes encoding killing proteins, e.g., the *gef* family, were developed (3, 5, 8, 9, 11, 14–17, 26). In a previous publication (5) we demonstrated how the regulatory gene expression system of the *meta*-pathway of the TOL plasmid could be combined with the *gef* gene in such a way that killing became a consequence of the absence of the substrate (and inducer) 3-methyl benzoate; the design was made and tested in *Escherichia coli*. Here we describe the efficacy of a modified version of this system in *P. putida* KT2440, opening up the possibility of transfer to a broad range of gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this work are listed in Table 1. All strains were grown at 30°C. *E. coli* strains were grown in LB, and *Pseudomonas* strains were grown in M9 minimal medium with either 0.5% (wt/vol) glucose or 5 mM *m*-methylbenzoate as the sole carbon source and supplemented with the A9 micronutrient solution previously described by Abril et al. (1). Appropriate antibiotics were added as required to select for the presence of plasmids and for integration of transposaseless minitransposons. Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 25; tetracycline, 10. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 2 mM. Solid media were as above, except that they were supplemented with 1.5% (wt/vol) agar.

Construction of the killing cassettes. A series of killing cassettes were constructed on the basis of the transposaseless mini-Tn*5* system described by Herrero et al. (10). Plasmid pLBJ60, a derivative of pUC18Not (7, 10) containing a fusion of a synthetic *lac* promoter, $P_{A1-04/03}$ (4), to the *E. coli gef* gene (17–19) (encoding the toxic Gef protein) and $lacI^{q1}$ (encoding the Lac repressor to control expression from the synthetic promoter), was digested with *Not*I, and a 2.2-kb fragment containing the above-described region was

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
P. putida		
KT2440	Wild type	K. N. Timmis
LBJ415	Tc^r, KT2440(pWWO)::$P_{A1-04/03}$[a]::*gef*, *lacI*^{q1}	This work
LBJ433	Km^r, Tc^r, *trp*, KT2440(pWWO)::$P_{A1-04/03}$::*gef lacI*^{q1}(Tc)::$P_{A1-04/03}$::*gef*(Km)	This work
KT2440(pCC102, pWWO)	Km^r	This work
LBJ466	Km^r, Tc^r, KT2440(pWWO, pCC102)::$P_{A1-04.03}$::*gef*	This work
LBJ488	Km^r, Tc^r, KT2440(pWWO, pCC102)::mini Tn*5*Tc	This work
E. coli		
HB101	*supE44 hsdS20* (r_B^- r_M^-) *recA56 galK2 galT22 metB1*	DTH collection[b]
MT102	F^-, *thi araD139 ara leu*Δ*7679* Δ(*lacIOPZY*) *galU galK* (r^- m^+)	Trier Hansen, Novo
NWL37	Gef^r Km^r	17
CC118 λ *pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE* (λ*pir*) *galK phoA2 thi*-1 *rspE rpoB argE* (Am) *recA1* λ*pir*	7, 10
Mv1190 λ *pir*	Δ(*lac-proAB*) *thi sup*E (λ *pir*)Δ(*srl-recA*)*306*::Tn*10* (F'::*traD362 proAB lacI*^{q1}ZΔM15) λ *pir*	7, 10
CSH36	*lacI lacZ*$^+$	B. Bachmann
Plasmids		
pWWO	Archetypal TOL plasmid	27
pRK600	Tra^+, Cm^r	7, 10
pCC102	*xylS2*, Pm::*lacI*, Km^r	5
pUT-Km	Ap^r, Km^r, mini-Tn*5* delivery system	7, 10
pUT-Tc	Ap^r, Tc^r, mini-Tn*5* delivery system	7, 10
pUC18Not	Ap^r	7, 10
pLBJ60	Ap^r, $P_{A1-04/03}$::*gef*, *lacI*^{q1} *oriV*	This work
pLBJ89	pUT-Km, $P_{A1-04/03}$::*gef*, Km^r, Ap^r	This work
pLBJ91	pUT-Tc, $P_{A1-04/03}$::*gef*, *lacI*^{q1}, Ap^r, Tc^r	This work
pLBJ95	pUT-Tc, $P_{A1-04/03}$::*gef*, Tc^r, Ap^r	This work

[a] $P_{A1-04/03}$ is a synthetic *lac* promoter (4).
[b] Strain collection at the Department of Microbiology, Technical University of Denmark.

cloned into the unique *Not*I site of pUT-Tc (7, 10) by using *E. coli* CC118 λ *pir* (7, 10). The resulting tetracycline-resistant plasmid was called pLBJ91 (Fig. 1). pLBJ60 was digested with *Mlu*I, and the resulting single-stranded ends were filled in with the Klenow enzyme and the four deoxynucleoside triphosphates and then further digested with *Sma*I. The plasmid was religated, removing the *lacI*^{q1} gene, and transformed into the Gef^r *E. coli* NWL37 (19). The resulting ampicillin resistance plasmid was called pLBJ88, and from this an 800-bp region, containing $P_{A1-04/03}$::*gef*, was isolated by digestion with *Not*I and cloned in pUT-Km (pLBJ89) and pUT-Tc (pLBJ95) (7, 10) with *E. coli* Mv1190 λ *pir* (7, 10) as the host strain (Fig. 1). All the plasmids (pLBJ89, pLBJ91, and pLBJ95) induced cell killing of the respective host when grown in the presence of IPTG.

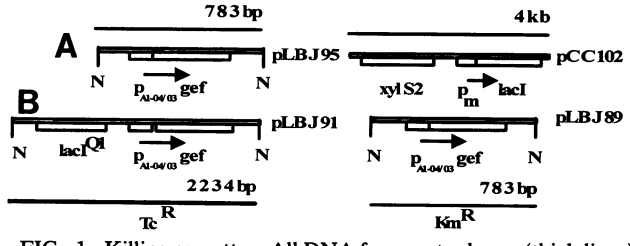

FIG. 1. Killing cassettes. All DNA fragments shown (thick lines) are integrated at the *Not*I site (marked with a N) of the pUT-Km or pUT-Tc mini-Tn*5*-transposon (7, 10) for the pLBJ suicide plasmids. pCC102 (5) is a kanamycin resistance plasmid. pLBJ91 and pLBJ95 are derivatives of pUT-Tc, and pLBJ89 is a derivative of pUT-Km. (A) *m*-Methylbenzoate-activated system; (B) activation of killing is controlled by the *lacI*^{q1} gene.

Testing of killing efficiency. As mentioned above, all constructions with an active containment system were tested on plates with and without IPTG. Bacteria were first streaked on plates containing selective antibiotics as well as IPTG and second on plates containing only selective antibiotics.

Triparental matings. Triparental matings were performed as described by Herrero et al. (10) to transfer the integration cassettes from the pLBJ plasmids onto the chromosome of *P. putida* KT2440. *E. coli* Mv1190 λ *pir*(pLBJ), *P. putida* KT2440(pWWO, pCC102) (plasmid pWWO allows growth on alkylbenzoates, and plasmid pCC102 carries a fusion of the TOL *meta*-cleavage pathway operon promoter [Pm] to *lacI* and the *xylS2* positive regulator), and *E. coli* HB101(pRK600) were used as the donor, the recipient, and the helper strain(s), respectively. The mixture of bacteria was laid onto LB plates supplemented with 5 mM *m*-methylbenzoate. After 12 to 24 h of incubation, *P. putida* KT2440(pWWO, pCC102) transconjugants harboring the mini-Tn*5* transposon derived from the suicide pLBJ plasmids were selected on minimal medium plates with the appropriate antibiotic and supplemented with 3-methylbenzoate as the sole carbon source.

Fluctuation test. Fluctuation tests (13) were performed to estimate the mutation rate of bacteria that make up an active biological containment system. Ninety vials, each containing 150 μl of M9 minimal medium cultures plus a limited amount of glucose (0.02% [wt/vol]), some with and some without 50 μM *m*-methylbenzoate (a concentration that allows full induction of the Pm promoter by the XylS2 regulator [21])

and appropriate antibiotics, were inoculated with 10 to 100 bacteria of the strain to be tested. After overnight growth, the average number of bacteria per culture (N_0) was estimated by plating out dilutions from two vials. To the rest of the cultures (88 vials), 50 μl of LB medium containing antibiotics and IPTG was added to a final concentration of 2 mM. The cultures were then incubated overnight. The number of vials in which no bacterial growth was observed (clear vials) (p_0) was counted, and the mutation rate (a) was estimated from the equation $a = -1/N_0 \ln (p_0/p)$ (13).

Soil experiments. Two sandy loam soils, a cambisol soil (0.63% [wt/wt] organic matter and 20.4% [wt/wt] $CaCO_3$) and a fluvisol soil (2.3% [wt/wt] organic matter and 6% [wt/wt] $CaCO_3$) as described previously (20) were used. Before use, the soils were sifted through a 4-mm-mesh metal sieve, and then 70-g samples of soil were placed in jars. Unless otherwise indicated, soils were sterilized in an autoclave under a vapor stream (120°C for 1 h) three times; the mass was allowed to cool completely between each step. All experiments were performed at a moisture content of about 10 to 15%. One milliliter of cells in 50 mM phosphate buffer was added to the jars containing sterile or nonsterile soils to a density of about 10^6 CFU/g of soil unless otherwise indicated. To estimate culturable cells (CFU), we added 10 g of soil to 90 ml of 50 mM phosphate buffer and shook the mixture at 30°C for 1 h to obtain the initial dilution. While still being shaken, this dilution was used to obtain a range of dilutions so that we could detect between 10^2 and 10^8 CFU/g of soil. In each CFU determination, at least three different dilutions were spread in duplicate on selective plates. The values given in Results represent the average numbers of readily countable CFU from all dilutions. Standard deviations were in the range of 5 to 25% of the given values.

RESULTS AND DISCUSSION

Construction of *P. putida* strains bearing a containment system. Triparental matings were performed with *P. putida* KT2440(pWWO, pCC102) as the recipient, *E. coli* Mv1190 λ *pir* harboring pUT-miniTn*5*Tc or pLBJ95 (pUT-miniTn*5* $TcP_{A1\text{-}04/03}$::*gef*) as the donor, and the conjugation helper *E. coli* HB101(pRK600). Tc^r *P. putida* KT2440 transconjugants [*P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc (LBJ488) and *P. putida* KT2440(pWWO, pCC102)::miniTn*5* $TcP_{A1\text{-}04/03}$::*gef* (LBJ466)] were selected on minimal medium with 5 mM *m*-methylbenzoate, tetracycline, and kanamycin. To test the functionality of the active biological containment system, we streaked bacteria on LB plates containing antibiotics (kanamycin and tetracycline), some with and some without 5 mM *m*-methylbenzoate. As expected, *P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc survived on plates with and without *m*-methylbenzoate, whereas *P. putida* KT2440(pWWO, pCC102)::miniTn*5*$TcP_{A1\text{-}04/03}$::*gef* did not grow on plates without *m*-methylbenzoate.

The survival of the constructed bacteria was also assayed in liquid culture medium. *P. putida* KT2440(pWWO, pCC102)::miniTn*5*$TcP_{1\text{-}04/03}$::*gef* and *P. putida* KT2440 (pWWO, pCC102)::miniTn*5*Tc were grown overnight in LB medium supplemented with 5 mM *m*-methylbenzoate. Bacteria (2 ml) were harvested from each culture, washed three times in M9 minimal medium, and finally resuspended in 200 ml of M9 minimal medium containing 0.5% (wt/vol) glucose. Four 50-ml subcultures were incubated with or without 5 mM *m*-methylbenzoate and 2 mM IPTG (Fig. 2) and continuously shaken at 30°C. The growth of the culture was monitored over time by measuring the optical density at 450 nm (OD_{450}). After a delay phase of 2 h, *P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc grew exponentially under all four conditions with similar growth rates and a generation time of around 70 min (not shown). Figure 2 shows that *P. putida* KT2440(pWWO, pCC102)::miniTn*5* $TcP_{A1\text{-}04/03}$::*gef* grew exponentially with a generation time of 67 min after a lag phase of 2 h when *m*-methylbenzoate was added to the culture medium. In contrast, cultures without *m*-methylbenzoate did not grow. Although *P. putida* KT2440 (pWWO, pCC102)::miniTn*5*$TcP_{A1\text{-}04/03}$*gef* appears to function according to its design, these results indicate that an excess of LacI repressor is synthesized in the presence of the effector *m*-methylbenzoate, since the additional presence of IPTG had no effect on cell growth. A further refinement of the control system may require the introduction of all elements on the chromosome. It is worth mentioning that cultures without *m*-methylbenzoate but supplemented with IPTG did not grow at all after prolonged (50-h) incubation, whereas the culture without *m*-methylbenzoate and without IPTG reached about $OD_{450} = 1$ in 48 h, probably because of the growth of bacteria which escaped death (see below).

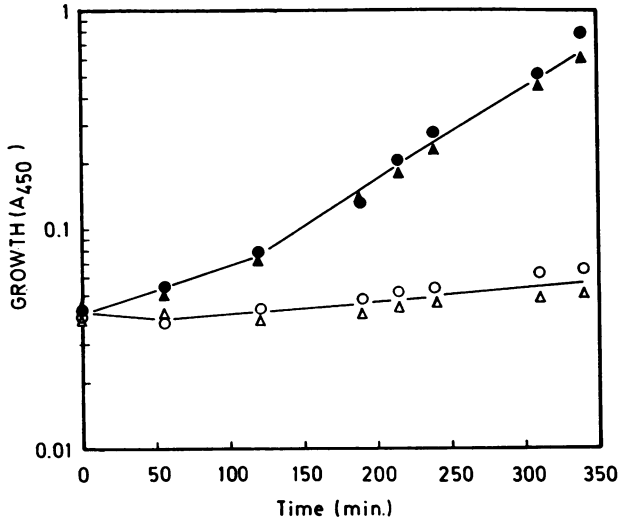

FIG. 2. Biological containment of *P. putida* 2440 based on the presence of *m*-methylbenzoate. *P. putida* KT2440(pWWO, pCC102)::miniTn*5*$TcP_{A1\text{-}04/03}$::*gef* was grown overnight on LB medium with 5 mM *m*-methylbenzoate, kanamycin, and tetracycline. Cells were then washed on M9 minimal medium as described in Materials and Methods and resuspended in the same medium with 0.5% (wt/vol) glucose in the absence of any other compound (○) or in medium supplemented with 2 mM IPTG (△), 5 mM *m*-methylbenzoate (▲) or 5 mM *m*-methylbenzoate and 2 mM IPTG (●). At the indicated times, the turbidity of the culture (A_{450}) was determined.

Analyses of bacteria escaping suicide and a solution to the problem. Since bacteria that survive killing in the above experiments were selected as resistant to kanamycin and tetracycline, loss of the plasmid comprising the regulatory elements and the transposon with the killing function was excluded. Plasmid pCC102 DNA was prepared from 40 independent Gef^r clones and used to transform *E. coli* CSH36, a *lacI lacZ*$^+$ strain. Transformants from the 40 independent transformations were selected on LB plates containing kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in the presence or absence of *m*-methylbenzoate. In all cases the colonies were blue in the absence and colorless in the presence of *m*-methylbenzoate.

TABLE 2. Rate of escape from killing of *P. putida* KT2440(pWWO) bearing one or two copies of the killing gene

Strain	No. of copies of killing gene	Rate of escape[a]
P. putida KT2440(pWWO, pCC102)::miniTn*5*Tc$P_{A1\text{-}04/03}$::*gef*	1	1.5×10^{-5}
P. putida KT2440::miniTn*5*Tc,*lacI*^{q1} $P_{A1\text{-}04/03}$::*gef*	1	1.2×10^{-6}
P. putida KT2440::miniTn*5*Tc$P_{A1\text{-}04/03}$::*gef lacI*^{q1}::miniTn*5*Km$P_{A1\text{-}04/03}$::*gef*	2	1.5×10^{-8}

[a] The rate of escape was calculated from a series of fluctuation tests carried out with the above-mentioned strains. The mutation rate is lowered by increasing the numbers of copies of the killing cassette. Approximately 10^6 CFU was used in each vial.

This suggested that bacterial escape from killing was not due to a malfunction of the regulatory control element. Fluctuation tests were carried out with *P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc$P_{A1\text{-}04/03}$::*gef* to estimate the rate at which bacteria escape from killing (see Materials and Methods). These analyses indicated that escape from killing occurs with a frequency of about 10^{-5} per cell per generation (Table 2).

To test whether the rate of escape from cell killing reflected the mutation rate of the killing system and whether this could be lowered, we constructed two new *P. putida* KT2440 (pWWO) strains. The first *P. putida* strain [*P. putida* KT2440 (pWWO)::mini-Tn*5*Tc$P_{A1\text{-}04/03}$*gef lacI*^{q1} (LBJ415)] contains one copy of the killing cassette (pLBJ91) under the control of *lacI*^{q1}, and the second [*P. putida* KT2440(pWWO)::mini-Tn*5*Tc$P_{A1\text{-}04/03}$*gef lacI*^{q1}::mini-Tn*5*Km$P_{A1\text{-}04/03}$*gef* (LBJ433)] contains two copies (pLBJ91 and pLBJ89) under the control of one *lacI*^{q1} gene. Fluctuation tests were carried out in LB medium, and the analyses indicated that escape from killing occurred at frequencies of 10^{-6} and 10^{-8} for one and two copies of the killing cassettes. The lower mutation rate obtained here for the one-copy system compared with the *m*-methylbenzoate-activated system is probably due to partial induction during the degradation of the high internal pool of LacI molecules when *m*-methylbenzoate is removed from the medium. The results suggest a way to reduce the number of bacteria escaping killing by duplicating the killing cassette.

Survival of contained and noncontained *P. putida* KT2440 (pWWO, pCC102) in different soils. The survival of *P. putida* KT2440(pCC102, pWWO)::miniTn*5*Tc and *P. putida* KT2440 (pCC102, pWWO)::miniTn*5*Tc$P_{A1\text{-}04/03}$::*gef* was assayed first in two different sterile soils. The bacteria were introduced, at a cell density of about 10^6 CFU/g of soil, into sterile cambisol and fluvisol soils (20), some of which were amended with 0.08% (wt/wt) *m*-methylbenzoate. Thereafter, the number of CFU was estimated by plating out bacteria on minimal medium with *m*-methylbenzoate as the sole carbon source and supplemented with tetracycline and kanamycin. By using these selective plates, growth of the introduced bacteria only was measured. We found that after 2 weeks *P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc was established in the unamended cambiol soil at a level of about 10^5 CFU/g of soil whereas in fluvisol soil the number of CFU increased to about 10^7 CFU/g of soil. In amended soils the number of bacteria after 2 weeks was about 1 order of magnitude higher (Table 3). In contrast, *P. putida* KT2440 (pWWO, pCC102)::miniTn*5*Tc$P_{A1\text{-}04/03}$::*gef* did not become established in cambisol soil whether *m*-methylbenzoate was present or not. In the fluvisol soil the behavior of the contained bacteria with the killing system was significantly different: in the unamended soil about 1% of the initial cell load was recovered, whereas in the presence of the substituted benzoate the number of bacteria increased up to 10^8 CFU/g of soil after 2 weeks (Table 3). These results indicate that introduction of the bacteria harboring a killing gene in a soil poor in organic matter and rich in calcium carbonate leads to rapid elimination of the bacteria, in contrast to the survival of the strain without the killing gene. Whether the strong nutrient depletion together with the killing gene leads to this sharp elimination of the bacteria is unknown. In contrast, in soil rich in organic matter, the number of cells in the presence of the pollutant was about 10^4 to 10^6 larger than in its absence, whereas for a control strain without the killing gene the ratio of survival in the presence and absence of *m*-methylbenzoate was only one order of magnitude.

The survival of *P. putida* KT2440(pWWO, pCC102)::mini Tn*5*Tc$P_{A1\text{-}04/03}$::*gef* with time was monitored in nonsterile fluvisol soil with or without 0.08% (wt/wt) *m*-methylbenzoate. The total viable indigenous population was counted on LB medium, whereas the introduced *P. putida* KT2440(pWWO, pCC102)::miniTn*5*Tc$P_{A1\text{-}04/03}$*gef* bearing the killing gene was monitored on selective minimal medium containing glucose, *m*-methylbenzoate, kanamycin, and tetracycline. It was observed that the total number of CFU was relatively constant with time in soils supplemented with the benzoate analog and in unsupplemented soils (about 10^8 CFU/g of soil) (not shown), which suggests that neither the introduction of *P. putida* KT2440 nor the *m*-methylbenzoate had a significant effect on the indigenous population. However, the presence of the pollutant in the soil had a noticeable effect on the survival and establishment of the introduced bacteria harboring the containment system. Bacteria were introduced into the two soils at an initial cell density of about 10^6 CFU/g of soil. In amended soil the number of bacteria increased rapidly to about 10^8 CFU/g of soil in 3 days, remaining at this level thereafter; however, in the unamended soil the number of bacteria decreased with time, falling below our detection limits after about 15 days (Fig. 3).

About 10^6 CFU of the noncontained *P. putida* KT2440 (pWWO, pCC102)::miniTn*5*Tc per g of soil was also introduced into nonsterile fluvisol soil either amended with 0.08% m-methylbenzoate or not amended. In contrast with the contained strain, the noncontained one established at about 10^7 and 10^8 CFU/g of soil in the unamended and amended fluvisol soils, respectively. We have therefore shown that a

TABLE 3. Establishment of *P. putida* KT2440 (pWWO, pCC102) with or without a biological containment system in sterile soils

Strain	Level of *P. putida* (CFU/g of soil) in:			
	Cambisol		Fluvisol	
	−	+	−	+
P. putida(pWWO, pCC102)::miniTn*5*Tc	1×10^5	1×10^6	1×10^7	1×10^8
P. putida(pWWO, pCC102)::miniTn*5*Tc $P_{A1\text{-}04/03}$::*gef*	<10	<10	1×10^4	2×10^8

[a] About 10^6 CFU of both strains per g of soil was introduced into sterile cambisol and fluvisol soils unsupplemented (−) or supplemented (+) with 0.08% (wt/wt) *m*-methylbenzoate. The number of bacteria was estimated after 2 weeks.

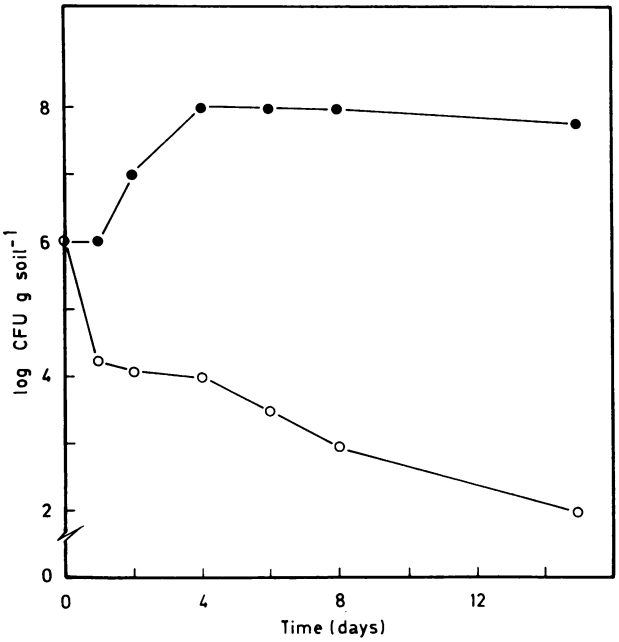

FIG. 3. Survival of *P. putida* KT2440(pWWO, pCC102) bearing a killing cassette (pLBJ95) in nonsterile soils. The bacteria were grown as in the legend to Fig. 2, except that about 10^6 CFU was introduced per g of nonsterile fluvisol soil unsupplemented (○) or supplemented (●) with 0.08% (wt/vol) *m*-methylbenzoate. The CFU per gram of soil was determined by making dilutions of the soil in M9 and plating on M9 minimal medium with 0.5% (wt/vol) glucose supplemented with kanamycin, tetracycline, and 5 mM *m*-methylbenzoate.

biological containment system designed for *P. putida* KT2440(pWWO) and based on conditional expression of the *E. coli gef* gene is functional and works within the expected genetic limits, although its possible use in a real scenario is needed to reduce the rate of escape from killing.

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