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Construction and Behavior of Biologically Contained Bacteria for Environmental Applications in Bioremediation

M. CARMEN RONCHEL,[1] CAYO RAMOS,[1] LARS B. JENSEN,[2] SØREN MOLIN,[2] AND JUAN L. RAMOS[1]*

Department of Biochemistry and Molecular and Cellular Biology of Plants, Consejo Superior de Investigaciones Científicas, E-18008-Granada, Spain,[1] and Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark[2]

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The survival of microorganisms can be predicted through the use of active biological containment systems. We have constructed contained *Pseudomonas putida* strains that degrade alkylbenzoates. The modified strain carries a fusion of the P_{lac} promoter to the *gef* gene, which encodes a killing protein. Expression from P_{lac} is controlled through a regulatory cascade, so that P_{lac} is switched on or off by the absence or presence of alkylbenzoates, respectively. Similar uncontained strains were also constructed and tested as a control. Contained and uncontained strains were genetically stable, and their survival and functionality in soil microcosms were as expected. Both contained and uncontained strains survived well in soils supplemented with alkylaromatics, whereas survival of the contained strain in soil microcosms without methylbenzoates was markedly reduced, in contrast to the control strain, which survived in these soils in the absence of alkylbenzoates. The TOL plasmid was transferred in soils between *Pseudomonas* strains but was not able to mobilize the elements of the containment system.

The potential risks associated with unintentional releases of genetically engineered microorganisms, as well as the unpredictability of their behavior in natural ecosystems, are subjects of considerable concern. Even greater is the concern being expressed over the next phase of gene technology, which involves the possible intentional release of genetically engineered microorganisms for agricultural, biomedical, and environmental applications (10). Most of our knowledge of the behavior of genetically engineered microorganisms comes from so-called microcosm studies, in which a piece of nature—soil, river sediment, polluted river water, activated sludge, etc.—has been removed from its natural location and brought to the laboratory, where most "environmental" conditions (temperature, day-night cycles, humidity, etc.) can be controlled. Microcosm studies have generally concluded that the benefits and risks associated with recombinant microorganisms are the same as those posed by the parental microorganism (12). However, conventional genetic procedures limit the interchange of DNA among closely related species, whereas molecular techniques allow a wider variety of genes to be introduced into microorganisms, usually with greater precision, which results in potentially more predictable microorganisms.

A way to increase the predictability of genetically engineered microorganisms is to provide them with active biological containment systems that form barriers that minimize the dissemination of the recombinant microorganisms themselves (1, 2, 7, 9, 11, 16) or that prevent dissemination of the rDNA (1, 4, 8) outside the target area. Equally important, active biologically contained microorganisms will minimize the impact on natural populations, because the survival of the introduced microorganism in a given niche will be limited in time. We have constructed biologically contained microorganisms by using the *gef* killing gene from *Escherichia coli* coupled to regulatory circuits for the metabolism of alkylbenzoates. This system allows the killing gene to turn on in the absence of chemical signals. The model system we have developed is made of two elements: a killing cassette and a regulatory cassette that controls the function of the killing cassette. The first element consists of a fusion of the P_{lac} promoter to the *gef* gene in a mini-Tn*5* element donor. Expression of the Gef protein results in the insertion of this protein into the cell membrane, which in turn causes the collapse of the membrane potential (10). In the current model system, the regulatory cassette is based on the XylS regulator and the P_m promoter for the *meta*-pathway of the TOL plasmid fused to the *lacI* gene (Fig. 1). In the presence of alkylaromatics, synthesis of the Gef protein is prevented and the cells remain alive, whereas in the absence of the effector, the Gef protein is synthesized and a large fraction of the cells die (3, 7). In this study, we show that *Pseudomonas putida* EEZ30, which carries the killing cassette on the host chromosome and the control element on a *mob*$^+$ *tra* plasmid, survives and functions in several soil and aquatic microcosms in accordance with its design.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The parental strain for the construction of contained and uncontained strains was *P. putida* EEZ29 (Table 1). This strain bears the TOL plasmid, pWW0, which allows it to grow on *m*-methylbenzoate (12, 14), and the Km^r plasmid pCC102, which carries the *xylS2* gene and the P_m::*lacI* fusion (3). Therefore, pCC102 bears the regulatory cassette of the biological containment system (Fig. 1). *P. putida* EEZ30, EEZ31, and EEZ32 are derivatives of *P. putida* EEZ29, and their relevant characteristics are given in Table 1. *P. putida* UWC1 is a Rif^r derivative of KT2440 (provided by M. Day, University of Cardiff, Cardiff, United Kingdom). *E. coli* CSH36 is a *lacI* strain (provided by B. Bachmann, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). *E. coli* CC118λPIR was used to replicate the pUT-based plasmid (6), whose replication requires the Pir protein.

E. coli strains were grown at 30°C in LB medium. *P. putida* strains were grown at 30°C in M9 minimal medium (3), with either 5 mM benzoate or *m*-methylbenzoate as the sole carbon source. pUT-Tc (mini-Tn*5*-Tc) (6), pLBJ95 (mini-Tn*5*-Tc-P_{lac}::*gef*) (7), and the Cm^r helper plasmid pRK600 (6) have been described previously. Antibiotics were added at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; and rifampin, 50 μg/ml.

Fluctuation tests. Fluctuation tests were done as previously described (7) to estimate the mutation rate of bacteria that escape from the active biological containment system.

Soil microcosm assays. Two sandy-loam soils, a cambisol soil (0.63% [wt/wt]

* Corresponding author. Mailing address: Estación Experimental del Zaidín, Apdo 419, E-18008 Granada, Spain. Phone: 34-58-121011. Fax: 34-58-129600.

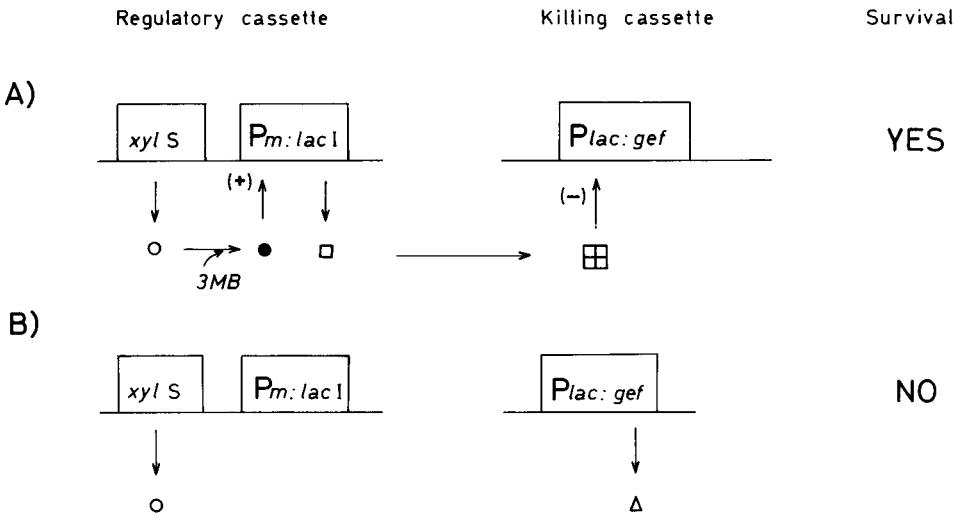

FIG. 1. Elements and functioning of a biological containment system. (A) The regulatory *xylS* gene is expressed constitutively (○) and becomes active in the presence of *m*-methylbenzoate (3MB) (●), promoting transcription from P_m. This leads to the synthesis of the LacI repressor (□), which forms tetramers that prevent expression from P_{lac}. (B) In the absence of *m*-methylbenzoate, the Gef protein (△) is made; this protein collapses the membrane potential, which leads to cell death.

organic matter, 20.4% [wt/wt] $CaCO_3$) and a fluvisol soil (2.3% [wt/wt] organic matter, 6% [wt/wt] $CaCO_3$), were used as described previously (13). Before use, the soils were sifted through a 4-mm-mesh metal sieve, and 90-g samples of soil were placed in the jars. A 1-ml volume of cell culture was added to the jars to a density between 10^4 and 10^8 CFU/g of soil; the densities were estimated as described previously (5, 13). *P. putida* EEZ30, EEZ31, and EEZ32 were enumerated on M9 minimal medium with *m*-methylbenzoate as the sole carbon source and supplemented with kanamycin and tetracycline as selective antibiotics. No indigenous bacteria able to grow in this medium were found. Bacteria able to grow on M9 minimal medium with 5 mM *p*-hydroxyphenylacetic acid (*p*OHPA) as the sole carbon source were used as an indicator of the indigenous population. The *P. putida* strains used in this study were not able to use *p*OHPA.

Aquatic microcosm assays. Natural waters from the River Maitena in the village of Güejar Sierra, Granada, Spain, and Atlantic Ocean coastal water collected at El Rompido Huelva, Spain, were used as aquatic microcosms. Portions (20 ml) of these waters were supplemented or not with 5 mM *m*-methylbenzoate, and bacteria were introduced to an initial cell density of about 10^6 CFU/ml.

RESULTS AND DISCUSSION

Construction of contained and uncontained *Pseudomonas* strains and their stability. Matings of *P. putida* EEZ29 with *E. coli* MV1190λPIR(pLBJ95) in the presence of HB101(pRK600) were done, and Km^r Tc^r clones of *P. putida* EEZ29 were selected on M9 minimal medium plus 5 mM *m*-methylbenzoate. Transconjugants appeared with a frequency of 10^{-5} per recipient. As expected, the transconjugants grew on the plates with the aromatic as the sole C source but not on plates without this compound, which confirmed that they were biologically contained bacteria.

TABLE 1. Relevant contained and uncontained strains constructed in this study

Strain	Relevant characteristic[a]
EEZ29	TOL, pCC102; Km^r $3MB^+$
EEZ30	Contained EEZ29: mini-Tn*5*::P_{lac}::*gef* on the host chromosome, Tc^r Km^r $3MB^+$
EEZ31	Uncontained EEZ29: mini-Tn*5* without the killing gene on the host chromosome, Tc^r Km^r $3MB^+$
EEZ32	Uncontained EEZ29: mini-Tn*5* without killing gene on the TOL plasmid, Tc^r Km^r $3MB^+$

[a] Km^r and Tc^r indicate resistance to kanamycin and tetracycline, respectively. $3MB^+$ indicates the ability to grow with *m*-methylbenzoate as the sole C source.

The TOL plasmid is able to mobilize host chromosome DNA with a frequency between 10^{-4} and less than 10^{-8} per recipient, depending on the location of the marker on the host chromosome (15). To select from among the transconjugants a clone in which the mini-Tn*5*-Tc-P_{lac}::*gef* was located in a position with low mobility, matings between different Km^r Tc^r P_{lac}::*gef* *m*-methylbenzoate-positive *P. putida* EEZ29 and Rif^r Km^r *P. putida* UWC1(pCC102) were done and Rif^r Km^r Tc^r transconjugants of the latter were selected on plates supplemented with *m*-methylbenzoate, rifampin, kanamycin, and tetracycline. The rate of transfer of the tetracycline marker was, in general, below 10^{-7} transconjugant per recipient. One clone whose tetracycline resistance marker transferred at a rate below 10^{-8} per recipient was selected for further studies. This strain was called *P. putida* EEZ30 (Table 1).

That the killing element in *P. putida* EEZ30 was not inserted on the TOL plasmid or on pCC102 was confirmed by the fact that the TOL plasmid transferred from *P. putida* EEZ30 to other *P. putida* recipients at a rate close to 1 whereas the Tc^r marker transferred at a rate below 10^{-8} per recipient. Furthermore, plasmid pCC102 prepared from this strain and transformed in *E. coli* CSH36 yielded Km^r colonies (around 10^5/μg of DNA) but no Tc^r or Km^r Tc^r transformants were found.

In the other series of matings, *P. putida* EEZ29 was mixed with *E. coli* MV1190λPIR(pUT-Tc) and the helper strain, *E. coli* HB101(pRK600) and Km^r Tc^r *m*-methylbenzoate-positive transconjugants were selected on M9 minimal medium supplemented with kanamycin, tetracycline, and 5 mM *m*-methylbenzoate. We searched for transconjugants that carried the mini-Tn*5*-Tc on the host chromosome and on the TOL plasmid. The latter were selected on the basis of their ability to cotransfer the TOL plasmid, which confers the ability to grow on *m*-methylbenzoate and resistance to tetracycline, whereas transconjugants harboring the insertion on the host chromosome were selected as unable to cotransfer the TOL plasmid and the Tc^r determinant. Two clones were chosen for further studies: *P. putida* EEZ32, which carries the Tc marker on the TOL plasmid (Table 1), and *P. putida* EEZ31, which carries the mini-Tn*5*-Tc on the host chromosome (Table 1) at a location that is mobilized by the TOL plasmid at a rate below 10^{-8} transconjugant per recipient.

The strains constructed were highly stable, as shown by the fact that pCC102 and the mini-Tn*5* were not lost after growth of the strain on LB medium for more than 50 generations in the absence of antibiotic pressure. The regulatory cassette in pCC102 remained functional after growth of *P. putida* EEZ30, EEZ31, and EEZ32 in the presence and absence of antibiotics; plasmid DNA prepared from these strains and used to transform the *lacI E. coli* CSH36 yielded 100% colorless transformants on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and *m*-methylbenzoate and 100% blue transformants on plates containing X-Gal but not *m*-methylbenzoate.

The rate of Gef escape in *P. putida* EEZ30 was determined by slightly modified fluctuation tests (10). The results showed that the rate of Gef-resistant colonies was on the order of 2.2×10^{-6} to 5.7×10^{-7} per cell per generation. The rate of escape from Gef killing was 2 to 4 orders of magnitude lower in this strain than in bacteria in which the killing element was located on plasmids (3, 7, 9). Why the bacteria bearing the killing element on the chromosome are more stable than those with the killing element on a plasmid is not known. However, the basal rate of transcription of the P_{lac}::*gef* fusion on the chromosome may be lower than when the fusion is present at a higher copy number, e.g., on plasmids. This may lead to instability and selection of clones without a functional P_{lac}::*gef*, which grow faster than those with this fusion or similar fusions (7, 9).

Survival of contained and uncontained *P. putida* bacteria in soil microcosms. Pots with 90 g of fluvisol soil were supplemented or not with 0.1% (wt/wt) *m*-methylbenzoate. Contained or uncontained *P. putida* strains were introduced in the pots, which were incubated at 17 to 22°C, because incubation at temperatures above 28°C had a noticeable negative effect on the survival of *P. putida* 2440 derivatives (13, 14). In these soils, the number of indigenous microbiota able to use *p*OHPA was about 1×10^7 to 2×10^8 CFU/g of soil. In experiments lasting 1 month, the numbers of these indigenous microorganisms in unamended and amended soils remained relatively constant regardless of whether contained or uncontained bacteria were introduced (Fig. 2A and B).

The behavior of the uncontained strain in the fluvisol soil was influenced by the number of bacteria introduced and by the presence or absence of *m*-methylbenzoate in the soil. When the control strains *P. putida* EEZ32 and EEZ31 were introduced at a cell density of about 10^4 to 10^5 CFU/g of soil, the number of bacteria remained relatively constant for 1 month in pots not supplemented with *m*-methylbenzoate. In contrast, in the supplemented soil, the number of bacteria increased to about 10^8 CFU/g of soil after 3 to 5 days and then declined slowly to about 10^7 CFU/g of soil 1 month after the bacteria were introduced (Fig. 2C). When this strain was introduced at a higher cell density (around 10^8 CFU/g of soil), the number of bacteria declined slowly in both supplemented and unsupplemented pots and was in the range of 5×10^6 CFU/g of soil by the end of the assay (results not shown).

Survival of the contained *P. putida* EEZ30 was also influenced by the presence or absence of *m*-methylbenzoate in the pots. At the lowest cell load (about 10^4 to 10^5 CFU/g of soil), the number of bacteria declined in the absence of *m*-methylbenzoate, so that after 1 month the number of bacteria was below 10^2 CFU/g of soil. In supplemented soils, the number of bacteria increased, reaching 10^7 CFU/g of soil 1 week later. Thereafter, the number of bacteria declined by 1 order of magnitude after 3 weeks (Fig. 2D). When the contained strain was introduced at a load of 10^8 CFU/g of soil, the number of bacteria declined both in the presence and in the absence of *m*-methylbenzoate. The rate of decrease was faster in the unsupplemented soil, so that after 1 month the difference in numbers was significant: about 10^7 CFU/g of soil in the supplemented soil versus 10^5 CFU/g of soil in the unsupplemented one (results not shown).

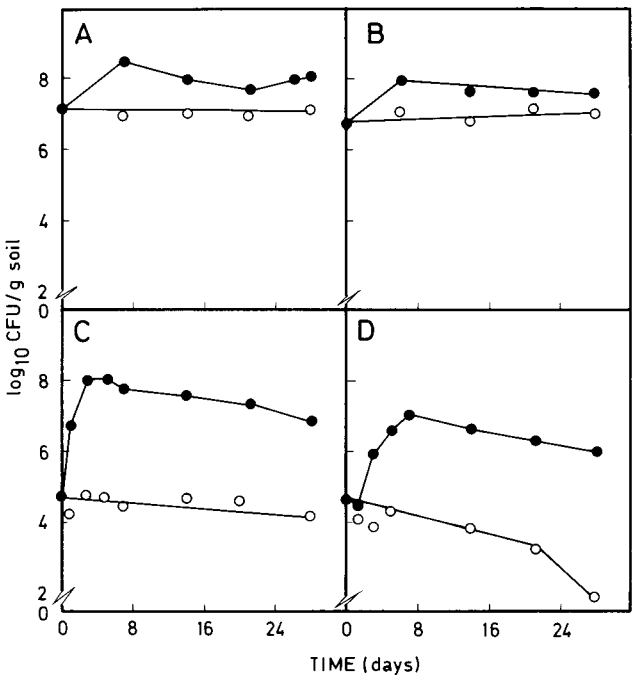

FIG. 2. Survival of autochthonous bacteria and contained and uncontained *P. putida* strains in a fluvisol soil. The soil was a fluvisol unamended (open symbols) or amended (solid symbols) with 0.1% (wt/wt) *m*-methylbenzoate. (A and B) Survival of indigenous microorganisms able to use *p*OHPA in soils in which *P. putida* EEZ32 (A) or *P. putida* EEZ30 (B) was introduced. (C and D) The control *P. putida* EEZ32 (C) and the contained *P. putida* EEZ30 (D) were introduced into this soil at about 5×10^4 CFU/g of soil.

Survival of the contained bacteria in soils with *m*-methylbenzoate was the result of the induction of the LacI protein, which prevented expression of the killing gene (Fig. 1). This was shown by the fact the bacteria mineralized *p*-methyl-[^{14}C]benzoate, as determined by $^{14}CO_2$ evolution, at a rate similar to that of the uncontained control strain and of the parent strain without pCC102 and the mini-Tn*5*-Tc element (5, 17).

The survival of the contained and uncontained strains in a cambisol soil poor in organic matter was also tested. The results showed that whereas the uncontained strain established in these soils at about 10^5 CFU/g of soil regardless of the presence of *m*-methylbenzoate, the number of cells of the contained strain in unsupplemented soil declined to below detection limits. When the cambisol soil was amended with *m*-methylbenzoate, the number of bacteria of the contained strains remained relatively constant at about 10^5 CFU/g of soil.

It was noted that the numbers of contained bacteria in both the fluvisol and cambisol soils without *m*-methylbenzoate did not begin to decline until several days after inoculation (Fig. 2). Early survival may result from the time needed to degrade the internal pool of LacI molecules, because the bacteria introduced in soils had been precultured with the benzoate effector (17).

Absence of lateral transfer of the elements of the containment system and transfer of the TOL plasmid in the fluvisol soil. Gene transfer in soil microcosms with and without 0.1% (wt/wt) *m*-methylbenzoate was assayed by simultaneously in-

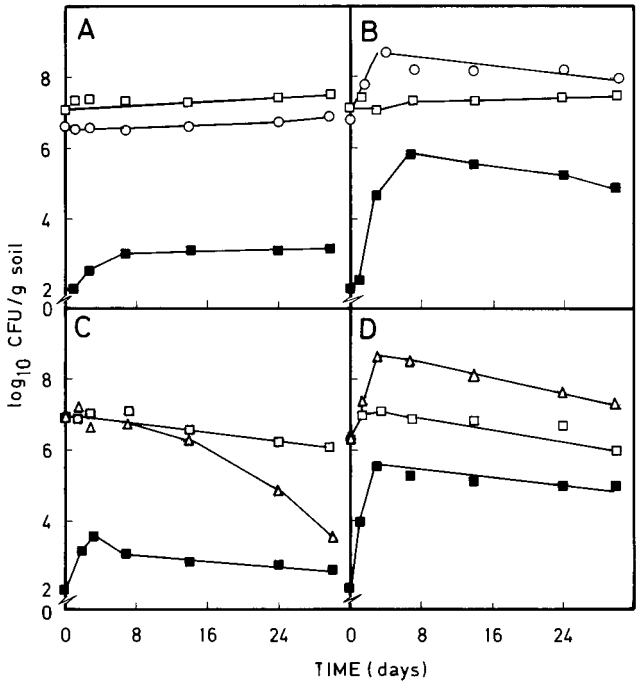

FIG. 3. Gene transfer in soils. The recipient strain was always *P. putida* UWC1 (□), and the transconjugants were *P. putida* UWC1 that received the TOL plasmid (■). The donor strain in panels A and B was the uncontained *P. putida* EEZ32 (○), and the donor strain in panels C and D was the contained *P. putida* EEZ30 (△). (A and C) Soils unsupplemented with *m*-methylbenzoate; (B and D) soils supplemented with 0.1% (wt/wt) *m*-methylbenzoate. At the indicated times, donors, recipients, and transconjugants were enumerated.

troducing into the soils the control strain, *P. putida* EEZ32, or the contained strain, *P. putida* EEZ30, as the donor and *P. putida* UWC1 as the recipient. Donor and recipient were each introduced at about 10^7 CFU/g of soil. At different periods, donors, recipients, and transconjugants were enumerated.

In unamended soils, the populations of donor *P. putida* EEZ32 and recipient *P. putida* UWC1 strains remained fairly constant (Fig. 3A). The number of transconjugants detectable 24 h later was about 10^2 CFU/g of soil, and the highest cell density of transconjugants reached was on the order of 10^3 CFU/g of soil (Fig. 3A). In this soil, similar numbers of UWC1 transconjugants were able to grow on *m*-methylbenzoate or were Tc^r (results not shown), suggesting that cotransfer of the TOL plasmid and the Tc^r determinant had occurred, as expected. When *P. putida* EEZ32 and UWC1 were introduced into the soil amended with *m*-methylbenzoate, the number of *P. putida* EEZ32 cells increased to about 5×10^8 CFU/g of soil whereas the recipient strain remained at about 10^7 CFU/g of soil (Fig. 3B). About 10^4 CFU of transconjugants per g of soil was detected after 24 h. In this soil, the highest cell density of transconjugants was on the order of 10^6 CFU/g of soil (Fig. 3B). As in the assay described above, all *m*-methylbenzoate-positive transconjugants were Tc^r. In this series of assays, we also tested whether the TOL plasmid was able to mobilize pCC102. We looked for Km^r Rif^r transconjugants of *P. putida* UWC1 on minimal medium with benzoate or *m*-methylbenzoate and found no Km^r transconjugants, which suggests that the TOL plasmid did not function as a helper for pCC102.

In the assays described above, the number of degraders of *p*OHPA in the conjugation pots remained relatively constant regardless of whether contained or uncontained bacteria were introduced and regardless of whether the soil was supplemented with *m*-methylbenzoate (data not shown). In these assays, we also tested whether the Tc^r-TOL plasmid in *P. putida* EEZ32 was transferred to the natural soil population of degraders of *p*OHPA. These transconjugants were sought on M9 minimal medium plates supplemented with tetracycline and 5 mM *p*OHPA as the sole carbon source. No Tc^r bacteria able to use *p*OHPA were found in unamended soils or soils supplemented with *m*-methylbenzoate, suggesting that the TOL plasmid was transferred at a very low frequency, if at all, to this indigenous bacterial population (data not shown).

In another series of assays in soils with and without *m*-methylbenzoate, *P. putida* EEZ30 was used as the donor strain and *P. putida* UWC1 was used as the recipient. Transconjugants of UWC1 able to grow on *m*-methylbenzoate were selected and found at a rate similar to that noted above. Figure 3C shows the number of donors, recipients, and transconjugants in unamended soils, and Fig. 3D shows the number of UWC1 transconjugants in amended soils. No Tc^r or Km^r transconjugants were found in these assays, suggesting that the transfer of the killing element of the containment system on either the host chromosome or the control element on pCC102 occurred at a very low frequency, if at all, in this soil.

TABLE 2. Survival in river water of *P. putida* with or without a biological containment system

Strain	Log cell count[a]			
	−3MB		+3MB	
	Initial	Final	Initial	Final
EEZ32	6.0	3.0	6.0	4.8
EEZ30	6.0	1.8	6.0	4.2

[a] The initial and final cell numbers are the $\log_{10}$ CFU per milliliter. The final counts refer to the number of CFU per milliliter 15 days after the introduction of bacteria in pristine water taken from the Maitena River. Standard deviations were in the range of 10 to 20% of the given values.

Survival of contained and uncontained *P. putida* strains in aquatic microcosms. To assay the behavior of the contained and uncontained strains in different habitats, two aquatic microcosms were considered, one consisting of pristine water from the Maitena River and the other consisting of seawater.

River water was supplemented or not with 5 mM *m*-methylbenzoate, and uncontained and contained *P. putida* bacteria were introduced at about 10^6 CFU/ml. These strains were not able to establish in this aquatic microcosm, and the number of bacteria decreased with time. The decrease in the number of bacteria of the contained strain was greater than that of the uncontained one, and it was most noticeable in river water not supplemented with *m*-methylbenzoate (Table 2). In seawater, none of the *P. putida* strains survived well. This was more evident in the absence of *m*-methylbenzoate, because the counts fell below our detection limits within 1 week after inoculation (results not shown).

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