Field Release of Biologically Contained Soil Bacteria for Environmental Applications in Bioremediation

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Abstract

The full exploitation of recombinant microbes for bioremediation of polluted sites will inevitably involve their release in the environment, where non-controllable conditions prevail. One way to increase the predictability of genetically engineered microorganisms (GEMs) is to provide them with a containment system biologically active. Active biological containment systems are based on genes that induce cell killing. We have developed a model system of chemical induction of suicide as a biological containment principle for the biodegradation of xenobiotics. The strategy used combines the killing function, based on the *Escherichia coli* porin-like protein Gef, with the regulatory system of the *meta*-cleavage pathway operon on the *Pseudomonas putida* TOL plasmid for the degradation of benzoates and alkylbenzoates. Transcription of the operon is driven from the P*m* promoter by the XylS regulator, but only in the presence of the substrate, i.e., 3-methylbenzoate (3MB). Two field trials have been performed in a controlled $100m^2$ site located in Granada (Spain). The experimental subsets consisted of groups of pots. One group of pots contained seeds coated by the control or the contained bacteria, and other series of pots were inoculated with the GEMs but were not sown. Some pots were supplemented with 3MB. *Zea mays* seeds were used in the field trial, initiated in May and completed in September 1995 ("summer release"). *Vicia faba* seeds were used in the field trial initiated in December 1995 and completed in April 1996 ("winter release"). The following items have been analyzed and are described in this paper: (1) survival of the contained and uncontained strains in pots supplemented or not with 3MB, (2) colonization of the rhizosphere of developing plants by the GEMs, (3) dispersion of the bacterial strains outside the inoculation area, and (4) effect of the introduced strains on indigenous soil bacteria.

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Introduction

Programed releases of recombinant microorganisms into environments where they cannot be physically contained are on the increase. Research on the bioremediation of dump sites and groundwaters, biological control and inoculants in agriculture, and live vaccines in biomedicine all required the release of microorganisms into open environments. However, there are still public and scientific concerns about such releases. Researchers have relatively few data on how recombinant microbes survive and multiply in the environment, or on how stable recombinant DNA is and how well it is transferred in natural environments. Like other research groups, we are attempting to provide answers to these questions, and at the same time are seeking solutions by designing active biological containment systems. Biological containment is not absolute, but it can help to reduce some of the uncertainties associated with microbial releases (Ramos *et al.*, 1995).

Active biological containment systems are based on killing genes, whose products interfere with cellular metabolism. Several killing proteins have been identified, and two of them have been used in active biological containment systems: nucleases and porin-like proteins. The most widely used porin-like proteins in biological containment are the members of the HOK family (HOK stands for *HO*st *K*illing). At least three homologous proteins belong to the HoK family: those encoded by the *hok* gene, which was located on the R1 plasmid; *relF*, and *gef*, which are *Escherichia coli* chromosome genes (Molin *et al.*, 1993). These three genes, which are highly homologous, encode small polypeptides of about 52 amino acids. All three genes have been used in biological containment with similar results.

We have developed a model system of chemical induction of suicide as a biological containment principle for the biodegradation of xenobiotics. This strategy combines the killing function, based on fusion of a modified promoter for the lactose operon (henceforth P*lac*) (Bujard *et al.*, 1987) to the *gef* killing gene, with the regulatory system of a degradative pathway (Contreras *et al.*, 1991; Ronchel *et al.*, 1995).

Construction and behavior of biologically contained bacteria

Pseudomonads, known for their metabolic agility, have considerable potential in bioremediation. The breakdown of 3-methylbenzoate (3MB), for example, is mediated by 13 genes in the *meta*-cleavage pathway operon on the TOL plasmid. Transcription of the operon is driven from the P*m* promoter by the XylS regulator, but only in the presence of the substrate 3MB (Worsey and Williams, 1974). The strategy for biological containment was to design bacteria that commit suicide when the 3MB is depleted and its catabolism switched off. We did this through a genetic construction that switched on the killing function when the TOL *meta*-cleavage pathway operon was no longer transcribed.

The system we have constructed consists of two elements. The regulatory element is based on the XylS regulator and the P*m* promoter. The P*m* promoter was fused to

the *lacI* gene, which encodes the LacI repressor. The killing element consists of a fusion of the P*lac* promoter to the *gef* killing gene in a mini-Tn*5* element donor (Ronchel *et al.*, 1995). In the presence of alkylaromatics, the XylS regulator drives transcription from the P*m* promoter to produce the LacI repressor. The LacI repressor, in turn, represses transcription of the P*lac::gef* unit. When 3MB is absent, XylS is inactive, LacI is not produced and Gef synthesis occurs. 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, leading to cell death.

We incorporated the containment system into two *Pseudomonas putida* strains: *P. putida* EEZ30 carries the killing cassette on the host chromosome and the control element on a *mob*$^+$ *tra*$^-$ plasmid (plasmid pCC102) (Contreras *et al.*, 1991), and strain *P. putida* CMC4 carries both control and killing elements on the host chromosome. The uncontained strain *P. putida* EEZ32 was also constructed and used as a control (Fig. 1).

The rate of Gef escape in *P. putida* EEZ30 and CMC4 was determined by slightly modified fluctuation tests (Molin *et al.*, 1993). The rate of mutation was in the order of 10^{-6} to 10^{-7} for EEZ30 and 10^{-8} for CMC4.

Contained and uncontained strains were genetically stable, and their behavior under laboratory conditions was as expected: the uncontained strain grew at a similar rate on minimal medium with or without 3MB; however, the contained strains grew only in medium with 3MB. When contained *P. putida* strains were tested in soil and aquatic microcosms with and without 3MB, their behavior was similar to that seen under laboratory conditions (see Ronchel *et al.*, 1995, for *P. putida* EEZ30 and EEZ32).

Field trials

Two field trials, approved by the Spanish Ministry of Public Works, have been performed in a controlled 100 m^2 site during the periods covering the summer of 1995 and winter of 1995-1996. The experimental site is located within the 2,000 m^2 experimental area at the Estación Experimental del Zaidín (CSIC, Granada, Spain). The experimental subsets in the area consisted of groups of pots. One group contained seeds covered by approximately 10^6 colony-forming units (CFU) per seed of the control or the contained bacteria, and other series of pots were inoculated with about 10^6 CFU of the GEM per gram of soil but without seeds. Some pots were supplemented with 0.01% 3MB. Care was taken to ensure that a distance of at least 1 m separated groups of pots with and without 3MB, as a precaution to avoid undesired side-effects due to the dispersion of the aromatic.

In the pots containing developing plants, bacterial counts were determined in the soil attached to the root (the rhizosphere) and in the rest of the soil (bulk soil). The contained and control strains were counted on minimal medium with 5 mM 3MB as the sole carbon source, and the appropriate antibiotics. No indigenous bacteria grew on these plates. Confirmation that the CFU/g of soil from the pots in which the contained strains were introduced indeed represented the contained strains was obtained by the fact that none of the bacteria selected on plates with 3MB were able to grow on minimal

medium with glucose plus IPTG, whereas all the control bacteria survived in the latter medium. We concluded that no mutants of the contained strain that might have escaped killing were established in soil.

For both field trials the population of indigenous microbes was calculated as the number of CFU of p-hydroxyphenylacetic acid utilizers per gram of soil, which are one of the predominant populations in the soil used.

During both field trials, five locations in the experimental field, in which no GEMs had been introduced, were used to monitor undesired dispersion of GEMs. Samples were taken every 14 days. Control or contained bacteria were never found outside the pots.

Summer release

Zea mays seeds were used in this field trial, which covered a period of 112 days starting on May 17 and ending in September 1995. Maximal and minimal temperatures in Granada during the summer release ranged between 25 and 45°C (day), and between 10 and 25°C (night).

The inocula used, *P. putida* EEZ30 and EEZ32, successfully colonized the roots of developing plants regardless of the presence of the aromatic in the soil. During this period bacteria could be detected in the rhizosphere of corn plants growing in amended soil during at least one month after they disappeared from the bulk soil (< 42 days). In the soils without 3MB the control strain colonized the rhizosphere at the same level as in soils containing the aromatic. However, colonization of the rhizosphere by the contained strain in the absence of 3MB was about 3 orders of magnitude lower than in soils containing the aromatic.

In the soils without plants none of the strains could be detected 7 days after the assay was started. Taken together, these results indicate a "protective effect" of the rhizosphere against mortality probably caused by high temperature.

During the assay the number of indigenous bacteria in the corn rhizosphere was in the order of 10^6 to 10^8 CFU/g of soil. In general, at any sampling time the number of bacteria found was one order of magnitude higher in soils containing 3MB than in soils without the aromatic. The number of indigenous microbes in the bulk soil was in the order of 10^5 to 10^7 CFU/g of soil, which is in general one order of magnitude lower than in the rhizosphere (Table 1). These results suggest that the indigenous microbes are well adapted to the high temperatures reached in soils in southern Spain during the summer.

Winter release

Vicia faba seeds were used in this field trial, which covered a period of 124 days starting in December 1995 and ending in April 1996. Maximal and minimal temperatures in Granada during this period ranged between 5 and 27°C (day), and between −3 and 13°C (night).

The contained strain used in this assay was *P. putida* CMC4, whose rate of Gef escape is one to two orders of magnitude lower than that calculated for *P. putida* EEZ30. *Pseudomonas putida* EEZ32 was used as a control strain.

Table 2 shows the number of bacteria present in the faba bean (*Vicia faba*) rhizosphere during the assay. In soils containing 3MB, both control and contained bacteria had colonized the rhizosphere to a cell load of 10^5 to 10^6 CFU/g of soil 25 days after the assay was started. A slight decline in the number of CFU/g of soil to 5 x 10^4 was observed on day 51 for both strains. Thereafter the contained bacteria started to decline, and at the end of the assay only 6 x 10^2 CFU/g of soil was found in the rhizosphere. However, on this day (124 days after initiation of the assay) the uncontained bacteria were found at a cell load of approximately 10^5 CFU/g of soil.

In soils without 3MB, control and contained bacteria colonized the bean rhizosphere to a cell load of about 10^4 CFU/g of soil 25 days after the assay was started. Thereafter the number of contained bacteria started to decline, and fell below our detection limit (10^2 CFU/g of soil) 51 days after initiation of the assay. From 61 to 117 days after initiation of the assay, this strain could be detected again in the rhizosphere at a cell load of approximately 10^3 CFU/g of soil. At the end of the assay the contained strain was not detectable. The number of uncontained bacteria in the faba bean rhizosphere fluctuated between 10^4 and 10^6 CFU/g of soil during the assay. At the end of the assay this strain was found at a cell load of approximately 10^5 CFU/g of soil.

Unsown soils with or without 3MB were inoculated with either the control or the contained bacteria to an initial cell load of approximately 10^6 CFU/g of soil. In soils containing 3MB, the number of both control and contained bacteria started to decline steadily immediately after inoculation, and after 25 days none of the strains were detectable. In soils without 3MB, the contained bacteria could not be detected 14 days after the assay was started. However, the uncontained bacteria was detectable in this soil during at least two weeks longer after the contained strain had disappeared.

During this field trial the behavior of the indigenous population of bacteria in the different experimental groups was similar to that observed during the summer release. In general, at any given sampling time the number of bacteria found was one order of magnitude higher in soils containing 3MB and/or plants than in soils without the aromatic. This confirms our previous finding that the amount of 3MB used in the assay (0.01%) is not toxic for the indigenous population of soil bacteria. Introduction of the control or the contained bacteria had no effect on the indigenous microflora.

References

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Table 1 Number of indigenous soil bacteria during the summer field release

		Number of bacteria (Log10 CFU/g of soil) Days after release							
		14		42		70		112	
GEM	3MB	R	BS	R	BS	R	BS	R	BS
None	+	7.7	6.4	8.6	6.5	7.2	6.0	6.5	5.1
	-	6.7	6.6	7.9	6.6	6.1	5.8	5.2	5.0
EEZ30	+	7.9	6.4	8.5	6.1	6.0	5.6	7.0	5.2
	-	7.0	5.8	6.8	6.5	6.7	6.0	6.2	5.0
EEZ32	+	7.4	6.7	8.1	6.2	6.7	6.2	7.0	5.1
	-	5.4	5.7	7.3	6.0	7.3	5.6	6.3	5.0

R, and BS; number of p-hydroxyphenyl acetic acid degraders present in the corn rhizosphere (R) and in the bulk soil (BS).

Table 2 Number of contained and uncontained bacteria in the faba bean rhizosphere during the winter field release

		Number of bacteria (Log_{10} CFU/g of soil)							
		Days after release							
GEM	**3MB**	**25**	**39**	**51**	**61**	**75**	**89**	**117**	**124**
CMC4	+	5.0	5.2	4.5	4.3	3.2	4.7	3.6	2.6
	-	3.8	3.7	n.d.	2.6	2.8	3.0	3.5	n.d.
EEZ32	+	5.7	5.2	4.5	5.3	4.6	3.8	5.2	4.9
	-	4.2	3.1	4.3	4.4	5.7	4.7	4.5	5.3

n.d., non detectable.

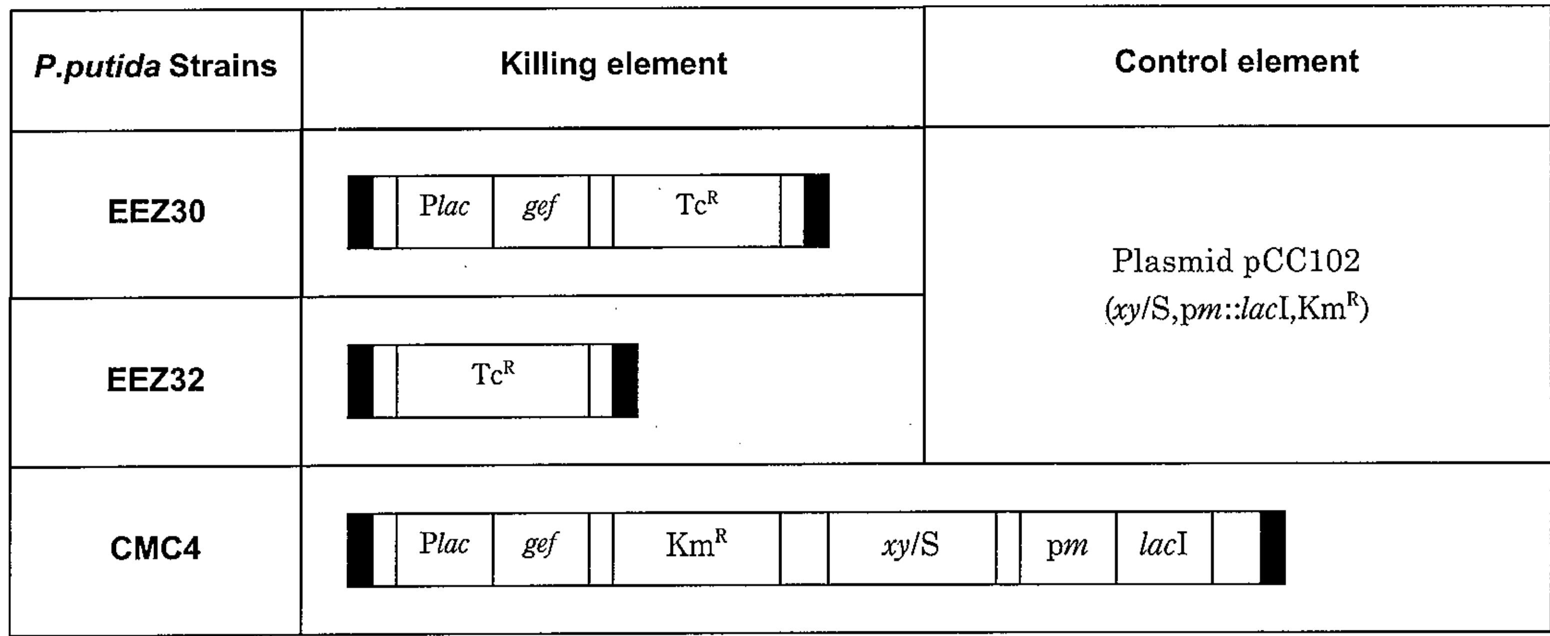

Fig.1 Elements of the different contained and uncontained *P. putida* strains.

Footnote to figure:
Tc^R, and Km^R; tetracycline and kanamycin resistance genes, respectively. Black bars represent the borders of the mini-Tn5 integrated on the host chromosome of each strain.