A dual lethal system to enhance containment of recombinant micro-organisms

Begoña Torres,† Susanne Jaenecke,** Kenneth N. Timmis,** José L. García† and Eduardo Díaz†

1Department of Molecular Microbiology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain
2Division of Microbiology, GBF-National Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

Active containment systems based on the controlled expression of a lethal gene are designed to increase containment of recombinant micro-organisms used for environmental applications. A major drawback in containment is the existence of mutations that generate surviving cells that cease to respond to the toxic effect of the lethal function. In this work the authors have developed for the first time a strategy to reduce the problem of mutations and increase the efficiency of containment based on the combination of two lethal functions acting on different cellular targets of major concern in containment, DNA and RNA, and whose expression is under control of different regulatory signals. To engineer the dual gene containment circuit, two toxin–antitoxin pairs, i.e. the colicin E3–immunity E3 and the EcoRI restriction–modification systems, were combined. The genes encoding the immunity E3 and the EcoRI methyltransferase proteins (antitoxins) were stably inserted into the chromosome of the host cell, whereas the broad-host-range lethal genes encoding the colicin E3 RNase and the EcoRI restriction endonuclease (toxins) were flanking the contained trait in a plasmid. This dual lethal cassette decreased gene transfer frequencies, through killing of the recipient cells, by eight orders of magnitude, which provides experimental evidence that the anticipated containment level due to the combination of single containment systems is generally achieved. Survivors that escaped killing were analysed and the mutational events involved were characterized.

INTRODUCTION

Horizontal gene transfer is a source of concern when genetically engineered micro-organisms are intended to be released in large quantities to the environment for biotechnological applications (Ramos et al., 1995; Wackett, 2000). Gene spread could be also undesirable in contained environments, e.g. a bacterial consortium in a fermentation tank, for process protection and process optimization reasons (Díaz et al., 1999). To make more predictable the behaviour of a genetically modified organism (GMO) introduced into a target habitat, its ability to spread new genetic information to potential recipients has to be reduced to avoid the appearance of undesired novel genetic combinations (gene containment). Moreover, the survival of the GMO has to be limited in time and space, i.e. engineering a controlled life cycle to reduce its dissemination and impact on the indigenous population of organisms (biological containment) (Molin et al., 1993; Ramos et al., 1995). Several gene and biological containment systems have been designed and they are based on a lethal function that, through a transcriptional and/or post-translational control element, becomes active in response to specific environmental signals (Molin et al., 1993; Díaz et al., 1999; Torres et al., 2000; Ronchel & Ramos, 2001; Davison, 2002). A general feature of the containment systems so far described is that a surviving subpopulation of cells, in the range of $10^{-7}$ to $10^{-3}$ per cell and generation depending on the particular system under study, ceases to respond to the toxic effect of the lethal function, which may be significant in environmental applications that use large quantities of cells (Molin et al., 1993; Szafranski et al., 1997). Analysis of the survivors has revealed that mutations are the main drawback in containment. Mutations that inactivate the containment system have been located either in the lethal function (Jensen et al., 1993; Munthali et al., 1996a; Torres et al., 2000) or in the control element (Bej et al., 1988; Knudsen et al., 1995).

A way to reduce the problem of mutations and increase the efficiency of containment is to engineer genetic circuits
with more than one lethal function (Davison, 2002). When the *gef* gene was used in a biological containment system responding to the absence of benzoate effectors, the rate of escape from killing decreased from $10^{-6}$ (one copy of *gef*) to $10^{-8}$ (two copies of *gef*) (Jensen et al., 1993). Duplication of the *relF* gene also increased plasmid containment by about three orders of magnitude (Knudsen & Karlström, 1991). Nevertheless, the reduction in the number of survivors by duplicating a lethal function is always smaller than that expected by theoretical calculations (Knudsen et al., 1995). This reduction in the efficiency of containment can be due to several factors such as: (i) homologous recombination and gene conversion between the two copies of the suicide function, (ii) presence of mutations that inactivate the regulatory element controlling the expression of both lethal genes, (iii) existence of mutations in the cellular target of the lethal function (Knudsen et al., 1995). To circumvent these limitations, the use of non-identical suicide functions with different cellular target sites and whose expression is under control of different regulatory circuits may be a suitable strategy.

Colicin E3 is a RNase encoded by the *colE3* gene that specifically cleaves the 16S rRNA, causing cell death (Zarivach et al., 2002). The *immE3* gene encodes the immunity E3 protein which binds stoichiometrically to colicin E3, preventing its RNase activity (Yajima et al., 1993). The *ecoRIR* gene of *Escherichia coli* (O’Connor & Humphreys, 1982). Protection of the host DNA from attack by this endonuclease is provided by the *EcoRI* methyltransferase encoded by the *ecoRIM* gene (Pingoud & Jeltsch, 1997). The *colE3/immE3* genes and the *ecoRIR/ecoRIM* genes have been previously used to design single containment systems that were shown to be efficient in a broad range of Gram-negative bacteria (Diaz et al., 1994; Munthali et al., 1996a; Torres et al., 2000). Since colicin E3 and the *EcoRI* endonuclease have different cellular targets, RNA and DNA respectively, and their corresponding antitoxins act at different levels, i.e. the toxin itself and the cellular target, respectively, they constitute ideal candidates for combination in a dual containment approach. In this work we present such a dual containment circuit that significantly reduces gene spread to generally achieve the anticipated level of containment.

**METHODS**

**Bacterial strains, plasmids, growth conditions, and transformation experiments.** The *E. coli* K-12 strains used were HB101Rif (Torres et al., 2000), XL-1 Blue (Sambrook et al., 1989), CC118::pir and S17-1::pir (de Lorenzo & Timmis, 1994). *E. coli* HB101::*immE3* is a derivative of strain HB101 that expresses the *immE3* gene constitutively from the chromosome and is therefore immune to colicin E3 (Diaz et al., 1994). Plasmid pVTRE is a low-copy-number chloramphenicol-resistance cloning vector that drives expression of cloned genes with the *Ptrc* promoter (Férez-Martín & de Lorenzo, 1996). pVTRE is a derivative of pVTRB that expresses the *ecoRIR* gene under the control of the *Ptrc* promoter (Torres et al., 2000). pUC18Sfi::colE3 is a pUC18Sfi derivative containing the *colE3* and *immE3* genes (Diaz et al., 1994). pHP450 is a pBR322 derivative harbouring the *Ω* interposon which contains the transcription-translation termination signals of gene 32 of phage T4 flanking the gene that confers streptomycin/spectinomycin resistance (*Smr/Spr*). Plasmid pSJ201 contains the *ecoRIM* gene cloned under control of the *Ptr* promoter from *λ* phage in a mini-*Tn5Smr/Spr* transposon (Torres et al., 2000). The RK2-Mob**+** RK2-Tra**+** plasmid pRK600 was used as helper in mobilization experiments (de Lorenzo & Timmis, 1994). Bacteria were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37°C. When required, antibiotics were added at the following concentrations: ampicillin, 100 mg ml$^{-1}$; chloramphenicol, 35 mg ml$^{-1}$; rifampicin, 50 mg ml$^{-1}$; spectinomycin, 100 mg ml$^{-1}$; and kanamycin, 50 mg ml$^{-1}$. *E. coli* cells were transformed by using the *BclI* procedure as described by Sambrook et al. (1989). Electroporation of *E. coli* cells was performed as previously described (Diaz et al., 1994). Transformants were selected on chloramphenicol-containing LB medium, except with plasmid pVTRECA, for which ampicillin-containing LB medium was also used.

**DNA manipulations and sequencing.** Plasmid DNA was prepared by the rapid alkaline lysis method (Sambrook et al., 1989). Genomic DNA was prepared as previously reported (Richards, 1987). DNA manipulations and other molecular biology techniques were essentially as described by Sambrook et al. (1989). DNA fragments were purified using the Gene-Clean Kit (BIO-101). Oligonucleotides were synthesized on an Oligo-100M nucleotide synthesizer (Beckman Instruments). Nucleotide sequences were determined directly from plasmids by using the dyeoxy chain termination method (Sanger et al., 1977). Standard protocols of the manufacturer for *Tag* DNA polymerase-initiated cycle sequencing reactions with fluorescently labelled dideoxynucleotide terminators (Applied Biosystems) were used. The sequencing reactions were analysed using a model 377 automated DNA sequencer (Applied Biosystems). Nucleotide sequence similarity searches were made by using the BLAST program (Altschul et al., 1990) via the National Institute for Biototechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

**Design of the *E. coli* HB101::*immE3ecoRIM* strain immune to both colicin E3 and the *EcoRI* endonuclease.** By means of RP4-mediated mobilization (de Lorenzo & Timmis, 1994), plasmid pSJ201 was transferred from *E. coli* S17-1::pir into *E. coli* HB101::*immE3*, a kanamycin-resistant strain. The resulting transconjugant strain, *E. coli* HB101::*immE3ecoRIM*, containing the *Ptrc::immE3* and the *Ptr::ecoRIM* fusions stably inserted into the chromosome, was selected for the transposon marker, spectinomycin, on kanamycin-containing LB medium. The resulting *E. coli* HB101::*immE3ecoRIM* strain constitutively expressed the *immE3* and *ecoRIM* genes as revealed by the cross-streak test and by protection of its chromosomal DNA from *EcoRI*-mediated cleavage in an *in vitro* DNA restriction assay (see below), respectively. *E. coli* HB101::*immE3ecoRIM* did not show any significant difference in growth characteristics from its parental HB101Rif counterpart as revealed by growth rate determinations and competition experiments, suggesting that none of the immunity functions appears to be detrimental to the host cell under the growth conditions used. The growth rate was determined by viable counting and by measuring OD$_{600}$ of the cultures along the growth curve. To perform the competition experiments, the immune and parental strains were inoculated together in the same medium, samples were taken at the exponential and stationary phase of growth and cell numbers were determined by plating on rifampicin-containing LB medium (selects for HB101Rif and HB101::*immE3ecoRIM* cells) and on kanamycin-containing LB medium (selects for HB101::*immE3ecoRIM* cells). The cell ratio of the two strains remained constant along the growth curve.

For the construction of the Pc::colE3 cassette, the promotorless colE3 gene was PCR-amplified from plasmid pUC18Sfi-colE3 by using primers Col5 [5'-CCGGATCCCTTGACAAACGCGTTGTTT-TATGTACAG-3']; the sequence spans from position -71 to -48 with respect to the ATG initiation codon of the colE3 gene (Masaki & Ohta, 1985); the engineered BamHI site is underlined; the engineered -35 consensus box of the Pc promoter is indicated in italics and Col3 [5'-GGTTCTAAGCCTCTCATAGATTTC-3']; the sequence spans the TGA stop codon (in italics) of colE3; the engineered XbaI site is double-underlined. The resulting 1.6 kb DNA fragment was digested with BamHI and XbaI and cloned into double-digested BamHI+XbaI pVTRB vector. The resulting plasmid, pVTRCol (Fig. 1), was isolated in E. coli HB101immE3 and expresses the colE3 gene under the control of the tandem Ptrc promoter and a synthetic promoter (Pc). A Smr/Spr DNA cassette flanked by transcription termination signals from phage T4 was cloned upstream of the Pc::colE3 fusion in plasmid pVTREC, giving rise to plasmid pVTRGof (Fig. 1).

To develop a dual lethal cassette, the BamHI+XbaI-digested Pc::colE3 fragment was cloned into the double-digested BamHI+XbaI pVTR4 plasmid. Using E. coli HB101immE3ecoRIM as recipient strain, a chloramphenicol-resistant transformant containing plasmid pVTREC, which expresses the Prc::ecoRIR and Pc::colE3 fusions in tandem in a chloramphenicol-resistant low-copy-number vector (Fig. 1), was identified. As revealed by growth rate determinations and competition experiments, pVTREC did not provide a significant growth disadvantage to the host cell HB101immE3ecoRIM when compared with the control plasmid pVTR4. The growth rate was determined by viable counting and by measuring OD600 of the cultures along the growth curve. To perform the competition experiments,

Fig. 1. Schematic representation of the construction of the contained plasmids. The thick and thin arrows show the promoters and the direction of transcription of the genes, respectively. Prc, trp/lac hybrid promoter; Pc, synthetic promoter. The lethal ecoRIR and colE3 genes are indicated by black and vertically striped blocks, respectively. The genes encoding ampicillin (Ap'), and streptomycin/spectinomycin (Sm'/'Sp') resistance are indicated with white and dotted blocks, respectively. The gene encoding ampicillin resistance was cloned with its own P3 promoter (Brosius et al., 1982). Cm', gene encoding chloramphenicol resistance. Primers Col5, Col3, Ap5' and Ap3' are detailed in Methods. T4, transcriptional terminator of gene 32 from phage T4; T1/T2 transcriptional terminators of the E. coli rmb operon; oriVpSC101, origin of replication of plasmid pSC101. Relevant restriction sites shown are: B, BamHI; H, HindIII; Hp, HpaI; N, NodI; X, XbaI. BamHI(Klenow) indicates digestion with BamHI followed by filling-in the protruding ends generated with E. coli DNA polymerase I Klenow fragment.
HB101immE3ecoRIM(pVTREC) and HB101immE3(pVTBR) cells were inoculated together in the same medium. Samples were taken at the exponential and stationary phases of growth and cell numbers were determined by plating on LB medium containing chloramphenicol and kanamycin [selects for both strains] and on LB medium containing chloramphenicol and spectinomycin [selects for HB101immE3ecoRIM(pVTREC)]. The cell ratio of the two strains remained constant along the growth curve.

To construct an ampicillin resistant (Ap') cassette, the bla gene encoding the β-lactamase from transposon Tn3 was PCR-isolated from plasmid pUC19 by using primers Ap5' [GGGGATCCGGTTCCTAGACGTCAGGTGGCAC], the engineered BamHI site is underlined; it hybridizes upstream of the P3 promoter of the bla gene (Brosius et al., 1982) and Ap3' [GGGGATCCGGTTCATGAGATTTATCTAAAAGG], the engineered BamHI site is underlined. The resulting 1-kb Ap' DNA cassette was digested with BamHI and cloned into BamHI-digested pVTREC. The resulting plasmid, pVTRECA (Fig. 1), was isolated in E. coli HB101immE3ecoRIM cells on ampicillin-containing LB plates.

Preparation of crude cell extracts and EcoRI endonuclease assay. To prepare crude extracts from E. coli HB101immE3ecoRIM containing plasmids pVTBR, pVTREC, and pVTRECA, cells were grown in chloramphenicol-containing LB medium to an OD600 of about 2. Cell cultures were then centrifuged (3000 g, 10 min at 20 °C), and cells were washed and resuspended in 0.05 M Tris/HCl (pH 7.6) containing 10% sucrose and 2 mM dithiothreitol, prior to disruption by passage through a French press (AminoCo) operated at 20000 p.s.i. The cell debris was removed by centrifugation at 26000 g for 30 min at 4 °C. The clear supernatant fluid was decanted and used as crude cell extract. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. EcoRI restriction endonuclease assays were performed as described previously (Torres et al., 2000). Crude extracts from cells containing pVTREC and pVTRECA, but not those from cells containing pVTBR, showed a pattern of restriction fragments indistinguishable from that obtained with purified EcoRI enzyme from a commercial source.

Assay for colicin E3 and immunity E3 activity. The production of immunity E3 protein in E. coli HB101immE3ecoRIM was checked qualitatively by the cross-streak test (Takagaki et al., 1973). Colicin E3 production by plasmids pVTREC, pVTRECA, and pVTRECA was assayed by the spot test (Masaki & Ohta, 1985) in which the degree of sensitivity to the colicin E3 present in crude extracts was determined as the highest dilution of the extract required to make a clear inhibition spot on the top agar inoculated with a colicin-sensitive indicator strain such as E. coli HB101.

RESULTS AND DISCUSSION

Principles and construction of a dual containment system

The gene containment system developed in this work is based on a double toxin–antitoxin genetic circuit. In the donor cell, two lethal functions (toxins) encoded by genes closely associated with the target DNA whose containment is required are inhibited by the cognate immunity functions (antitoxins). The genes encoding the immunity functions are not linked to the target DNA, and therefore co-transfer of toxin and antitoxin is a highly unlikely event. On the contrary, transfer of the target DNA to a non-immune recipient cell will be accompanied by transfer of the lethal genes, thus causing cell death and preventing further spread of this particular trait. To engineer such a containment system, we have used the colicin E3/immunity E3 (Masaki & Ohta, 1985) and the EcoRI endonuclease/EcoRI methyltransferase (Pingoud & Jeltsch, 1997) toxin–antitoxin pairs.

To design an E. coli host cell immune to both colicin E3 and the EcoRI endonuclease, the ecoRI gene expressed under control of the strong Pr promoter from the λ phage (Torres et al., 2000) was stably inserted into the chromosome of E. coli HB101immE3, a strain expressing the immE3 gene constitutively, and therefore immune to colicin E3. Since the resulting E. coli HB101immE3ecoRIM strain did not show any significant difference in growth characteristics from its parental HB101rif counterpart (see Methods), none of the immunity functions appears to be detrimental to the host cell under the conditions used.

On the other hand, we have previously cloned and expressed the ecoRIR lethal gene under the control of the broad-host-range Prrc promoter in the low-copy-number pVTBR vector, giving rise to the contained pVTRE plasmid (Fig. 1) (Torres et al., 2000). To clone and express the colicin E3 lethal function in an analogous system, plasmid pVTREC was constructed (Fig. 1). To develop a dual lethal cassette controlled by two different regulatory elements, we designed plasmid pVTRECA. This plasmid harbours a 4.5 kb NolI cassette containing the ecoRIR gene under control of the Lact repression Prrc promoter and the colE3 gene under control of the constitutive Pc promoter (Fig. 1). As anticipated, whereas crude extracts from HB101immE3ecoRIM (pVTRECA) showed colicin E3 activity by the spot test and EcoRI endonuclease activity in DNA restriction assays (see Methods), extracts from the control strain HB101immE3ecoRIM(pVTBR) did not. As shown with pVTRE (Torres et al., 2000), plasmids pVTREC and pVTRECA did not provide a significant growth disadvantage to the host cell HB101immE3ecoRIM when compared with the control plasmid pVTBR under the conditions used (see Methods). To demonstrate the functionality of the Pc promoter, transcriptional termination signals from the Ω interposon were cloned upstream of the Pc::colE3 fusion in plasmid pVTREC, giving rise to plasmid pVTRECA (Fig. 1). E. coli HB101immE3ecoRIM(pVTRECA) cells showed colicin E3 production, confirming that the Pc promoter was indeed driving the expression of the colE3 gene.

Single containment versus dual containment

To assess whether a dual containment system based on two different lethal functions acting on different cellular targets and controlled by different regulatory elements is significantly more efficient than containment systems based on each single lethal function, we compared the transformation frequencies of plasmids pVTREC, pVTREC, pVTRECA, and the control plasmid pVTBR using a recipient strain, E. coli XL-1 Blue, that lacks the immE3
and ecoRIM genes. Containment efficiency was measured as the reduction in plasmid transfer with respect to the transfer frequency of the pVTRB control plasmid. Electrotransformation was used as mechanism of plasmid transfer since it allows a high efficiency of DNA uptake in E. coli and it has been previously used to measure the efficacy of containment systems, showing containment levels similar to those obtained using conjugation as the mechanism of plasmid transfer (Diaz et al., 1994; Torres et al., 2000). As shown in Table 1, the efficacy of plasmid containment was about $10^4$ with the EcoRI-based containment system and $10^5$ with the colicin E3-based containment system. Since plasmid pVTRColIT behaved similarly to plasmid pVTRCol (Table 1), the expression of the colE3 gene from the Pc promoter seems to be as efficient as that from the tandem Ptrc-Pc promoters. The efficacy of containment of plasmid pVTRC (about $10^6$) was higher than that of plasmids pVTRE and pVTRCol (Table 1), demonstrating that the combination of the ecoRIR and colE3 genes enhances gene containment.

Nevertheless, the level of containment achieved by the combination of two different lethal functions with different regulatory signals did not reach the level expected by theoretical calculations, which predict that the efficiency of a dual containment system should be the product of the containment efficiencies due to each individual lethal function (Knudsen et al., 1995). To investigate the reason for the reduced efficiency of the dual containment system, we examined several E. coli XL-1 Blue transformants that were selected from three different electrotransformation experiments and that survived acquisition of plasmid pVTREC. Electrophoretic analyses revealed that plasmids isolated from ten different survival clones were smaller than the parental pVTREC plasmid (7–9 kb) (Fig. 2). Moreover, sequence analyses showed the existence of deletions that included both lethal genes and, in most cases, the lacIq regulatory gene (Fig. 2). Interestingly, inactivation of the lethal functions by integration of insertion sequences (IS) (Mahillon & Chandler, 1998), the most frequent mechanism

<table>
<thead>
<tr>
<th>Donor plasmid</th>
<th>Lethal gene</th>
<th>Selection marker*</th>
<th>No. of transformants†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVTRE</td>
<td>ecoRIR</td>
<td>Cm</td>
<td>1906 ± 538</td>
</tr>
<tr>
<td>pVTRCol</td>
<td>colE3</td>
<td>Cm</td>
<td>117 ± 28</td>
</tr>
<tr>
<td>pVTRColIT</td>
<td>-</td>
<td>Cm</td>
<td>145 ± 27</td>
</tr>
<tr>
<td>pVTREC</td>
<td>+</td>
<td>Cm</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>pVTRECA</td>
<td>+</td>
<td>Cm</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>pVTRECA</td>
<td>+</td>
<td>Ap</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

*Cm, chloramphenicol; Ap, ampicillin.
†The values indicate the number of antibiotic-resistant transformants obtained in the total volume of the electrotransformation (1 ml). Electrotransformation experiments were performed as described in Methods using 10 ng plasmid and E. coli XL1-Blue as recipient strain. Data are the means of values from three independent experiments ± SD. Control: 10 ng of control plasmid, pVTRB (ecoRIR, colE3), produced $6 \times 10^7 ± 2 \times 10^7$ transformants in the total volume of the electrotransformation.

![Fig. 2. Analyses of the plasmids isolated from E. coli clones that have acquired the pVTREC plasmid. Genes are indicated with blocks. The Ptru and Pc promoters are also indicated. Black blocks and continuous lines represent DNA regions that are present in the analysed plasmids; striped blocks and discontinuous lines represent deleted regions. The number of amino acids encoded by the 3' end of the truncated colE3 gene present in plasmids isolated from clones M3 to M8 is also indicated. oriV, origin of replication of plasmid pSC101.](http://mic.sgmjournals.org)
of survival of the EcoRI-based gene containment system in \textit{E. coli} (Torres et al., 2000), was not observed with the dual containment circuit developed in this work. In contrast, deletions involving both lethal genes in pVTREC led to the formation of clones that escaped killing. The different mechanism of mutation in the host cells to inactivate a single lethal function versus a dual lethal cassette reflects the fact that whereas IS-dependent inactivation of two lethal functions controlled by different regulatory signals would require two mutational events, simultaneous deletion of the two lethal functions requires only a single mutational event. Our results show that the frequency of deletion events in a particular organism will determine the efficiency of containment when using a lethal cassette harbouring more than one killing gene. A genetic circuit such as the one presented here can be also of great utility to study the role of mutations in microbial adaptation to adverse conditions.

Inactivation of a dual containment system via a single deletion event involves the loss of the DNA region located between the lethal genes. Therefore the cloning and expression of a particular trait flanked by two lethal genes would assure that the spread of the trait to potential recipients will be reduced even in case of inactivation of the dual containment system via deletion. To demonstrate this, we have cloned the \textit{bla} gene, encoding the \beta-lactamase of the \textit{Tn3} transposon as a model trait (Brosius \textit{et al.}, 1982), in the \textit{ecoRIR–colE3} intergenic region of plasmid pVTREC. The expression of the \textit{colE3} and \textit{ecoRIR} genes in the resulting plasmid, pVTRECA (Fig. 1), was confirmed by the spot test and by \textit{in vitro} DNA restriction assays, respectively. Plasmid pVTRECA was transferred to \textit{E. coli} XL-1 Blue cells by electroporation, and transformants were selected by growth on rich medium containing either chloramphenicol (selects for the Cm’ marker of the plasmid, which is not flanked by the lethal genes) or ampicillin (selects for the contained \textit{bla} trait). As observed with plasmid pVTREC, chloramphenicol-resistant transformants with pVTRECA appeared with a frequency of about $10^{-6}$ compared to that of the control plasmid pVTRB (Table 1), and all harbourered plasmids of smaller size than pVTRECA. The fact that none of these chloramphenicol-resistant transformants was able to grow in ampicillin-containing medium, and none showed colicin E3 and EcoRI endonuclease activities, is in agreement with the existence of deletions embracing both lethal genes and the target trait to plasmid pVTRECA. When three different electrottransformation experiments carried out with plasmid pVTRECA were plated on ampicillin-containing LB medium, we only observed the appearance of two transformants, indicating that the frequency of transfer of the target trait (\textit{bla} gene) was as low as $10^{-6}$ (Table 1). Thus, these data reveal that the horizontal spread of a target gene flanked by the \textit{ecoRIR} and \textit{colE3} genes in a dual containment cassette is reduced by a factor ($10^6$) that is close to the anticipated containment level due to the combination of colicin E3 (provides a containment of $10^3$) and the EcoRI endonuclease (provides a containment of $10^4$).

When the two \textit{E. coli} XL-1 Blue ampicillin-resistant transformants that survived acquisition of plasmid pVTRECA were analysed, we observed the existence of a similar plasmid whose size was larger than 8-9 kb. Sequence analysis of this plasmid showed the presence of two IS1A insertion sequences (Umeda \& Ohtsubo, 1991) that had integrated in both the \textit{colE3} and \textit{ecoRIR} genes, but that maintained the \textit{bla} gene unmodified. The existence of two IS sequences in the mutated pVTRECA plasmid caused high instability, and different plasmid rearrangements were observed after several rounds of cultivation of the ampicillin-resistant clones that escaped killing (data not shown). In summary, these data show that two different mutational events, e.g. integration of IS elements within the lethal genes, rather than a single mutation, e.g. deletion of both lethal genes, are necessary to inactivate a dual containment cassette designed to prevent gene spread of a target trait flanked by the lethal genes.

**Conclusion**

We have demonstrated here that the combination of different lethal functions acting on different cellular targets and controlled by different regulatory signals is a valuable strategy to increase containment. Engineering the multiple containment system in mini-transposon vectors, which themselves exhibit non-detectable transfer frequencies when integrated into the host chromosome (de Lorenzo \& Timmis, 1994), should decrease further any spread of cloned traits to ecologically insignificant levels that cannot be detected even under optimal gene transfer conditions (Munthali \textit{et al.}, 1996b). This work also reveals that in a biological containment system the lethal genes should be placed at different locations to avoid their inactivation through a single deletion event. It was shown previously that the efficiency of a biological containment system can be reinforced by using a host strain with a genetically engineered background (Ronchel \& Ramos, 2001). Therefore, the combination of such a strategy with a multiple lethal system like the one described here may be a suitable approach to achieve efficient programmed suicide for increasing containment of novel recombinant micro-organisms.

**ACKNOWLEDGEMENTS**

The help of E. Aporta with oligonucleotide synthesis, A. Díaz, G. Porras and S. Carbajo with sequencing, and the technical assistance of E. Cano, M. Carrasco and F. Morante are gratefully acknowledged. This work was supported by EU contract QLRT-2001-02884; by the Red del CSIC de Biorremediación y Fitorremediación, and by grants BMC2000-0125-C04-02 and GEN2001-4698-C05-02 from the Comisión Interministerial de Ciencia y Tecnología.

**REFERENCES**


