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ENVIRONMENTAL BIOTECHNOLOGY

A fluorescent, genetically engineered microorganism that degrades organophosphates and commits suicide when required

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Abstract One way to reduce the potential risk of genetically engineered microorganisms (GEMs) to the environment is to use a containment system that does not interfere with the performance of the GEM until activated. Such a system can be created by inserting a suicide cassette consisting of a toxin-encoding gene controlled by an inducible promoter. We constructed a GEM that can degrade organophosphorus compounds, emit green fluorescence, and commit suicide when required by putting the genes that control these different functions under different promoters. The genes for enhanced green fluorescent protein (EGFP) and organophosphorus hydrolase (OPH) were cloned downstream of the lambda P_L promoter in the plasmid pBV220. These genes could be expressed freely as long as the GEM was metabolizing because the repressor sequence *cIts857* had been deleted. The extracellular nuclease gene of *Serratia marcescens*, without its leader-coding sequence, provided the suicide mechanism. This was put under the control of the T7 promoter to form a suicide cassette activated by the presence of an environmental signal, in this case, arabinose. To improve the reliability of this containment system, the suicide cassette was duplicated within the conditional suicide plasmid. The plasmid carrying the EGFP and OPH fusion genes and that containing the suicide cassette were compatible and coexisted in the same host.

Keywords Genetically engineered bacteria · Suicide · Organophosphorus hydrolase · Green fluorescent protein

Introduction

Genetically engineered microorganisms (GEMs) have the potential to be an effective tool for cleaning up certain kinds of environmental contamination. However, their use has been limited because of the risk of genes moving from GEMs to other organisms and possible interference by GEMs with the distribution and growth of indigenous microbial populations. To minimize these risks, effective ways of both monitoring and limiting the survival of GEMs are required. Several methods for detecting the presence of GEMs in the environment have been developed, such as gene probes (Matheson et al. 1997), polymerase chain reaction (PCR; Khan et al. 1998), monoclonal antibodies (Ramos-Gonzalez 1992), bioluminescence (Ripp et al. 2000), and green fluorescent protein (GFP; Wu et al. 2000). Of these, only GFP can provide a direct indication of the presence or absence of GEMs without special instruments. GFP is a natural protein found in bioluminescent jellyfish that emits bright green fluorescence when exposed to UV light (Shimomura et al. 1962). Enhanced green fluorescent protein (EGFP) is a man-made red-shift variant

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of GFP that fluoresces when exposed to visible light, and its fluorescent intensity is 35 times stronger than that of GFP at 488 nm (Cormack et al. 1996). Because it requires no cofactors except oxygen (Lindow 1995) and can be fused with many other proteins without losing its capacity for fluorescence (March et al. 2003), EGFP is an ideal tool for detecting GEMs in the environment (Leff and Leff 1996; Gory et al. 2001).

Active biological containment (ABC) systems are designed to control the survival of bacterial populations. They are often based on lethal genes (e.g., porin-like proteins, nuclease, or streptavidin) that are triggered by specific physical or chemical signals in the environment, such as isopropyl-β-D-thiogalactopyranoside (IPTG; Knudsen et al. 1995), 3-methyl benzoate (Ronchel and Ramos 2001), or changes of temperature (Ahrenhotz et al. 1994). Although many virtual containment systems have been modeled on computers, few in vivo systems have actually been created (Jensen et al. 1993; Torres et al. 2003). An ABC system should be designed specifically for the particular GEM intended for release.

In this paper, we describe how we used the co-transformation of two plasmids containing different functional genes and promoters to successfully incorporate a containment system into a GEM that expressed a fusion protein of EGFP and organophosphorus hydrolase (OPH, E. C. 8.1.3.1). One of the plasmids was a conditional suicide plasmid containing two copies of the lethal nuclease gene of *Serratia marcescens* without the leader-coding sequence. By releasing nuclease, this plasmid was designed to cause degradation of the GEM's cellular DNA, thereby decreasing the likelihood of horizontal gene transfer from the GEM to other microorganisms in the environment (Ahrenhotz et al. 1994; Balan and Schenberg 2005). The other plasmid was a compatible carrier of the EGFP and OPH fusion gene, which was placed downstream of lambda P_L promoter and endowed the GEM with the capacity for both green fluorescence and to degrade organophosphorus compounds (OPs).

OPs are a broad class of neurotoxic chemicals frequently used as pesticides or chemical warfare agents. The removal of OP contamination is a global priority. OPH, discovered in the soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* spp. (Munnecke 1980), is able to hydrolyze a number of OPs (Chae et al. 1994; Lai et al. 1995) and is one of the most common enzymes used in GEMs for cleaning up OP residues (Wang et al. 2002; Kang et al. 2006).

An ideal GEM should survive only in the presence of its target substrate and die as soon as this has been sufficiently degraded. In a perfect GEM, the expression of lethal genes would be triggered by declining substrate concentration; however, we were unable to find a gene expression system controlled by OPs or OP-related substances. Therefore, we used a cheap and nontoxic gene inducer, arabinose, to trigger the expression of the lethal gene.

Materials and methods

Plasmid construction

Construction of the suicide mechanism The *S. marcescens* nuclease gene, minus its leader peptide-coding nucleotide sequence, was amplified using PCR from pAH12 (kindly gifted by Dr. W Wackernagel, Oldenburg University, Germany) and cloned in the *Nco*I and *Xho*I restriction sites of the pET-28b vector (primers used were no. 1 and no. 2, see Electronic supplementary material Table 1). The fragment containing the sequences of the kanamycin resistance gene, the *Serratia* nuclease gene, and T7 promoter were amplified by PCR (primers used were no. 3 and no. 4, see Electronic supplementary material Table 1) and ligated with a fragment of p15A origin that had been amplified by PCR from the plasmid pLysS (primers used were no. 5 and no. 6, see Electronic supplementary material Table 1). The resultant plasmid was designated pSS.

Using PCR, the fragment containing the sequences of the *Serratia* nuclease gene and T7 promoter was amplified again (primers used were no. 7 and no. 8, see Electronic supplementary material Table 1) and cloned in pSS to produce a plasmid with two copies of the *Serratia* nuclease gene, pDS (Fig. 1a).

Construction of the EGFP-OPH fusion protein The *egfp* gene, with its stop codon deleted, was amplified using PCR from pEGFP-N3 (Clontech, Palo Alto, CA, USA) and was cloned in the *Eco*RI and *Bam*HI restriction sites of plasmid pBV220 (kindly gifted by Ms R. Liu, Tianjin Medical College, China; primers used were no. 9 and no. 10, see Electronic supplementary material Table 1). The resultant plasmid was designated pBV-EGFP. Primers were designed to amplify the whole sequence of pBV-EGFP, except the *cIts857* gene which encodes the repressor protein for transcriptional control on plasmid pBV220 (primers used are no. 11 and no. 12, see Electronic supplementary material Table 1). The amplified fragment was self-circularized with T4 DNA ligase to generate the plasmid pL-EGFP.

The *opd* gene, which expresses OPH, was amplified from pGEM-T Easy-opd obtained from Dr. C. L. Qiao (Institute of Zoology, Chinese Academy of Sciences, Beijing, China; primers used were no. 13 and no. 14, see Electronic supplementary material Table 1). The PCR-amplified *opd* gene was then inserted between the *Bam*HI

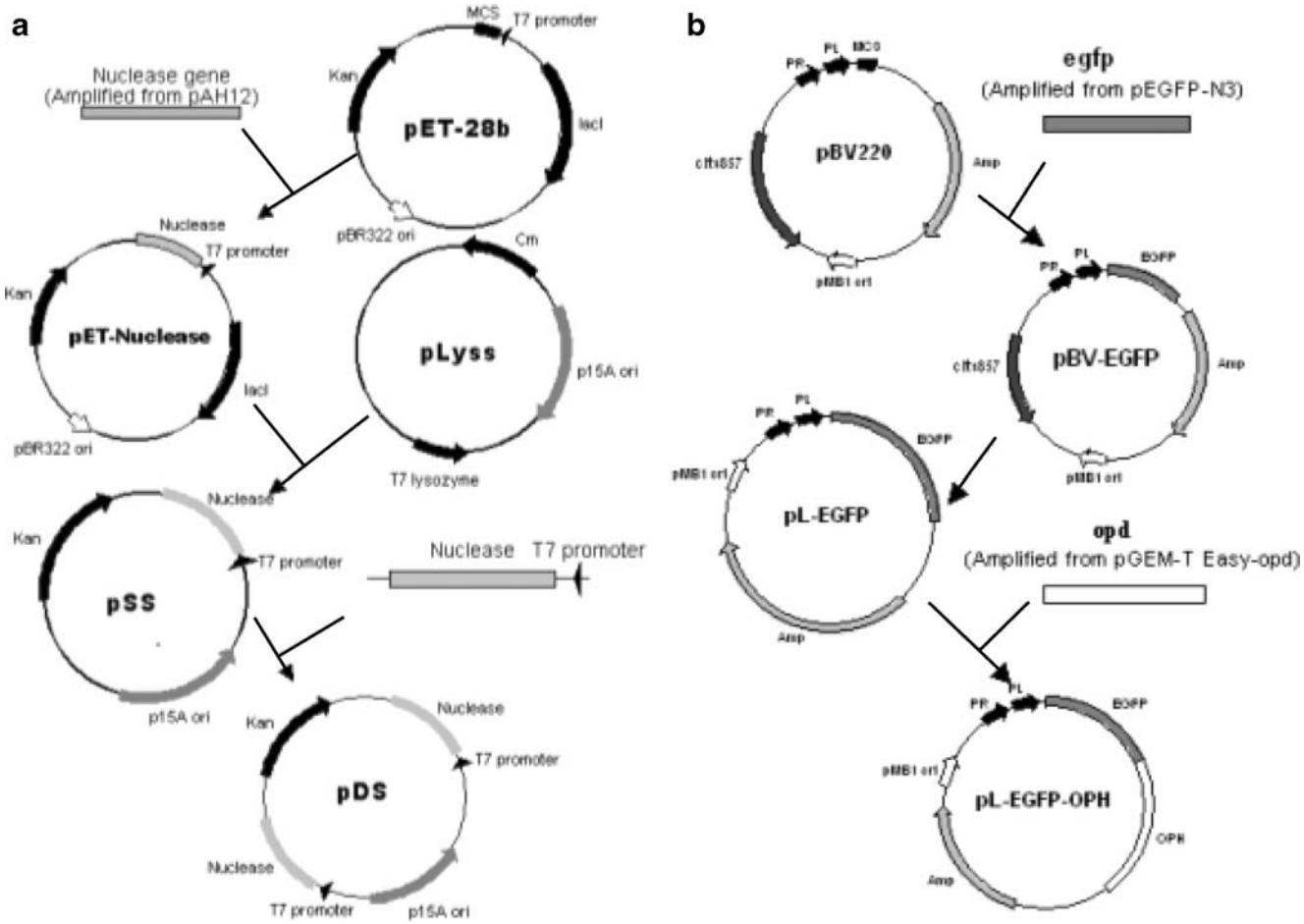


Fig. 1 Construction map of the recombinant plasmid. **a** Construction map of the containment plasmid pDS. The truncated nuclease gene of *Serratia*, the sequence of the kanamycin resistance gene, the T7 promoter, and the fragment of p15A origin were ligated together to generate the plasmid pSS. Then, a second copy of the sequence of the nuclease gene and T7 promoter was cloned in pSS to form the plasmid pDS. **b** The construction map of the plasmid pL-EGFP-OPH. The *egfp* gene without the stop codon was inserted into the plasmid pBV220 to make the plasmid pBV-EGFP. The whole sequence of pBV-EGFP except the *cIts857* gene was amplified by PCR and self-circularized to generate the plasmid pL-EGFP. Then, the *opd* gene was inserted to form the plasmid L-EGFP-OPH. P_R lambda P_R promoter, P_L P_L promoter, *MCS* multiple cloning sites, *ori* origin

and *Pst*I sites of pL-EGFP to obtain the plasmid pL-EGFP-OPH (Fig. 1b).

Construction of BL21AI-GOS

The plasmids pL-EGFP-OPH and pDS were sequentially transformed into the *Escherichia coli* strain BL21-AI™ (F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm araB::T7RNAP-tetA* (Invitrogen, Carlsbad, CA, USA) using the calcium chloride procedure (Sambrook and Russell 2001), producing a GEM (hereafter, BL21AI-GOS) that can emit green fluorescence, degrade OPs, and commit suicide when induced to do so. The ability to emit green fluorescence and degrade OPs is constitutive in this organism; only its suicide response requires induction. The inducer is arabinose because the used *E. coli* strain has the T7 RNA polymerase gene downstream the arabinose-inducible *araBAD* promoter.

Comparison of the efficiency of single- and dual-suicide systems

Overnight cultures of cells harboring either pDS or pSS (hereafter, BL21AI-DS or BL21AI-SS), and a control culture of cells without plasmids, were transferred (2%, *w*/*v*) into liquid Luria–Bertani (LB) medium. Cells of BL21AI-DS and BL21AI-SS were placed in medium containing 50 μg kanamycin per milliliter and the control culture in a non-selective medium. All cultures were then incubated at 28°C until their optical density (OD_{600}) was between 0.5 and 0.8 at the mid-growth phase. Each culture was then divided into two parts; in one part, the suicide response was induced by adding 0.01% (final concentration, *w*/*v*) arabinose, while the other part was not induced. Cultures were sampled every 2 h for 10 h, after which the optical density of all samples was measured and a plate experiment performed as follows:

The samples were diluted with non-selective LB medium till their OD_{600} reached 0.2, after which they were diluted 1:200 with the same medium. Finally, 150 μl of the final dilution of each culture was spread on an LB plate containing 50 μg kanamycin per milliliter in the case of cells harboring plasmids and on a non-selective plate in the case of the control culture. Plates were cultivated overnight (16–24 h) at 28°C, after which the number of bacterial colonies on them was counted.

Changes in BL21AI-GOS fluorescence and population growth curves

Seed cultures of BL21AI-GOS were transferred (2%, *w*/*v*) into liquid LB medium containing 50 μg kanamycin per milliliter and 50 μg ampicillin per milliliter and grown at 28°C in a shaker. When their optical density (OD_{600}) at the mid-growth phase reached between 0.5 and 0.8, the culture was separated into two parts. The suicide response was induced in one part by adding 0.01% (final concentration, *w*/*v*) arabinose, while the other part was not induced to serve as a control. Both parts continued to be incubated at 28°C and were sampled every 1.5 h over a 15-h period. The OD_{600} and fluorescence of samples were measured with a Beckman DU800 spectrophotometer (Foulerton, MN, USA) and Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan; excitation wavelength=488 nm, emission wavelength=509 nm, bandwidth=5 nm), respectively.

After 15 h of incubation, a plate experiment was performed as described previously, except that different selective plates were used and the colonies were viewed under both a Leitz DMIRB fluorescent microscope (Leica, GmbH, Wezlar, Germany) and by the naked eye in daylight.

Preparation of total cell protein

Cells of different cultures were centrifuged at 10,000×*g* for 1 min, washed with 50 mM Tris–HCl buffer (pH 7.8), resuspended completely in the same buffer (200 μl vs. starting volume of 1 ml culture), then sonicated on ice for about 2 min with an ultrasonic processor (Sonics & Materials, Danbury, CT, USA; power level=2–3, amplitude=40 Hz). The OPH activity of the resultant total cell protein mixtures (TCP) was later assayed. Protein concentration was determined using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

Western blotting analysis

Equal amounts of TCP (10 μg) were mixed with 4× sample buffer (250 mM Tris–HCl, pH 6.8), 40% (*w*/*v*) glycerol, 300 mM DTT, 8% (*w*/*v*) sodium dodecyl sulfate (SDS), 10% (*w*/*v*) β-mercaptoethanol, and 0.02% (*w*/*v*) bromophenol blue and boiled at 100°C for 3 min. Electrophoresis was performed on the resultant solution on 10% SDS-polyacrylamide gels (Laemmli 1970). After electrophoresis, gels were blotted onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) at 75 V for 120 min using the wet transblotting method. A primary anti-GFP monoclonal antibody (Clontech) was used at a 1:1,000 dilution. The secondary antibody, used at a 1:5,000 dilution, was a horseradish peroxidase-conjugated anti-mouse IgG (Sigma, St Louis, MO, USA). The Western blots were developed using standard ECL reagents (Pierce, Rockford, IL, USA) and imaged using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

OPH activity assay

The OPH activity of TCP and whole cells was assayed in 1 ml of 50 mM Tris–HCl buffer (pH 7.8) containing 3 μl of 50 mM parathion (99% purity, dissolved in ethanol). The TCP assay was performed on 10 μg of TCP and whole cell assay on a culture of whole cell suspension (WCS) with an OD_{600} of 0.1. The control was a culture without OPH activity. Change in absorbance (at 400 nm) was monitored continuously with a Beckman DU800 spectrophotometer for 2 h at 30°C.

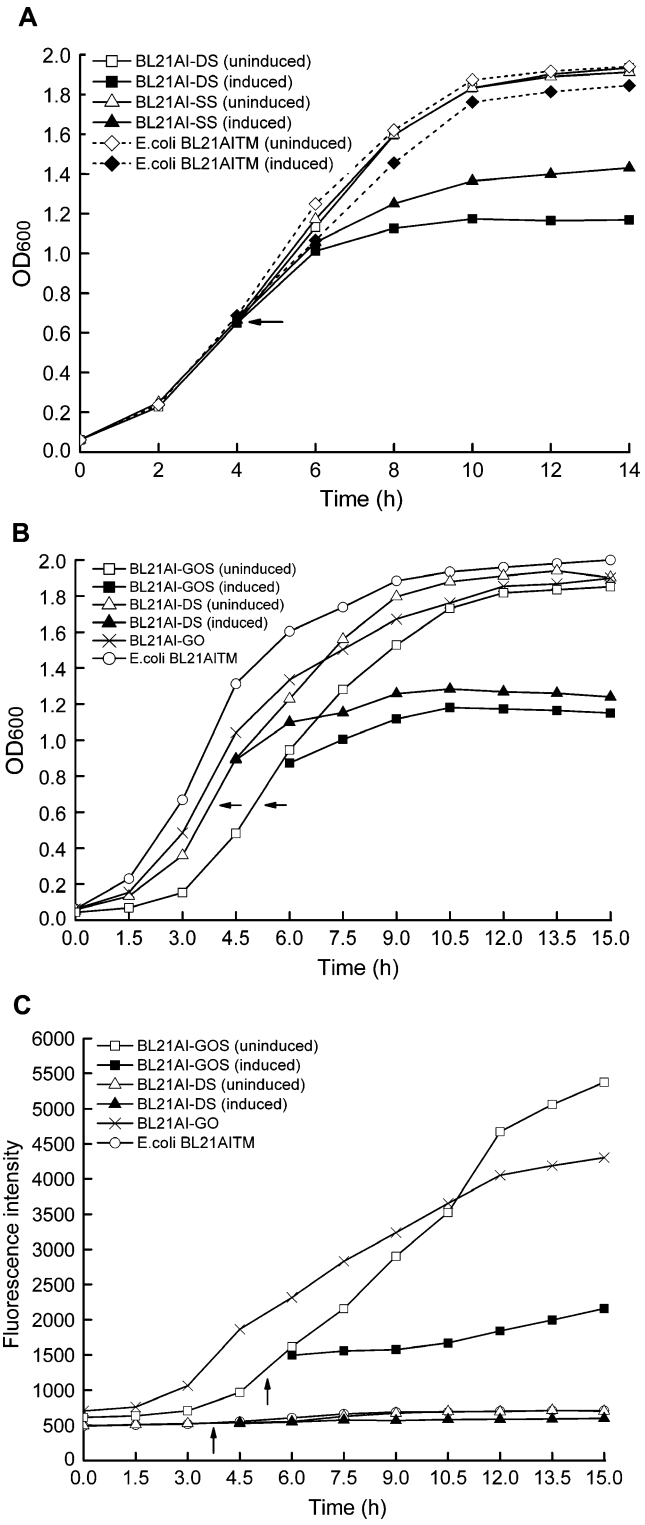
Results

Comparison of the effectiveness of dual (pDS) and single (pSS) suicide cassette plasmids

The growth of BL21AI-DS was distinctly slower than BL21AI-SS after induction of the suicide response with arabinose (Fig. 2a). The former had almost stopped propagating 4 h after the addition of arabinose, while the latter did not stop propagating until about 6 h later. This indicated that the pDS plasmid, which contained two copies of the lethal gene, was more effective in stopping cell division than the

Fig. 2 Growth curves and intensity of fluorescence of BL21AI-DS, BL21AI-SS, *E. coli* BL21AI™, BL21AI-GOS, and BL21AI-GO. **a** Growth curves of the bacteria. Cultures of BL21AI-DS, BL21AI-SS, and *E. coli* BL21AI™ were divided into two parts when the value of OD_{600} was between 0.5 and 0.8. The suicide response was induced in one of these by adding 0.01% arabinose, while the other was an uninduced control. The OD_{600} of all cultures were measured every 2 h for 10 h after induction. **b**, **c** Growth curves (**b**) and fluorescence intensity (**c**) of BL21AI-GOS, BL21AI-DS, and BL21AI-GO. Cultures of BL21AI-GOS and BL21AI-DS were divided into two parts when their optical density (OD_{600}) was in the range of 0.5–0.8. The suicide response was induced in one part, while the other served as a non-induced control. The optical density and intensity of fluorescence of all cultures were monitored every 1.5 h with a spectrophotometer and a fluorescence spectrophotometer, respectively. The *arrow* indicates the start point of induction

pSS plasmid, which contained only one copy. This was confirmed by the plate test. The number of both BL21AI-DS and BL21AI-SS colonies decreased on plates after induction. However, 10 h after induction, colonies of BL21AI-DS had almost all died, while a few colonies of BL21AI-SS continued to survive (Table 1). The control plates, colonies of BL21AI-DS, BL21AI-SS, and BL21AI™ that had not been induced, or BL21AI™ with arabinose, remained densely covered by >20,000 colonies (data not shown).



Growth curves and change in intensity of fluorescence

BL21AI-GOS grew more slowly than those of cells harboring just one plasmid. Following induction, the growth of cells containing the pDS plasmid (BL21AI-DS and BL21AI-GOS) was slower than the controls (Fig. 2b).

The intensity of fluorescence of uninduced BL21AI-GOS cultures, and that of cells harboring pL-EGFP-OPH (hereafter, BL21AI-GO), increased quickly, but the fluorescence of BL21AI-GOS cultures increased more slowly after induction, almost exactly matching its post-induction growth curves (Fig. 2c). Cells without the pL-EGFP-OPH plasmid displayed only a basal level of fluorescence.

To verify the effectiveness of the suicide mechanism, samples of each culture were collected at 15-h intervals and spread on different selective plates. No living colonies of BL21AI-GOS were found after induction on plates containing kanamycin, or both kanamycin and ampicillin, while 118±26 colonies were found on the plate containing ampicillin. The plates with BL21AI-GOS cultures that had not been induced were densely covered by about 20,000 colonies (data not shown).

Colonies of BL21AI-GOS and BL21AI-GO emitted strong green fluorescence when viewed under a fluorescent microscope (Fig. 3a, b). Green fluorescence was also detectable in these colonies with the naked eye in daylight (Fig. 3c, d). The Western blot test confirmed the existence of the fusion protein EGFP-OPH in BL21AI-GOS and BL21AI-GO (Fig. 4).

Degradation of parathion by BL21AI-GOS

OPH can hydrolyze parathion to *p*-nitrophenol, which displays a strong absorption band at 400 nm (OD_{400}) at pH 7.8. Because this absorbance is proportional to the moles of *p*-nitrophenol produced, it can be used to calculate how much parathion has been hydrolyzed. The results showed that TCP and WCS of BL21AI-GOS and BL21AI-GO all displayed catalytic activity. During the detection process, the

Table 1 Number of surviving colonies of BL21AI-DS and BL21AI-SS on plates after different periods of induction

Induction period (h)	2	4	6	8	10
BL21AI-DS	51±25	4±3	1±0	1±0	0±0
BL21AI-SS	1,097±191	80±10	27±2	17±2	2±1

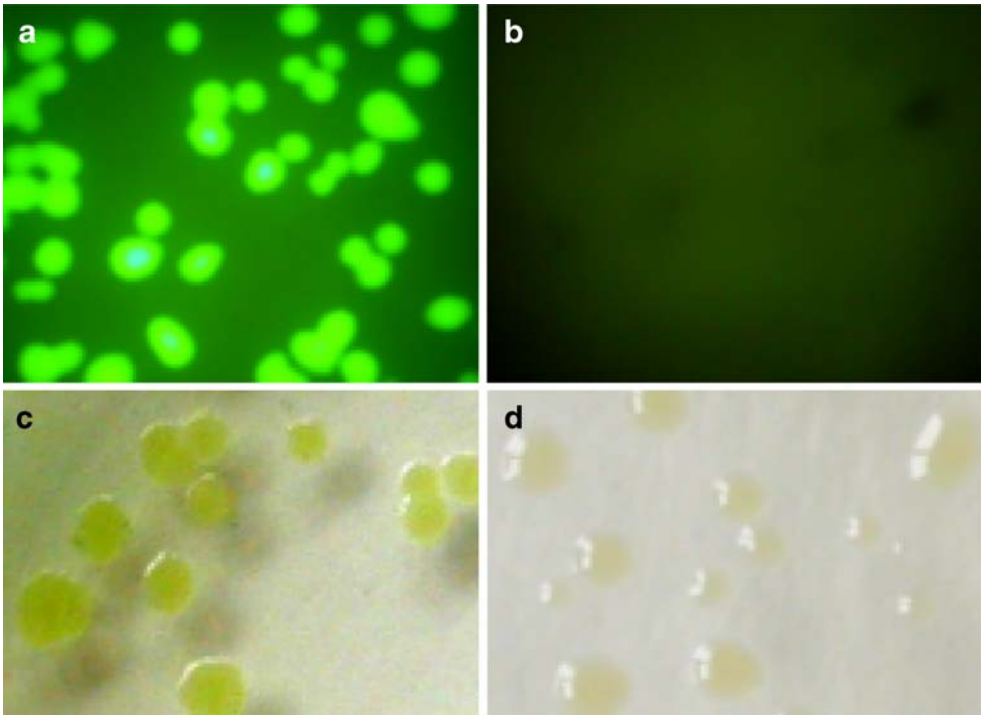


Fig. 3 Photographs of BL21AI-GOS. **a**, **b** Colonies of BL21AI-GOS (**a**) and *E. coli* BL21AI™ (**b**) on LB agar plates photographed through a fluorescent microscope (magnification, 10× 4). **c**, **d** Colonies of BL21AI-GOS (C) and *E. coli* BL21AI™ (D) on LB agar plates photographed directly in daylight (All photos were taken with a Nikon 4500 digital camera)

control organism (plasmid-free *E. coli* BL21AI™) degraded little amount of parathion (Fig. 5).

Both TCP and WCS of BL21AI-GOS showed better degradation ability than that of BL21AI-GO. After the first 50 min, about 37.26% and 20.06% of parathion had been degraded by TCP and WCS of BL21AI-GOS, respectively, compared to about 23.35% and 9.86% by TCP and WCS of BL21AI-GO, respectively. The initial hydrolysis rate of TCP of BL21AI-GOS and BL21AI-GO were about 1.12×10^{-4} and 0.70×10^{-4} μmol min^{-1} μg^{-1} protein, respectively.

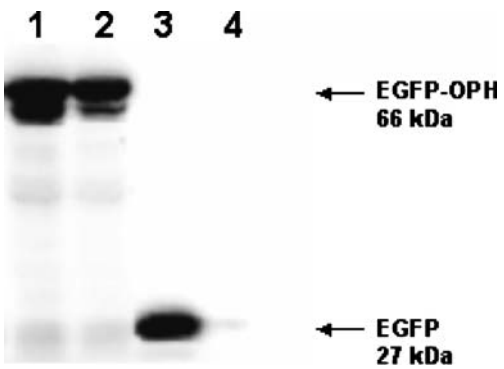


Fig. 4 Western blotting analyses of the total cell protein of BL21AI-GOS. Equal amounts of the total cell protein of BL21AI-GOS, BL21AI-GO or cells harboring pL-EGFP and plasmid-free *E. coli* BL21AI™ were separated by SDS-PAGE and detected with anti-GFP antibody and anti-mouse IgG antibody. *Lane 1* to *lane 4* samples of BL21AI-GOS, BL21AI-GO or cells harboring pL-EGFP and plasmid-free *E. coli* BL21AI™, respectively

Discussion

IPTG has been the most commonly used chemical inducer for the suicide of GEMs (Bej et al. 1988; Knudsen et al. 1995), but it is both relatively expensive and toxic. Finding a cheaper and safer inducer would therefore be beneficial for both researchers and the environment. The efficient suicide plasmid we constructed can be induced by arabinose, which is both relatively cheap and non-toxic. In this plasmid, the lower copy replicon p15A, the strong

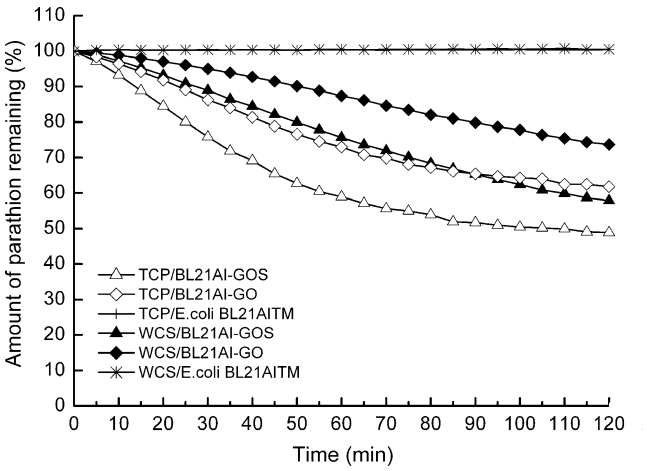


Fig. 5 Degradation of parathion by total cell protein and whole cells of BL21AI-GOS, BL21AI-GO, and plasmid-free *E. coli* BL21AI™. The assay of both total cell protein activity (*TCP*) and whole cell suspension activity (*WCS*) was performed at 30°C for 2 h and the amount of degradation of parathion was monitored continuously

T7 promoter, and dual-suicide cassettes were used to decrease the basal expression of the lethal gene, thereby improving the effectiveness of the suicide mechanism.

The duplication way was ever employed in suicide mechanism based on the lethal *E. coli rel*F gene (Knudsen et al. 1995). Both the previous study and our study showed that duplication of the lethal gene resulted in a quicker response than that obtained from a single copy. However, the *rel*F gene used in the previous study is a porin-like protein that may facilitate the release of recombinant DNA into the environment (Klemm et al. 1995), while the nuclease used in our study can destroy the genetic material of GEM, thereby decreasing the risk of recombinant DNA transfer (Ahrenhotz et al. 1994; Balan and Schenberg 2005).

The suicide mechanism was incorporated into BL21AI-GO, which had the pL-EGFP-OPH plasmid. The latter had a high copy replicon pMB1, which was compatible with the pDS plasmid. To make OPH gene expression independent of an inducer, we cloned the EGFP-OPH gene fusion downstream of the lambda pL promoter and deleted the copy of the lambda *cIts857* repressor from the plasmid pBV220. Luckily, no obvious growth burden was apparent when the functional plasmid pL-EGFP-OPH was transformed into the host.

The desired protein was the fusion protein EGFP-OPH, which has the capacity for both green fluorescence and OPH activity. After the resultant GEM had been incubated on a plate for several days, green fluorescence was visible with the naked eye in daylight. This feature allows colonies expressing the EGFP fusion protein to be identified on agar plates without a special light source, thereby greatly simplifying the screening of colonies. When the plasmids pL-EGFP-OPH and pDS were co-transformed into the host, its growth rate was slower than that of cells carrying just one. However, a slower growth rate did not reduce BL21AI-GOS's functional efficiency. The fluorescence and OPH activity of BL21AI-GOS was stronger than that of BL21AI-GO. This may be due to the difference in copy number, which could result in the difference in expressing quantity of desired protein.

The growth and fluorescence of BL21AI-GOS decreased following induction with arabinose. The plate experiment showed that the dual suicide system was more effective than the single system. However, although no colonies survived after induction on the plate containing kanamycin, about 0.5% colonies continued to survive on the plate containing ampicillin. Since kanamycin resistance was carried by the pDS plasmid, this indicates that some of BL21AI-GOS lost this plasmid during incubation. Although antibiotics and antibiotic resistance genes have traditionally been used for the selection and maintenance of recombinant plasmids in hosts such as *E. coli*, it is unpractical to use antibiotics to keep plasmid in GEMs in the field. To avoid the spread of antibiotic resistance traits to environmental microbes, it should have no antibiotic resistance genes in an ideal GEM. In recent years, there have been some reports about antibiotic-free plasmid selection and maintenance (Cranenburgh et al. 2001, 2004). In this new method, the lac operator sequence was used as a selectable element. When the strains are transformed with a multicopy plasmid containing the lac operator, the operator competitively titrates the LacI repressor and allows expression of *dap*D, an essential chromosomal gene, from the lac promoter. This new way of plasmid selection and maintenance may be applied to the design of GEMs to avoid the use of antibiotics and improve plasmid retention, which is also a good way to integrate the target genes in the host chromosome.

Besides plasmid retention, there was another problem which is the field induction of the suicide function. It seems to have some difficulties for large-scale spread of inducer at effective concentration in environment. However, a "population control" circuit that autonomously regulates the density of an *E. coli* population has recently been built by coupling gene expression to cell survival and death using cell–cell communication (You et al. 2004). By this way, the cell death rate was regulated by a bacterial quorum-sensing system, which needed no inducer during the whole course. It would be a great progress if one could apply this system to GEMs.

In summary, we believe that the present work has contributed with a novel strategy for containment of genetically modified strains of *E. coli*. We have also provided the first verification that duplication of the nuclease gene increases the effectiveness of the suicide response, a hypothesis questioned by Dr. Wackernagel in 1994 (Ahrenhotz et al. 1994). On the other hand, by deleting the cI repressor gene, we have made possible the constitutive expression of a heterologous gene under control of the lambda pL promoter. These findings have the potential to significantly improve both the efficiency and safety of GEMs. Further work is required to solve the problem of plasmid stability and the field induction to ensure usefulness of the suicide GEM in the environment.

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