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Use of Colicin E3 for Biological Containment of Microorganisms

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The genetic determinant of the lethal antibiotic colicin E3 was cloned under the control of a tightly regulated promoter in the absence of the gene for its cognate inhibitor. Combination of this killing cassette with a stringent regulatory element provided a substrate-dependent conditional suicide system that was exploited for the biological containment of a *Pseudomonas putida* strain. The lethality of a single gene copy and the distinct and universal cellular target of the antibiotic suggest colicin E3 as an ideal candidate for combination with other lethal functions to design highly efficient containment systems for microorganisms.

Environmental biotechnology involves the release of large quantities of microbial monocultures into particular habitats. However, the variable conditions inherent in the open environment render difficult the prediction of the behavior and fate of microbes in the field and have raised concerns about the potential environmental impact of such microbial releases (18). To increase the predictability of such microorganisms, different strategies have been developed to reduce the potential transfer of new genes from the introduced organisms to the indigenous microbiota, i.e., gene containment, and to restrict the released organism to the target habitat for a limited period of time, i.e., biological containment (1, 9, 10, 13, 15). Active biological containment systems are based on lethal functions whose expression is under the control of a regulatory element that directly accommodates the task of the microorganism in such a way that the lethal function does not interfer with the performance of the host cell but induces suicide once the application has been completed (13).

Colicin E3 is a 58-kDa RNase which cleaves 16S rRNA 49 nucleotides from its 3′ end, thereby eliminating the sequence complementary to the Shine-Dalgarno sequence of the mRNA, inactivating the 30S ribosomal subunit and inhibiting protein synthesis (7). In colicin E3-producing cells, the RNase activity is neutralized by the acidic 9.3-kDa immunity E3 protein encoded by the *immE3* gene (7). Although colicins naturally have narrow activity ranges, limited to *Escherichia coli* and closely related species, determined by the need for specific cell surface receptors for colicin binding, the target sequence for the colicin E3 RNase is universally conserved among the 16S-like rRNAs in all three primary kingdoms (12). As a result, ribosomes from distant bacterial genera (7), from chloroplasts (16), and even mammalian ribosomes (17) are inactivated by this RNase in cell extracts. Furthermore, we have recently shown that when colicin E3 is synthesized within target cells in the absence of immunity E3 protein, it has an efficient, broad-range lethal function (5).

In our colicin E3-based gene containment system, the lethality of a *colE3*-bearing promiscuous plasmid was neutralized by simultaneous expression in the recombinant cell of a chromosomally integrated *immE3* gene (5). We report here the further development of the colicin E3 lethal system to produce a colicin E3-based biological containment system through the construction of a viable ColE3$^+$ *immE3*$^+$ microorganism by coupling the expression of a single copy of the *colE3* gene to a stringent regulatory element derived from the *Pseudomonas putida* TOL catabolic pathway for the biodegradation of toluene and xylenes.

To engineer the colicin E3-based killing cassette, a 1.6-kb *Eco*RI-*Hin*dIII fragment containing the *colE3* gene without its native promoter (5) was subcloned into an *Eco*RI-*Hin*dIII double-digested pUHE24-2 vector (2) and the ligation mixture was used to transform E3 immune strain *E. coli* CC118*immE3*$^+$ (5). The resulting plasmid, pMMS1 (Fig. 1A), contains the *colE3* gene under the control of synthetic broad-host-range *lac* promoter $P_{A1/04/03}$, which is efficiently repressed by the LacI repressor (8). Two transcriptional terminators, t_0 from phage lambda and T1 from the *E. coli rrnB* operon, are located downstream of *colE3* and flank a chloramphenicol resistance selection gene. Figure 1B shows plasmid pCC102, which contains the regulatory element of the containment system, a fusion between the P_m promoter of the TOL *meta*-cleavage pathway operon and the *lacI* gene encoding the LacI repressor plus the *xylS2* gene, which encodes a positive regulator (XylSthr45) of P_m (3). The rationale of this strategy of containment is that in the presence of effectors of XylSthr45, such as 3-methylbenzoate (3MB), the LacI protein is synthesized and expression of the colicin E3 lethal function is prevented; once such an effector becomes depleted (through degradation) or the bacteria move to locations outside the polluted area, expression of the killing element is induced and the cells die. To construct $P_{A1/04/03}$::*colE3*-containing fusions in a variety of microorganisms intended to be released into the environment, plasmid pMMS3 was constructed (Fig. 1A) by using *E. coli* CC188*immE3*$^+$λpir (a CC118*immE3*$^+$ strain lysogenized with the λpir phage) as the host strain. Plasmid pMMS3 carries, within a mini-Tn*5* transposon which allows stable insertion of the killing cassette into the chromosomes of a variety of gram-negative bacteria (4), the $P_{A1/04/03}$::*colE3* fusion flanked by genes conferring resistance to streptomycin-spectinomycin and chloramphenicol. To integrate the killing element into the chromosome of *P. putida* KT2442 harboring pCC102, we performed triparental filter matings (4) by using *E. coli* CC118*immE3*$^+$ λpir(pMMS3) as the donor and *E. coli* HB101(pRK600) (4) as the helper. *P. putida* KT2442(pCC102)::mini-Tn*5*Sm/Sp ($P_{A1/04/03}$::*colE3*) transconjugants, hereafter designated *P. putida* KT2442-CSE3, were selected on M9 minimal agar plates (14) supplemented with 0.2% citrate, 1 mM 3MB, 50 μg of

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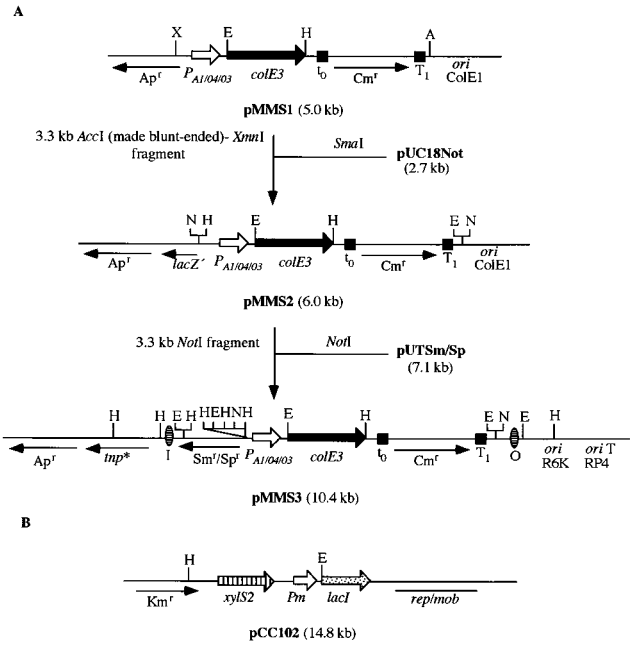

FIG. 1. Design of the lethal and regulatory elements of the containment system. (A) Strategy used to construct the lethal element. Plasmid vectors pUC18Not and pUTSm/Sp have been described previously (4). ⊖, 19-bp I and O termini of Tn*5*. *tnp**, gene devoid of *Not*I sites encoding Tn*5* transposase. (B) Regulatory element. Plasmid pCC102 has been reported by Contreras et al. (3). Arrows represent the promoters and the direction of gene transcription. Black squares are the t_0 and T_1 transcriptional terminators. Replication origins *ori*-ColE1 and *ori*R6K and RP4-mediated mobilization functions *mob* and *ori*TRP4 are also indicated. Relevant restriction sites: A, *Acc*I; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; X, *Xmn*I. Antibiotic resistances: Ap^r, ampicillin; Cm^r, chloramphenicol; Km^r, kanamycin; Sm^r/Sp^r, streptomycin-spectinomycin.

kanamycin per ml, and 1 mg of spectinomycin per ml, which were incubated at 30°C. Control strain *P. putida* KT2442 (pCC102)::mini-Tn*5*Sm/Sp, hereafter designated *P. putida* KT2442-CS, was obtained in a similar way by using *E. coli* CC118λpir(pUTSm/Sp) (4) as the donor.

To assess the efficacy of the colicin E3-based conditional suicide system, we analyzed the survival of *P. putida* KT2442-CSE3 and KT2442-CS in LB medium (14) supplemented with either 0.1 mM 3MB (induces *lacI* expression) or 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (*lacI* expression prevented and internal pool of LacI repressor inactivated). As shown in Fig. 2, while control strain *P. putida* KT2442-CS grew in both media at similar rates, contained strain *P. putida* KT2442-CSE3 grew normally in the presence of 3MB but not in medium supplemented with IPTG. These results show that the colicin E3-based conditional suicide system functioned and was regulated as intended.

It should be noted that cultures of *P. putida* KT2442-CSE3 incubated in the presence of 2 mM IPTG resumed growth after prolonged (16 h) incubation (data not shown), which suggests that a subpopulation of cells escaped suicide. By plating these cultures onto LB–kanamycin–0.1 mM 3MB and LB–kanamycin–2 mM IPTG plates, we determined that the fraction of cells surviving suicide was about 5×10^{-4}. To study the kinetics of suicide, aliquots of cultures of *P. putida* KT2442-CSE3 incubated in the presence of 2 mM IPTG were taken at different times and plated on LB–kanamycin–0.1 mM 3MB plates to identify cells still able to form colonies. The greatest reduction in CFU, about 3 orders of magnitude, was obtained 4 h after addition of IPTG (data not shown). To gain insight into the mechanism of survival of the lethal action of colicin E3, 20 suicide-negative mutants of *P. putida* KT2442-CSE3 were analyzed. All contained a plasmid identical in size to pCC102, which ruled out the possibility of loss of the regulatory element. To test whether this element was still functional in the mutants, plasmids were transformed into *E. coli* CSH36, a *lacI* $lacZ^+$ strain (Cold Spring Harbor Laboratory) and transformants were selected on LB-kanamycin plates containing the chromogenicsubstrate5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in the presence or absence of 5 mM 3MB. In all cases, blue and colorless colonies were detected in the absence and presence of 3MB, respectively, demonstrating that the regulatory element functioned as expected since 3MB induced the expression of P_m::*lacI*. By the method of Herschman and Helinski (6), we determined the basal levels of colicin E3 activity in cell extracts of mutants and parental *P. putida* KT2442-CSE3 incubated with 0.1 mM 3MB. While extracts from the parental strain gave a clear zone of growth inhibition when spotted onto a lawn of sensitive *E. coli* HB101 (14), extracts from the suicide-negative mutants did not. These results strongly suggest that spontaneous mutations in the killing element of the containment system are responsible for the small subpopulation surviving colicin E3 induction, a finding also reported for containment systems based on other lethal functions (8, 10, 13). Experiments done as described by Luria and Delbrück (11) indicated that the mutation rate to IPTG resistance was about 10^{-5} per cell per generation (data not shown).

Interestingly, when cultures of *P. putida* KT2442-CSE3 grown in the presence of 0.1 mM 3MB were resuspended in LB-kanamycin medium lacking both 3MB and IPTG, growth

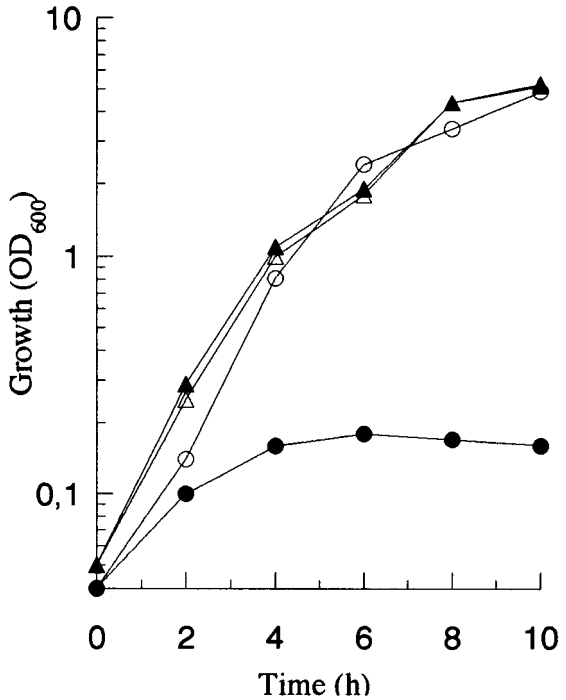

FIG. 2. Effects of 3MB and IPTG on the growth of contained and control bacteria. Cultures of *P. putida* KT2442-CSE3 (○, ●) and *P. putida* KT2442-CS (△, ▲) were grown overnight at 30°C in LB medium containing 50 μg of kanamycin per ml and 0.1 mM 3MB, washed three times, and resuspended in fresh LB-kanamycin medium to an optical density at 600 nm (OD_{600}) of 0.05. One half of each culture received 0.1 mM 3MB (○, △), whereas the other half received 2 mM IPTG (●, ▲).

was resumed (data not shown). However, when after 5 h of subsequent growth (five doublings) the culture was again resuspended at an optical density at 600 nm of 0.04 (about 3×10^6 CFU/ml) in fresh LB-kanamycin medium, its behavior was similar to that of cultures incubated in the presence of 2 mM IPTG (Fig. 2); i.e., there was inhibition of growth. This suggests that when 3MB is removed from the medium, *P. putida* KT2442-CSE3 cells have a large internal pool of LacI molecules which is diluted out or turned over only after several generations of growth. Addition of IPTG directly inactivates the LacI repressor in this pool and hence leads to much faster colicin E3 induction and subsequent arrest of growth. This particular behavior of the colicin E3-based containment circuit, not reported with the Gef lethal function (8), facilitates its use as a generation time-dependent suicide system. That is, the pool of LacI protein repressing the colicin E3 lethal function present at the time of disappearance of the benzoate inducer would presumably allow survival of the host cells long enough to complete their biotechnological purpose in the environment once they have been released. The feasibility of this concept will be investigated further.

In summary, we have reported here that the gene that encodes the universal lethal function of colicin E3 can be used in a single copy, stably integrated in the bacterial chromosome and in the absence of the E3-specific inhibitor, to engineer a conditional suicide system for biological containment of bacteria. Although we have used the response of *P. putida* to 3-MB as a model system, the colicin E3-based containment circuit can also be used to contain other microorganisms and can be engineered to respond to alternative environmental signals in accordance with the particular biological function to be achieved. Colicin E3 RNase thus becomes an interesting candidate for combination with other lethal functions acting on different cellular targets, e.g., the membrane-acting Gef family of proteins (13), to reduce the frequency of escape from the killing function in a multiple-suicide system and thereby increase the efficiency of containment. The validity of these systems remains to be assessed in the habitats for which they were designed.

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REFERENCES

1. **Ahrenholtz, I., M. G. Lorenz, and W. Wackernagel.** 1994. A conditional suicide system in *Escherichia coli* based on the intracellular degradation of DNA. Appl. Environ. Microbiol. **60:**3746–3751.
2. **Bujard, H.** Personal communication.
3. **Contreras, A., S. Molin, and J. L. Ramos.** 1991. Conditional-suicide containment system for bacteria which mineralize aromatics. Appl. Environ. Microbiol. **57:**1504–1508.
4. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
5. **Díaz, E., M. Munthali, V. de Lorenzo, and K. N. Timmis.** 1994. Universal barrier to lateral spread of specific genes among microorganisms. Mol. Microbiol. **13:**855–861.
6. **Herschman, H. R., and D. R. Helinski.** 1967. Purification and characterization of colicin E2 and colicin E3. J. Biol. Chem. **242:**5360–5367.
7. **Jakes, K. S.** 1982. The mechanism of action of colicin E2, colicin E3 and cloacin DF13, p. 131–167. *In* P. Cohen and S. van Heyningen (ed.), Molecular action of toxin and viruses. Elsevier Biomedical Press, Amsterdam.
8. **Jensen, L. B., J. L. Ramos, Z. Kaneva, and S. Molin.** 1993. A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli gef* gene. Appl. Environ. Microbiol. **59:**3713–3717.
9. **Klemm, P., L. B. Jensen, and S. Molin.** 1995. A stochastic killing system for biological containment of *Escherichia coli*. Appl. Environ. Microbiol. **61:**481–486.
10. **Knudsen, S., P. Saadbye, L. H. Hansen, A. Collier, B. L. Jacobsen, J. Schlundt, and O. H. Karlström.** 1995. Development and testing of improved suicide functions for biological containment of bacteria. Appl. Environ. Microbiol. **61:**985–991.
11. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28:**491–511.
12. **Noller, H. F.** 1993. On the origin of the ribosome: coevolution of subdomains of tRNA and rRNA, p. 137–156. *In* R. F. Gesteland and J. F. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Ramos, J. L., P. Andersson, L. B. Jensen, C. Ramos, M. C. Ronchel, E. Díaz, K. N. Timmis, and S. Molin.** 1995. Suicide microbes on the loose. Bio/Technology **13:**35–37.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Schweder, T., K. Hofmann, and M. Hecker.** 1995. *Escherichia coli* K12 *relA* strains as safe hosts for expression of recombinant DNA. Appl. Microbiol. Biotechnol. **42:**718–723.
16. **Steege, D. A., M. C. Graves, and L. L. Spremulli.** 1982. *Euglena gracilis* chloroplast small subunit rRNA. J. Biol. Chem. **257:**10430–10439.
17. **Suzuki, H.** 1978. Colicin E3 inhibits rabbit globin synthesis. FEBS Lett. **89:** 121–125.
18. **Wilson, M., and S. E. Lindow.** 1993. Release of recombinant microorganisms. Annu. Rev. Microbiol. **47:**913–944.