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Development and Testing of Improved Suicide Functions for Biological Containment of Bacteria

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We have developed very efficient suicide functions for biological containment based on the lethal *Escherichia coli relF* gene. The suicide functions are placed in duplicate within a plasmid and arranged to prevent inactivation by deletion, recombination, and insertional inactivation. The efficiency of this concept was tested in a plasmid containment system that prevents transfer of plasmids to wild-type bacteria. Protection against plasmid transfer was assayed in test tubes and in rat intestine. Protection was efficient and refractory to inactivation by mutation and transposons. The efficiency of the suicide system was also tested in soil and seawater. We show that unprecedented suicide efficiency can be achieved in soil and seawater after suicide induction by IPTG (isopropyl-β-D-thiogalactopyranoside). More than 7 orders of magnitude reduction in suicide bacteria was achieved.

As genetically engineered bacteria gain more widespread use in bioremediation, agriculture, and medical industry, built-in containment systems become more attractive. If the environmental impact of introduced or escaping bacteria can be minimized by suicide systems triggered by preprogrammed conditions, then the use of these bacteria becomes of less concern.

Descriptions of such suicide systems have been published previously (see reference 17 for a review). These systems have been based on lethal genes from *Escherichia coli* and have been triggered by tryptophan deficiency (18), plasmid transfer (11, 18), IPTG (isopropyl-β-D-thiogalactopyranoside) (2), and degradation of xenobiotic compounds (6). However, the deficiency of all these suicide systems is their inefficiency: a substantial fraction (often 10^{-4}) of bacteria is allowed to escape suicide even in model tests under optimal laboratory conditions. We have previously identified the factors limiting the efficiency of a *relF*-based suicide system to be mutational inactivation and selection of mutants (11). The mutation rate of a single suicide function was determined to be 10^{-6} per cell per generation. Leaky repression of suicide was demonstrated to lead to growth inhibition of suicide-positive populations, causing the selection of mutants that have lost suicide function. We described a two-plasmid system of duplicated suicide functions with a resulting reduction in mutation rates (11). This principle was applied to a suicide system in *Pseudomonas putida* in which two suicide systems were placed on the chromosome (9). This report describes the preparation and testing of a new single-plasmid suicide system that overcomes the problems of mutation and mutant selection. With efficiencies as high as those demonstrated, the concept of biological containment could gain more widespread use. To demonstrate the usefulness of this new system, we show its application to a plasmid containment system. Plasmid containment addresses the concern of transfer of genetically engineered traits to wild-type bacteria in the environment (21). The new plasmid containment system is tested both under controlled laboratory conditions and in a more complex environment, the rat intestine. We also test the new suicide functions in soil and seawater by inducing them with IPTG.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1. Plasmid pSK360 contains two *relF* suicide genes transcribed from the synthetic promoter $P_{A1\text{-}03/04}$ that are identical to previously described single-suicide functions (11). Plasmid pSK360 was constructed from (starting from bp 1 of pSK360): nucleotides (nt) 1 to 86 from pUHE21-2 (12), nucleotides AAT TCCC from pUC9 (1), nt 1075 to 1350 from the *relB* operon (1), nucleotides GCAGCCA from pUC9, nucleotides CAAG from *Hin*dIII linker, nucleotides CT from an *Xba*I linker, nt 1120 to 140 from pUHE21-2, nucleotides TGGCTGC from pUC9, nt 1350 to 1075 from *relB* operon, nucleotides GGGAATT from pUC9, nt 85 to 1 from pUHE21-2, nt 3414 to 3230 from pUHE21-2, nt 3832 to 1425 from pACYC184 (5), nucleotides AG from *Xba*I linker, nucleotides CTTG from *Hin*dIII linker, nt 3254 to 3848 from pBR322 (7), and nt 2903 to 3414 from plasmid pUHE21-2. A detailed description of plasmid pSK360, including DNA sequence and restriction sites, can be obtained from the authors upon request (or can be retrieved via Internet Gopher site dna.cedb.uwf.edu). The replication origin between the *Pvu*II sites of pSK360 was replaced by plasmid pBOE93 (a kanamycin-resistant RSF1010 derivative [18]), opened at the *Xmn*I site to yield the fusion plasmid pSK360::pBOE93. The control fusion with plasmid pBR322 was constructed with the *Eco*RI site of both plasmids to yield pBR322::pBOE93. BJ19 is a nalidixic acid-resistant derivative, selected on a nalidixic acid plate, of a wild-type *E. coli* isolated from rat feces.

Media. All media were NY rich media (11) unless noted otherwise. Solid media were NY medium with agar and antibiotics added (tetracycline, 20 mg/ml, and ampicillin, 100 mg/ml). IPTG (no. I5502; Sigma Chemical Co., St. Louis, Mo.) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma no. B4252) were added to concentrations of 1 mM and 40 mg/ml, respectively, where indicated. Filters used on top of solid media were nitrocellulose BA85.

Matings. Host strain BD3432 was mated on an NY plate as described previously (11) with either recipient CSH55 or the control recipient BD3434 with *lac* repressor.

Triparental matings. Donor strain BD3349 was grown to an optical density at 436 nm of 0.5 in NY medium, and 0.5 ml was mixed with 0.5 ml of recipient strain BD3347, harboring either pDW205, pSK360, or control plasmid pBR322 with the same optical density, and incubated without agitation for 1 h at the bottom of a 50-ml bottle at 37°C. A second recipient strain, XAC, was added (0.5 ml), and incubation was continued for 1 h. Ten milliliters of NY medium was then added, and incubation was continued for 2 h with agitation. Aliquots were plated on NY medium plus nalidixic acid plus kanamycin and NY medium plus nalidixic

TABLE 1. Bacterial strains and plasmids

E. coli strains or plasmid	Genetic markers	Origin	Reference
E. coli strains			
HB101	(*coliB*) *hsdS20*(r_B^-,m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Str^r) *xyl-5 mtl-1 supE44*		3
MC1000	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK strA*		4
BD3364	HB101/F′ *lacI*^{q1} *lacZ*::Tn*5* (*kan*)		11
S17.1	*recA1 pro thi hsdR hsdM*$^+$ (RP4)		20
BD3432	S17.1/pR2172 + pBOE::pSK360		This work
BD3379	S17.1/pBOE::pBR322		This work
BD3346	MC1000 *lacI*^{q1}		11
BD3347	MC1000 *lacI*^{q1} *recA1 srl*:Tn*10*(*tet*)		11
CSH50	Δ(*lac-pro*) *ara strA thi*		15
CSH55	Δ(*lac-pro*) *supE nalA thi*		15
BD3434	CSH55/pR2172		This work
BD3349	CSH50/F′ *lacI*::Tn*5*(*kan*) *lacZ*$^+$		This work
XAC	Δ(*lac-pro*) *argE*(Am) *nalA rif*		16
BJ19	Nal^r derivative of rat intestine isolate		This work
BD3392	BD3347/pSK360		This work
Plasmids			
pUHE21-2	*bla* $P_{A1-03/04}$	ColE1	12
pACYC184	*cat tet*	p15A	5
pSK360	*bla tet* 2 × ($P_{A1-03/04}$-*relF*)	ColE1	This work
pDW205	*bla lac* P_{UV5}-*relF*	ColE1	11
pR2172	*bla lacI*^{q1} (pBR322 *tet*::*lacI*^{q1})	ColE1	11
pBOE93	RSF1010 *kan*	IncQ	17
pBR322	*bla tet*	ColE1	7
pSK360::pBOE93	*bla tet kan*	IncQ	This work
pBR322::pBOE93	*bla tet kan*	IncQ	This work

acid plus ampicillin plus tetracycline, counting plasmid transfer rate as the number of transferred plasmids per transferred F′.

Enhanced triparental mating. The enhanced triparental mating experiment was performed as the triparental mating experiment described above except for the following. Transconjugants from the first mating were enriched on NY medium plus kanamycin plus tetracycline overnight before mixing with the second recipient. This modification increased the rate of control plasmid transfer, thereby lowering the detection limit. The transfer rate of the suicide plasmid was below the detection limit, so the detection limit is given.

Fluctuation experiment (14). Twenty-two cultures of approximately 1,000 cells of BD3364/pSK360 in 10 ml of NY medium were grown to 5×10^8 cells per culture. The cultures were harvested by centrifugation and plated on agar plates containing the inducer IPTG and the chromogenic β-galactosidase substrate X-Gal as described previously (11).

Suicide kinetics. BD3364/pSK360 was plated on nitrocellulose filters (BA85) on NY plates containing IPTG. At intervals, filters were transferred to plates without IPTG for incubation.

Cointegrate transfer. Donor strain BD3432, harboring cointegrate pSK360::pBOE93, was mated on a plate as described previously (11) (0.2 ml each of donor and recipient was mixed on NY plates and incubated for 12 h before resuspending and plating dilutions on selective plates) with either recipient strain CSH55 or BD3434 (*lacI*^{q1}).

Plasmid transfer in the rat intestine. To allow sufficiently high concentrations of donor and secondary host strains to detect transfer, germ-free (Han:Wist; Zentralinstitut für Versuchtierzucht, Hannover, Germany) rats were caged individually in isolators and fed KGy-irradiated Altromin 1314 (Brogaarden Gentofte, Denmark) and water ad libitum. They were fed donor strain BD3432 (1 ml of 10^8 cells per ml) by gavage three times over 7 days. The density of donor bacteria in the feces reached 10^8 CFU per g of feces. Then, on day 12, a recipient (BJ19) was added, and transconjugants were monitored in the feces for 7 days. Feces were sampled from the rectum, and dilutions were plated on selective plates. As a control, the plasmid fusion pBR322::pBOE93 in S17.1 was added on day 7 in control experiments. Plasmid transfer rates are averages of five experiments.

Seawater experiments. Seawater was collected from Santa Rosa Sound (estuary, 2.6% NaCl) in the vicinity of Pensacola Beach, Fla., and filter sterilized (0.22-μm-pore-size cellulose acetate; Corning Corp., Corning, N.Y.). BD3392 cells were grown in Luria broth (LB), inoculated at less than 10^4 cells, harvested at the exponential phase, washed in seawater, and resuspended in seawater to about 10^7 cells per ml. The seawater was incubated in separate tubes (1 ml in capped autoclaved Pyrex 9820 tubes [18 by 150 mm]; Corning) at 30°C without shaking. To half of the tubes, IPTG was added to a final concentration of 0.5 mM. To half of the tubes with and without IPTG, glucose was added to a final concentration of 2% (wt/vol). Samples were taken periodically (two tubes were sacrificed for each sampling), diluted in 0.9% NaCl, and plated on LB plus ampicillin (100 μg/ml; Sigma) plus tetracycline (20 μg/ml; Sigma) plates (LAT plates; for total CFU determination). Low colony counts were determined either by plating 50 ml directly from the seawater on LAT plates or by filtering one to five tubes, each containing 1 ml, and layering the filter (0.2-μm-pore-size, 47-mm polycarbonate membrane filters; Poretics Corp., Livermore, Calif.) on LAT plates. The dilution factor obtained by plating directly from seawater containing IPTG (a 500-fold dilution) was sufficient to enable growth of IPTG-sensitive cells.

Samples were also plated on the same type of plates supplemented with 1 mM IPTG (LATI plates) to score for suicide-minus mutants.

Experiments in nonsterile seawater were performed in the same way, except that the seawater was not filter sterilized and samples were not taken in duplicate.

In experiments to test suicide in large cultures (see Fig. 7), 10 ml of seawater was inoculated with cells from outgrown cultures (stationary phase, $>10^9$ cells). These seawater tubes were incubated at room temperature and sampled once each day.

Soil experiments. One gram of potting soil (Hyponex Corp., Marysville, Ohio; sieved through a 2-mm-pore-size aluminum mesh) was added to glass culture tubes with caps (same as those described above) and sterilized by autoclaving. BD3392 cells were grown in LB medium plus antibiotics as described above, harvested in the exponential phase, and resuspended in sterile 0.9% NaCl. Then, 0.3 ml of cells in saline solution was added to each tube, and the tubes were incubated at 30°C. To some of the tubes, IPTG and/or glucose was added, together with the cells, to estimated final concentrations in soils of 2 mM and 2% (vol/vol), respectively. The added liquid made the soil moist without any liquid phase. CFU counts were determined by adding 3 ml of sterile 0.9% NaCl to a tube (two tubes were sacrificed for each sampling), vortexing for 60 s, letting settle for 60 s, and diluting in 0.9% NaCl. Dilutions were plated on LAT for total CFU and on LATI for enumerating suicide-minus mutants.

Statistical treatment of data. All sampling for Fig. 5 and 6 was performed in duplicate, sacrificing two tubes for each datum point. The mean value and standard deviation of these two numbers were used in the figures. When one sampling yielded colonies and the duplicate did not, the observed CFU and the detection limit were used for the mean and standard deviation calculations.

For practical reasons, IPTG was mixed with resuspended bacteria and glucose on ice and then immediately added to the seawater or soil experiments. The first sample was taken as quickly as possible (within 5 min), and yet the CFU counts in the tubes with IPTG were slightly lower than the CFU counts in the tubes with no IPTG. These results indicate that suicide had already begun before the first sample was taken, probably in the tubes while the sample was being extracted. The figures show the same CFU counts at time zero for experiments with and without IPTG because all experiments were inoculated from the same culture. The plating of samples directly from cultures containing IPTG diluted the IPTG 500 times and did not prevent growth of IPTG-sensitive cells. This was determined by comparing the colony counts on plates with direct plating with the colony counts on plates where the sample had been diluted 100 times prior to dilution (i.e., diluting the IPTG 100 times). In all experiments reported, the two numbers were comparable when corrected for the dilution factor, showing that the IPTG concentration had no effect on colony counts in this concentration range.

RESULTS

Preparation of a suicide plasmid with duplicated lethal system. To reduce the inactivation rate of the suicide function, a suicide gene was duplicated within a plasmid to yield plasmid pSK360 (Fig. 1). The suicide function is based on the lethal *relF* gene from *E. coli* (8). The function of this gene remains elusive, but it rapidly kills the cell when expressed from a foreign promoter. We used the synthetic promoter $P_{A1-03/04}$ (12), which is efficiently repressed by the *lac* repressor. The two copies of this suicide function were arranged in such a way that no single mutational event (deletion in particular) can lead to inactivation of both. A deletion including *ori* would lead to loss of plasmid replication, and a deletion including the *bla* and *tet* genes would lead to loss of selective markers (as well as any gene that is to be contained, which should be cloned in one of these markers). As depicted in Fig. 2, suicide can be induced within the host cell by adding the gratuitous inducer of the *lac* operon, IPTG. After such an IPTG induction, the fraction of cells surviving suicide can be counted by plating on media in the absence and presence of IPTG. To obtain a statistical

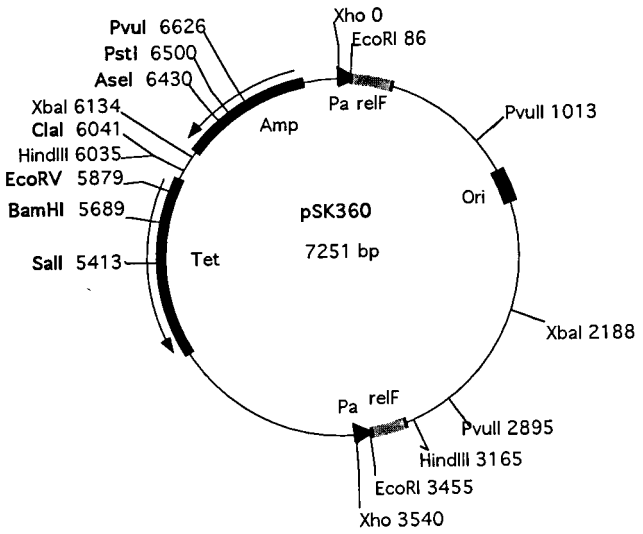

FIG. 1. Plasmid pSK360 containing duplicated killing functions. Unique sites suitable for cloning are indicated in boldface letters.

measure of the survival rate, a Luria-Delbrück fluctuation test was performed (11, 14): several IPTG plates were counted in parallel to quantify the zero fraction of the Poisson distribution describing the chance of mutation.

A fluctuation test was performed with BD3364/pSK360 as described in Materials and Methods. The host strain BD3364 contained the high-yield *lac* repressor gene $lacI^{q1}$, which is necessary for suicide repression, on an F′ plasmid. The mutation rate to IPTG resistance was calculated to be 10^{-8} per cell per generation (mutants were scored on two of nine plates, with 10^8 cells plated on each plate). However, the presence of the chromogenic substrate X-Gal in the plates allowed us to distinguish colonies that survived because of inactivated suicide functions (the chromosomal *lacZ* gene allowed X-Gal cleavage and, hence, a blue phenotype after IPTG induction) (Fig. 2) from colonies with superrepressor mutations in the *lacI* gene, rendering the repressor insensitive to IPTG (resulting in a white phenotype since the chromosomal *lacZ* could not be induced). Repeated experiments showed only white colonies (i.e., superrepressor mutants) and never any blue colonies. From the absence of blue colonies on all plates (21 plates) and the number of cells plated (5×10^8 per plate), the inactivation rate of both suicide functions is estimated to be less than 10^{-10} per cell per generation. This number is relevant for applications that do not rely on IPTG-induced suicide, for example, suicide after plasmid transfer.

The fluctuation tests described above were performed in a *recA* host. To determine whether homologous recombination between the suicide functions catalyzed by RecA has any effect, the fluctuation test was repeated in a $recA^+$ strain (BD3346). In this experiment, blue colonies occurred at a rate of approximately 10^{-8} per cell per generation, indicating that both suicide functions were inactivated at this rate. The homologous recombination, which the $recA^+$ strain allows, should lead only to inversion of the origin of replication. The increased frequency of loss of both suicide functions could be explained by mutation of one killer gene and then by loss of the other via gene conversion in connection with the recombination. One caveat is that the $recA^+$ and *recA* strains used for comparison are not otherwise isogenic. Thus, the difference in inactivation rate cannot be attributed conclusively to the RecA protein.

Suicide kinetics. The fluctuation experiments described above, in which suicide is defined as the absence of colonies on

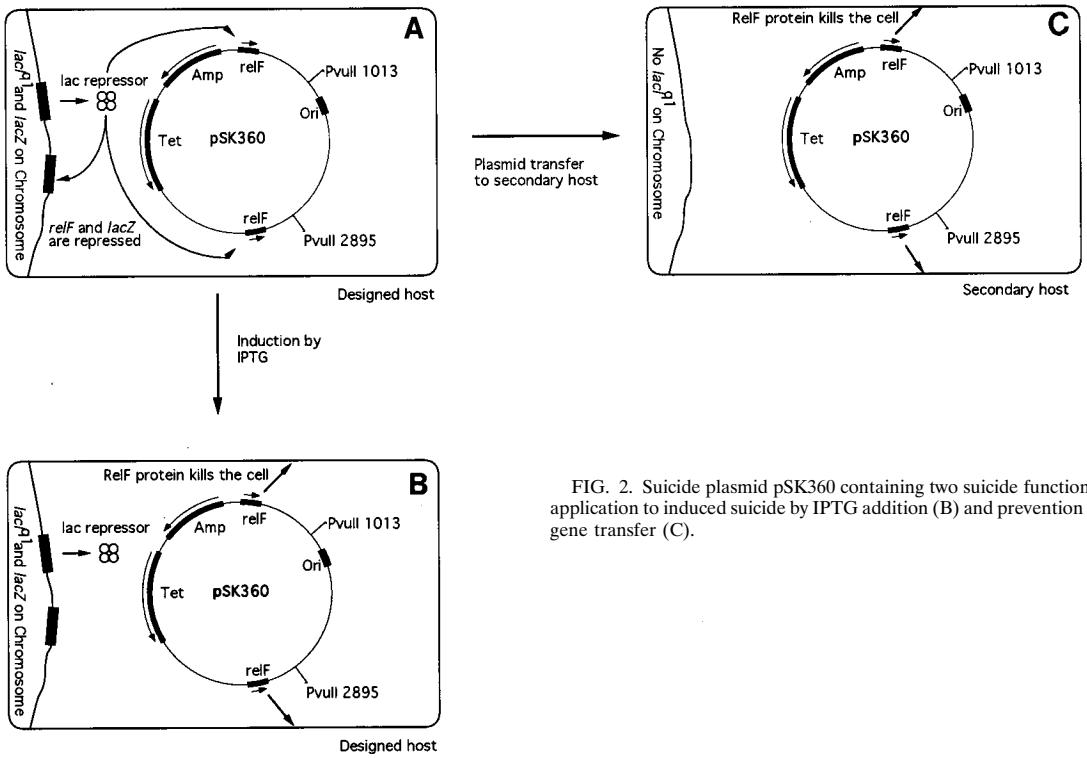

FIG. 2. Suicide plasmid pSK360 containing two suicide functions (A) and its application to induced suicide by IPTG addition (B) and prevention of horizontal gene transfer (C).

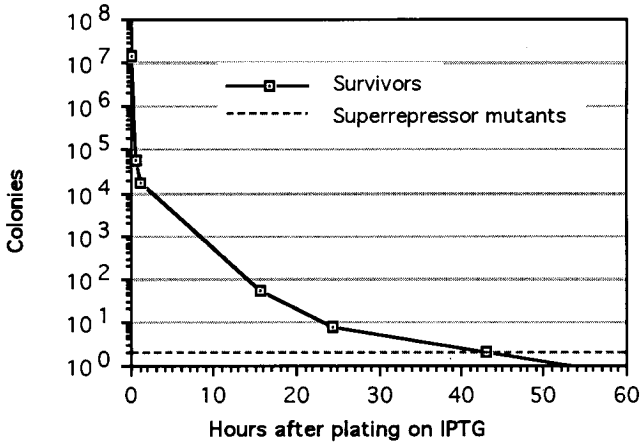

FIG. 3. Suicide kinetics after IPTG induction of strain BD3392, which carries plasmid pSK360. After different incubation times on IPTG plates, cells on filters were moved to plates without IPTG, and colonies formed after incubation were counted.

plates containing IPTG, do not provide any information on the kinetics of suicide nor do they distinguish between bacteriostatic and bacteriocidal effects. To gain insight into the exact kinetics of the suicide process, BD3392 cells were plated on filters placed on plates containing IPTG. At different times, these filters were transferred to plates without IPTG to identify cells that were still able to form colonies (Fig. 3). The results confirm that the suicide function is bacteriocidal but that it takes 40 to 50 h before the most persistent fraction of 10^{-8} cells disappears. Superrepressor mutants form colonies on IPTG plates but remain constant in number throughout the experiment and can be distinguished from nonmutated colonies that do not form until after they are transferred to plates without IPTG.

Application to plasmid containment. The suicide plasmid pSK360, as described above, is derived from the plasmid pBR322. It is not self-transmissible, but there are several avenues of transfer to secondary hosts. Such transfer may be of concern if the plasmid carries cloned genes that can be transferred to secondary hosts in the environment. Plasmid pBR322 can be transferred to secondary hosts by transduction, transformation, or mobilization by conjugative plasmids (11, 13). Suicide functions on the plasmid can prevent or reduce the spread of recombinant DNA by killing transconjugants following transfer when suicide functions are derepressed in secondary hosts that lack *lacI*^{q1} (Fig. 2).

Prevention of cointegrate-mediated plasmid transfer. (i) Nutrient agar. In a triparental mating experiment, an F′ plasmid carrying two transposons entered the cell harboring suicide plasmid pSK360 or control plasmid pBR322. Transfer of pSK360 or pBR322 (by transposon-mediated cointegrate formation with F′ [10]) to the recipient was monitored by selecting for ampicillin and tetracycline resistance. Transfer of pBR322 (measured as the number of transferred plasmids per transferred F′ plasmid) was readily detectable (1×10^{-4}), whereas transfer of suicide plasmid pSK360 was below the detection limit ($<4 \times 10^{-7}$). For comparison, the single suicide function in plasmid pDW205 offered little protection against plasmid transfer (transfer rate, 10^{-5}), probably because the chance of transposon integration into the suicide function was very high (about 1 in 10 if random insertion were assumed).

To determine if any limit in the protection against plasmid transfer could be detected, the potential for transfer was enhanced by studying the transfer of a premade cointegrate between pSK360 and a self-transmissible RSF1010 derivative. This fusion yielded a conjugative suicide plasmid. Although the transfer rate of the pSK360::pBOE93 to the recipient with the *lacI*^{q1} repressor (control; BD3434) was very high (10^{-1} transconjugants per donor [BD3432]), transfer to the recipient that did not contain the repressor (CSH55) was below the detection limit ($<10^{-7}$ transconjugants per donor).

(ii) Rat intestine. The efficiency of the plasmid containment was tested in the rat intestine to demonstrate the efficiency of the suicide function in preventing the spread of recombinant DNA in vivo. The *E. coli* host has its normal habitat in mammalian intestines. Thus, we chose to test our plasmid containment in the rat intestine. However, to allow colonization of the intestine by the debilitated laboratory strains which we were using, germfree rats without detectable microflora had to be used. Figure 4A shows the mean results of five rat experiments using the control plasmid pBR322::pBOE93. The host with the control plasmid colonized the intestine (as monitored by colony counts on diluted rat feces); the recipient (nalidixic acid resistant) was then administered. Transconjugants were detected at a level of 10^2 to 10^3 CFU/g of feces from the day after administration of the secondary host to the end of the experiment. However, when the same experiment was performed with the contained plasmid (Fig. 4B) (mean of five experiments), no transconjugants were detected, although the same concentration of host and secondary host as that in the control experiment was used.

It should be noted, however, that the donor with the contained plasmid appears to compete more poorly with the recipient than the donor with the control plasmid. To compensate for differences in donor concentrations between control experiment and contained experiment, transconjugants per donor were calculated. Pooling all samples after addition of recipient BJ19, a total of 3.4×10^3 donors with the control plasmid and a total of 7.5×10^7 transconjugants with the control plasmid were detected. Thus, the ratio of transconjugants to donors in the control experiment was 4.6×10^{-5}. In the experiment with contained plasmid, no transconjugants were detected (detection limit, 1), but a total of 1.0×10^8 donors was detected. Thus, the ratio of transconjugants to donors was less than 1.0×10^{-8} or at least 550 times less than that in the control experiment.

Suicide induction in soil and seawater. Initial experiments examined the efficiency of suicide in sterile soil. Figure 5 shows the effect of IPTG on the viability in soil of strain BD3392, which carries pSK360. Without IPTG, CFU counts remained nearly stable for the 28 h of the experiment. With IPTG, CFU counts dropped below the detection limit after 28 h (the last datum point is below the detection limit, which is equal to 0.2 CFU/g of soil; see Discussion). The decline in CFU after the addition of IPTG is more than 7 orders of magnitude. No significant difference was detected in the presence of glucose. Samples were also plated on LATI plates to detect suicide-minus mutants. No mutants were detected during the experiment (a detection limit of 66 CFU/g of soil).

The effect of IPTG on the viability of strain BD3392 in sterile seawater is shown in Fig. 6. Again, CFU counts dropped dramatically in the presence of IPTG, although the rate of suicide was lower than that observed in soil. Adding glucose and IPTG led to an increased rate of suicide. The reduction in CFU was more than 6 orders of magnitude after the addition of IPTG. No suicide-minus mutants were selected on LATI plates during the experiment (a detection limit of 24 CFU/ml).

In all of the experiments presented above, no suicide-minus

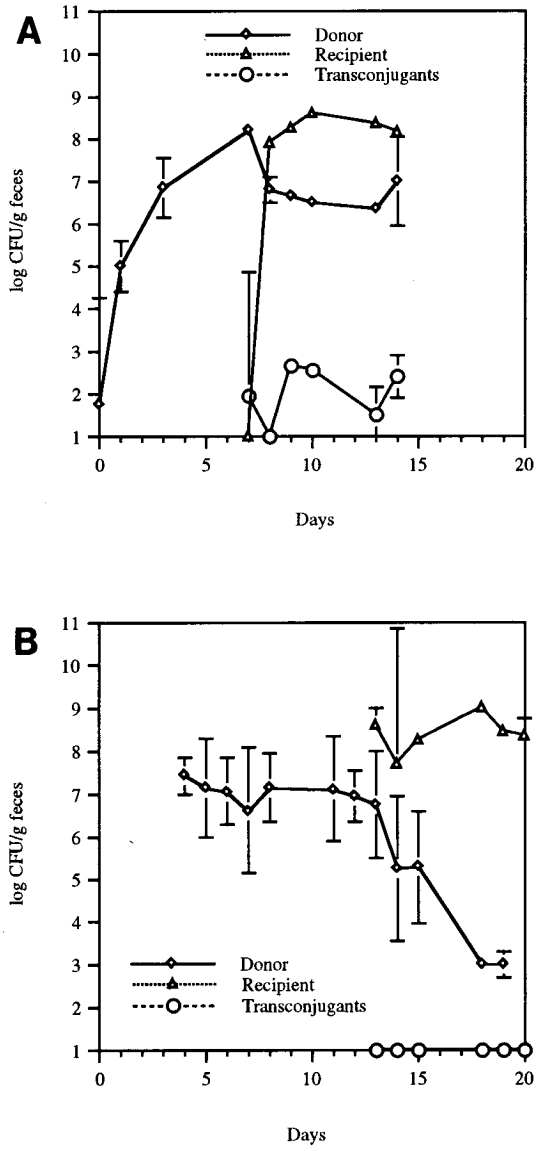

FIG. 4. Plasmid transfer in rat intestine. (A) Donor contains control plasmid pBR322:pBOE93; recipient is *E. coli* BJ19 (average of five rat experiments); (B) donor contains containment plasmid pSK360::pBOE93 (average of five rat experiments). The detection limit was 1 log(CFU) in all experiments. Datum points on the *x* axis [1 log(CFU)] indicate determinations that were below the detection limit.

mutants were detected. To demonstrate how selection of such mutants would have affected the population dynamics in sterile seawater, an overnight culture large enough to contain spontaneous suicide-minus mutants (above 10^9 cells, a culture is likely to contain mutants that arise at a rate of 10^{-8} per cell per generation) was used for inoculating the seawater. These mutants were monitored by plating on LATI, enabling growth of only suicide-minus mutants. After an initial decline in total CFU (detected by plating on LAT) and a rapid growth of mutants, mutants constitute the majority of the about 10^4 cells in the tube after 6 days (Fig. 7). In the absence of IPTG, the mutant population followed the trend of the total population of cells: after an initial small increase, mutant counts dropped below the detection limit 3 days after the inoculation of the tubes.

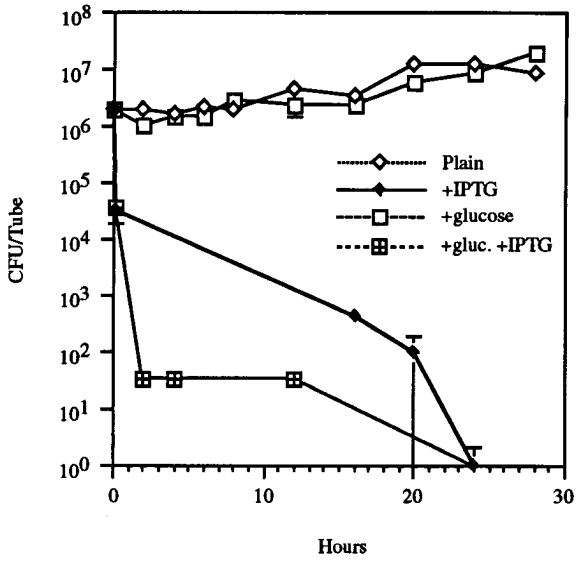

FIG. 5. Effect of IPTG and glucose on viability in sterile soil of strain BD3392, which carries plasmid pSK360. Samples labeled plain were taken from soil with no amendments. Viability was determined as CFU on LAT plates. Vertical bars indicate standard deviations from duplicate experiments. The detection limit was 0.2 CFU per tube. Datum points on the *x* axis indicate determinations that were below the detection limit.

Figure 8 shows the decline in CFU in nonsterile seawater with and without glucose. Without glucose, the decline in CFU occurs at about the same rate with and without IPTG, presumably reflecting competition and predation from indigenous species. In the presence of glucose, the introduced cells are stabilized for a short time. The addition of IPTG leads to rapid suicide.

DISCUSSION

We have shown in this report how an efficient suicide plasmid can be designed by duplication of a suicide function within

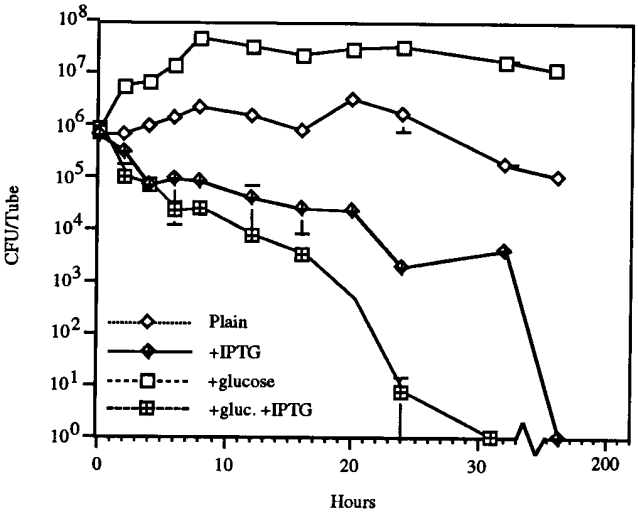

FIG. 6. Effect of IPTG and glucose on viability of strain BD3392 in sterile seawater. Viability was determined as CFU on LAT plates. Vertical bars indicate standard deviations from duplicate experiments. The detection limit was 0.2 CFU per tube. Datum points on the *x* axis indicate determinations that were below the detection limit. Note that the *x* axis is broken between 30 and 200 h.

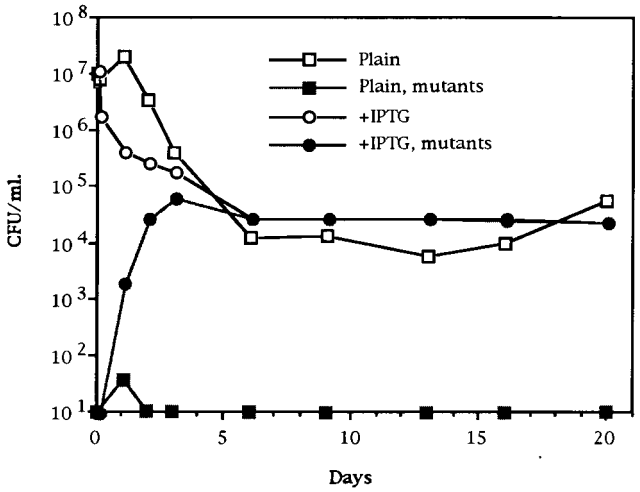

FIG. 7. Effect of IPTG on viability of strain BD3392 in sterile seawater. Spontaneous suicide-minus mutants were present in the inoculum. Viability was determined as CFU on LAT plates. Mutants (filled squares and circles) were detected as CFU on LATI plates. The detection limit was 5 CFU/ml. Datum points on the *x* axis indicate determinations that were below the detection limit.

a plasmid. Furthermore, we have shown how two suicide functions provide protection against inactivation by transposons (compare cointegrate-mediated transfer rates of pDW205 and pSK360) and that transfer of a plasmid from its host strain to a secondary host can be efficiently prevented (diminished by at least a factor of 10^6) under laboratory conditions. As one example of environmental conditions very different from optimal growth conditions in the laboratory, we tested transfer in rat intestine. Also in this situation, transfer was reduced below the detection limit (at least 550 times below the transfer rate of the control plasmid). Thus, we propose that the plasmid pSK360 can be used as a general cloning vector with reduced transfer potential wherever risk concerns warrant it. While no system can provide 100% protection, an efficient system as we describe it can be a significant element in any risk evaluation. The plasmid would not be useful as a cloning vector if the suicide functions disturbed the cell during normal handling. We have previously shown that a single, well-repressed suicide function has no measurable effect on exponential growth in the laboratory (11). The suicide functions have no measurable effect on exponential growth in the laboratory (11). The suicide functions should also be useful for cloning on the chromosome, to protect against the transfer of genes cloned on chromosomes.

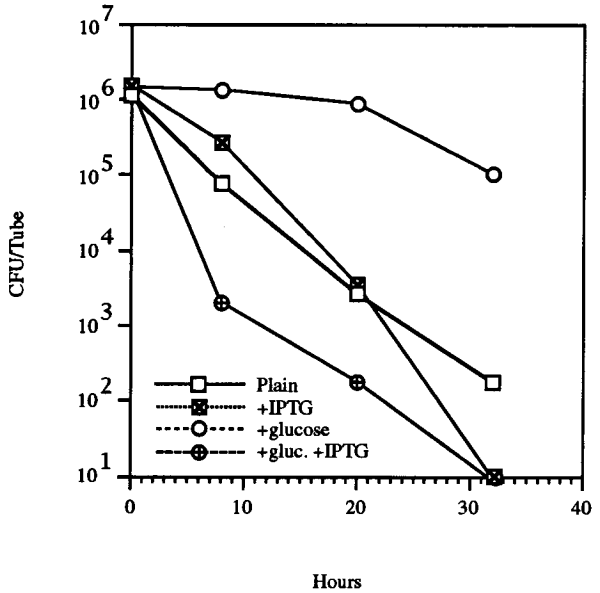

FIG. 8. Effect of IPTG and glucose on the viability of strain BD3392 in nonsterile seawater. Viability was determined as CFU on LAT plates. The detection limit was 20 CFU per tube. Datum points on the *x* axis indicate determinations that were below the detection limit.

The concept of basing containment of a given cloned gene on the expression of a lethal gene has the advantage that expression of the cloned gene in a foreign host bacterium is likely to result in expression of the lethal gene as well. In secondary hosts where the lethal gene is not expressed, the cloned gene is unlikely to be expressed and is then of less concern. Even in a foreign host recognizing *E. coli* promoters but containing the wild-type *lacI* gene, suicide results because of inadequate repression in the presence of moderate numbers of repressor molecules (11).

If the target of the containment is a host and not a plasmid, the suicide functions should be placed on the chromosome of the host. Such a system has been demonstrated by Jensen et al. (9). We have tested the efficiency of such host containment by IPTG induction of our plasmid-based dual suicide system inside its host. By analyzing the reasons for survival in complex environments such as soil and seawater, we have been able to achieve much more efficient suicide than that previously reported.

We interpret our results as follows. If suicide-minus mutants were present in the inoculum of the sterile seawater, IPTG-induced suicide of wild-type cells would eliminate competition and cause rapid proliferation of these mutants (Fig. 7). In seawater without IPTG, suicide-minus mutants had no apparent growth advantage (Fig. 7). In all other experiments (Fig. 5, 6, and 8), mutants were not detected, and all wild-type cells containing the suicide plasmid committed suicide. Thus, the efficiency of suicide (i.e., the fraction of survivors after suicide induction) appears to be limited only by the mutation rate. If the inoculum is prepared without mutants (see Materials and Methods), then all inoculated cells commit suicide. That limits the inoculum to about 1/(10× mutation rate) cells, or about 10^7 cells in our case, where the mutation rate is about 10^{-8} per cell per generation.

Whereas the suicide efficiency is dependent only on mutation rate, the suicide rate (i.e., the kinetics with which the suicide-induced cells die) depends on other factors. One such factor is nutrient availability, as demonstrated by the addition of glucose to seawater (Fig. 6).

In soil experiments, we cannot exclude the possibility that cells adhering to soil particles and resisting extraction survive. The recovery from soil immediately after inoculation is 85% (data not shown), but we have not determined the extraction efficiency after 1 week of incubation. In seawater we have no such problems, because the contents of an entire tube can be plated.

This interpretation of our results explains previously published results in sterile soil microcosms. Recorbet et al. (19) reported that the reduction in CFU after sucrose addition to *E. coli* cells with a sucrose-induced suicide gene, *sacB*, is approximately 3 orders of magnitude. They also reported that suicide-minus mutants constituted a fraction of 10^{-5}, which might have arisen at a rate of 10^{-6} per cell per generation, a rate which we have previously found for a single suicide function of a com-

parable gene size (11). Thus, mutants were highly likely to be present in an inoculum of 5×10^6 per microcosm, and their results show a selection of these mutants from an undetermined (but low) number to approximately 5×10^4 mutants per microcosm after induction of suicide.

Similarly, a paper by Bej et al. (2) reported a reduction of 2 orders of magnitude in CFU after induction of a single *lacP-hok* suicide function with IPTG.

This selection of mutants, however, may not occur in nonsterile microcosms or in the environment because of competition and predation. Indeed, Recorbet et al. reported a rapid elimination of suicide survivors from nonsterile microcosms.

Thus, the limit in efficiency of suicide is determined largely by the mutation rate of the suicide system. Under sterile conditions, suicide is poor if the inoculum is large enough to contain mutants. Under nonsterile conditions and, perhaps, in the environment, the die-off of the introduced microbes by predation and competition may reduce the population size, and proportionally the number of suicide mutants, to levels that will prevent survival following induction of the suicide system.

In this report, we have used two measures to describe suicide efficiency: the difference in CFU counts before and after suicide induction and the difference in CFU counts between tubes with and without IPTG. To facilitate comparisons between different systems, we suggest using the former number to report suicide efficiency.

When contemplating bacterial containment by activation of suicide functions following field applications, a number of issues must be addressed. (i) Plasmid-borne suicide functions will contain (eliminate) plasmid-borne genes, including prevention of plasmid transfer (6), but will not contain the hosts that have lost the plasmid. If the host has to be contained, the suicide functions have to be moved to the chromosome. A chromosomal insertion of the *sacB*-based suicide system has been demonstrated successfully (19). (ii) Induction by IPTG, lactose, or sucrose, which work in small microcosms, may not be practical on a larger scale because of the quantities involved and the potential difficulty of reaching all released bacteria. One solution could be to link induction of suicide to the degradation of a xenobiotic, as suggested by Contreras et al. for the P_m(TOL)-*gef*-based system (6). (iii) *E. coli*, which we have used in our present studies, may not be the bacterium of choice if competition with indigenous soil bacteria is desired. (iv) Mutation rates described in this and previous reports have been determined during rapid exponential growth. If the prevailing mutation type is caused by errors in replication, such a mutation rate, expressed per generation, could be valid in the environment. Potential stress-induced mutations, however, could affect the prediction of the number of mutants. Mutation rates under stress would need to be evaluated. In a first attempt to obtain such data, we allowed the seawater experiment described in Fig. 7 to stand for 10 days after the last datum point was taken, for a total of 30 days. The total CFU counts remained around 10^4 during this period, but no suicide-minus mutants were detected. These results indicate that stress (starvation)-induced mutations in this bacterium under these conditions had not occurred.

The mutation rates of our dual suicide system are not the lowest obtainable. Based on calculations presented by Knudsen and Karlström (11), a single suicide system will have a mutation rate of about 10^{-6} per generation. A dual system with a common regulation, such as the one used here, will have a rate of about 10^{-8} or lower, depending on whether transdominant regulatory mutations occur. A dual suicide system with independent regulation systems (induced, for example, by lactose and sucrose), might have a rate approaching 10^{-12}. A triple suicide system, with independent regulation and independent targets, could have an even lower mutation rate. Although multiple suicide systems that are totally independent of each other remain to be tested, it seems plausible that very efficient suicide in the environment can be achieved.

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