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Development of Efficient Suicide Mechanisms for Biological Containment of Bacteria

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To optimize plasmid containment, we have systematically investigated the factors that limit the killing efficiency of a suicide system based on the *relF* gene from *Escherichia coli* controlled by inducible *lac* promoters and placed on plasmids. In induction experiments with this suicide system, killing efficiency was unaffected by temperature and growth medium; there was no requirement for great promoter strength or high plasmid copy number. We could demonstrate that the factors limiting killing were the mutation rate of the suicide function and the reduced growth rate caused by a basal level of expression of the suicide gene during normal growth, which can give a selective growth advantage to cells with mutated suicide functions. The capacity of the plasmid-carried killing system to contain the plasmid was tested in transformation, transduction, and conjugational mobilization. The rate of plasmid transfer detected in these experiments seemed too high to provide adequate biological containment. As expected from the induction experiments, plasmids that escaped containment in these transfer experiments turned out to be mutated in the suicide function. With *lac*-induced suicide as a test, the efficiency of the system was improved by tightening the repression of the suicide gene, thereby preventing selection of cells mutated in the killing function. Reduction of the mutational inactivation rate of the suicide system by duplication of the suicide function augmented the efficiency of the suicide dramatically. These results permit the construction of extremely efficient biological containment systems.

In applications involving genetically engineered bacteria, it is sometimes desirable to use genetic debilitation to lessen requirements of physical containment. There are at least two fundamentally different approaches to construct biological containment systems: a passive mechanism based on debilitation of a strain to such an extent that it can no longer survive outside a laboratory environment (e.g. *Escherichia coli* strain χ1776 [11]) and an active mechanism which relies on suicide of the cell under certain conditions. The second approach has the advantage that the suicide mechanism may be incorporated into different "healthy" laboratory strains or production strains. The use of healthy strains greatly facilitates the utilization of biologically contained bacteria and allows the incorporation of the suicide mechanism into laboratory or production strains with special desired features. The condition triggering suicide may be transfer of plasmids to wild-type strains, reduced growth temperature outside controlled areas, reduced growth rate outside controlled areas, or absence of a chemical compound outside controlled areas (20).

Depending on the level of containment desired, there can be different demands on the efficiency of an active suicide function. The fraction of cells surviving induction of the suicide function provides the basis of a safety evaluation. The National Institutes of Health guidelines (21) define physical and biological containment levels. Combinations of these are stipulated for each type of work. For instance, EK2 biological containment level demands that no more than 1 in 10^8 cells perpetuate a cloned fragment outside the laboratory.

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Biological containment systems described previously (3, 20) have all suffered from the disadvantage that a considerable fraction of cells survives the induction of the suicide function. We therefore set out to investigate what factors limit the efficiency of active suicide systems. The results were obtained with the *E. coli relF* gene as a suicide gene, using *lac*-derived promoters. The results can, however, be extended to other similar containment systems. The *E. coli relF* gene is homologous to the previously described suicide genes *hok* and *gef* (23). We demonstrate that an efficient suicide system can be constructed and identify what properties are important for an efficient system.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Plasmids. The plasmids used are listed in Table 2. All cloning experiments were performed by using standard procedures (18). The model containment plasmid pDW205 shown in Fig. 1 contains nucleotides 1058 to 1260 from *lacL8UV5* (2), nucleotides 1073 to 1348 from *relB* (1) and the 4,937-bp *Hin*dIII-*Eco*RI fragment of plasmid pKCL11 (5). The runaway replication plasmid pSK330 (shown in Fig. 2), which harbors the killing function, was constructed from pDW205 and pMR60. pMR60 was a gift from M. Roland and was constructed by inserting a pUC19 polylinker *Sal*I-*Eco*RI fragment into pOU82 (14) digested with *Sal*I and *Eco*RI. pDW205 was digested with *Bam*HI and *Ase*I partially, and the p_{UV5}-*relF*-carrying *Ase*I-*Bam*HI fragment was cloned into pMR60 *Ase*I-*Bam*HI.

Determination of killing efficiency. The number of inducer-resistant cells was determined by plating cells on NY agar plates containing 100 μg of ampicillin per ml and 1 mM IPTG

TABLE 1. Bacterial strains

E. coli strain[a]	Genotype	Derivation or reference
MC1000	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK strA*	6
NF1815	MC1000 *recA1*	N. Fiil
NF1830	NF1815(F′ *lacI*^{q1} *lacZ*::Tn*5*)	N. Fiil
HB101	F′ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44*	4
BD3364	HB101(F′ *lacI*^{q1} *lacZ*::Tn*5*)	NF1830 × HB101
HfrC	*metB relA1 ton*	7
BD3342	HfrC *lacI*^{q1} *lacZ*::Tn*5* (Kan^r)	Transduction of Kan^r from NF1830 to HfrC[b]
KL14	Hfr *thi*	16
KL16	Hfr *thi*	16
BD3345	MC1000 *lacI*^{q1} *lacZ*::Tn*5* (Kan^r)	BD3342 × MC1000[c]
BD3346	MC1000 *lacI*^{q1} *lacZ*$^+$	BD3345 Kan^s *lacZ*$^{+d}$
XAC	Δ(*lac-pro*) *argE*(Am) *nalA rif*	19
BD71	Δ(*lac-pro*) *argA nalA thi relB2 supE*	B. Diderichsen
BD76	*argA nalA supE*	KL14 × BD71[e]
S17.1	*recA1 pro thi hsdR hsdM*$^{+f}$	24

[a] All strains are derived from *E. coli* K-12.
[b] Using T4GT7 transduction according to reference 28. Selected on AB plus 0.2% glucose, 50 μg of methionine per ml, and 20 μg of kanamycin per ml.
[c] Selected on AB plus 0.2% glucose, 40 μg of leucine per ml, and 20 μg of kanamycin per ml.
[d] Selected as red colonies on MacConkey lactose plates (12a).
[e] Selected on AB plus 0.2% glucose, 40 μg of arginine per ml, and 10 μg of nalidixin per ml.
[f] S17.1 contains the transmobilizing plasmid RP4 integrated into the chromosome. It can supply conjugation factors to plasmid pBOE93.

(isopropyl-β-D-thiogalactopyranoside). The total number of viable cells plated was determined by plating dilutions on the same plates without IPTG.

Transformation experiments. Transformation was performed by standard procedures (18). A total of 200 μl of competent cells (approximately 10^{10} cells per ml) was mixed with 1 μl of plasmid DNA (50 ng/μl) and diluted in NY medium (27) before plating on selective plates.

Transduction experiments. Plasmid transduction with T4GT7 was performed as in reference 26.

Mobilization experiments. Plasmid mobilization by fusion to the mobilizable plasmid pBOE93 was performed by mixing drops of donor and recipient cultures on nonselective plates followed by growth overnight and resuspension and plating of dilutions on selective plates as described in reference 20.

RESULTS

Evaluation of efficiency of the suicide function. The basic assay, described in Materials and Methods, to determine the killing efficiency relies on induction of the promoter p_{lacUV5} transcribing the suicide gene *relF* by plating on agar plates containing IPTG. By counting the number of colonies, *M*, on these plates and dividing by the total number of cells plated, *N*, the fraction of surviving cells, *M*/*N*, is determined. This assay was repeated with variations in single parameters to determine whether or not any of these parameters influenced the killing efficiency (Table 3). The results indicate that there is no important effect caused by any of the parameters tested. The striking variation between repeats of the experiment, however, pointed at mutation as the important factor determining survival, as elaborated in the Discussion.

Mutation rate of suicide system. Surviving colonies from experiments in the preceding section were analyzed to find the reason for survival. The suicide plasmids were isolated and transformed to strain NF1815 without the *lac* repressor. It turned out that the plasmids were able to transform this strain to an efficiency equal to that of a control plasmid, pBR322. Thus the plasmids had lost their suicide function, an observation pointing to the possibility that cells surviving on IPTG plates all contained plasmids with spontaneous mutations in the suicide function. To test this hypothesis, a Luria-Delbrück experiment (17) was performed. Eighteen 0.2-ml cultures were inoculated with approximately 100 cells of NF1830(pSK355) each. When the number of cells per culture reached 10^6, the cultures were plated on agar plates containing IPTG. After incubation, one plate had 49 colo-

TABLE 2. Summary of plasmids used

Plasmid	Origin	Genes	Additional comments	Derivation[a] or reference
pACYC184	p15A	*cat tet*		8
pBOE93	RSF1010	*kan bla mob*	*trans*-mobilizable	20
pBR322	ColE1	*bla tet rop*		10
pBR327	ColE1	*bla tet*		10
pDW205	pBR327	*bla* $p_{lacL8UV5}$-*relF*		This work
pKCL11	pBR327	*bla* $p_{lacL8UV5}$-*lacZ*		5
pKK2247	p15A	*kan tet*		5
pMR60	R1	*bla* cI857	Replication runaway + polylinker	pOU82
pOU82	R1	*bla* cI857	Replication runaway	14
pR2172	pBR322	*bla lacI*^{q1}		22
pSK330	R1	*bla* cI857 $p_{lacL8UV5}$-*relF*	Replication runaway	pMR60
pSK342	p15A	*tet* $p_{lacL8UV5}$-*relF*		pKK2247
pSK355	pBR327	*bla* $p_{A1/03/04}$-*relF*	Synthetic promoter/operator	pUHE21-2
pUHE21-2	pBR322	*bla* $p_{A1/03/04}$-′*cat*	Synthetic promoter/operator	15

[a] Derivation of the plasmids is described in the text and is shown in detail on the plasmid maps (Fig. 1 and 2).

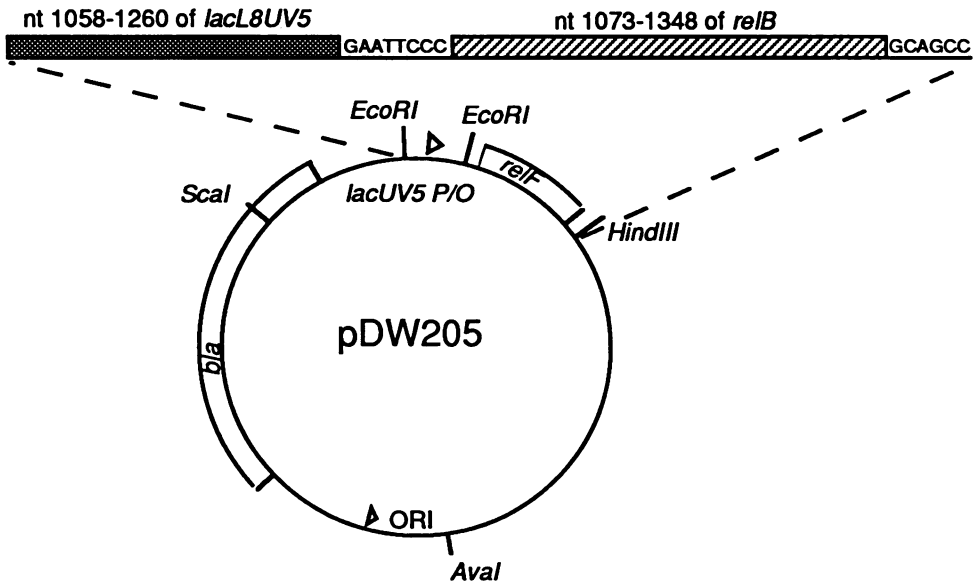

FIG. 1. Model containment plasmid pDW205. Sources of the DNA sequences are listed in the text.

nies, one had 5 colonies, one had 2 colonies, and five plates had only 1 colony each, whereas 10 plates had no colonies at all. This gives a mean number of 3.4 colonies per plate with a variance of 123, clearly suggesting that the mutations had occurred before plating and had resulted in clones of different sizes. By using the Poisson distribution, it is also possible to estimate the mutation frequency, R, per cell per generation from the fraction of cultures, P_0, with no mutants: $R = -(\ln 2/N) \ln P_0$; $R = -(\ln 2/5 \times 10^5) \ln (10/18) = 1 \times 10^{-6}$ per cell per generation. However, there is a difference from the system that Luria and Delbrück analyzed in that the mutating gene resides on a multicopy number plasmid. Because the mutation to an inactive suicide function is recessive, there is a segregation lag before detection of the mutation. The effect of this segregation lag is analyzed in the Appendix. This analysis suggests that the mutation rate per cell estimated in the Luria-Delbrück experiment underestimates the mutation rate per gene copy by approximately 1 order of magnitude.

Enrichment of mutants due to increased growth rate. If mutation is the key factor in inactivation of the suicide function, then the selective growth advantage that mutants can have may constitute a problem. We therefore investigated whether or not mutant selection might occur. Table 4 lists the growth rates in different media of cells harboring different suicide plasmids. It is seen that suicide plasmids in which the *relF* gene is transcribed from the p_{lacUV5} promoter confer a growth disadvantage to the cell when compared with control plasmids without suicide function. Consequently, a suicide plasmid in which the suicide function is mutated might gain a selective advantage over the nonmutated plasmids. In the Appendix the analysis of Luria and Delbrück is extended to include this selective growth advantage of the mutant. The results of this analysis show that a growth disadvantage beyond 5% can be quite detrimental to suicide systems. From Table 4 it is seen that suicide plasmid pSK355 in which the suicide gene is transcribed by the tightly controlled synthetic *lac*-repressible promoter $p_{A1/04/03}$ (15) fulfills the demand of a growth disadvantage of no more than 5%.

Evaluation of suicide function used for plasmid containment. To establish whether the mutation rate of the suicide functions would be the limit in actual containment systems of plasmids, transfer experiments were performed with model plasmid pDW205. The suicide function on this plasmid is repressed by a $lacI^{q1}$ gene on the chromosome of the host. If the plasmid is transferred to other (wild-type) strains, the suicide function is derepressed, leading to suicide of the secondary host. This mechanism was tested in three different model transfer experiments.

(i) Transformation. A culture of the designed host strain BD3346 harboring the contained plasmid pDW205 was lysed, and the plasmids were purified. Then the plasmids were transformed to wild-type strains and to the designed host strain. The transformation efficiency was compared with that of an uncontained control plasmid, pBR322. The transformation frequency of the plasmid was reduced several orders of magnitude below the transformation frequency of the uncontained control plasmid (Table 5).

(ii) Transduction. Strain NF1830 harboring the contained plasmid pDW205 was infected with bacteriophage T4GT7, which efficiently transduces plasmids. The infected cells were lysed, and the lysate was used to infect wild-type strains. The cells that had received a plasmid via the bacteriophage were selected by ampicillin resistance. As an internal control of the transduction efficiency, transduction of a chromosomal marker was also monitored.

The same experiment was performed with the host strain harboring an uncontained control plasmid. Plasmid transmission was reduced several orders of magnitude when the plasmid carried the containment construction (Table 6).

(iii) Mobilization in conjugation. A cointegrate between the mobilizable plasmid pBOE93 and the containment vector pDW205 was constructed in vitro, using the unique *Hin*dIII sites of both plasmids. A fusion between pBOE93 and pBR322, using the unique *Eco*RI sites of both plasmids, served as a control. As the conjugational donor *E. coli* S17.1, which contains the *trans*-mobilizing plasmid RP4 integrated into the chromosome, was used. This strain can transfer pBOE93 fusion plasmids to a broad range of strains. S17.1

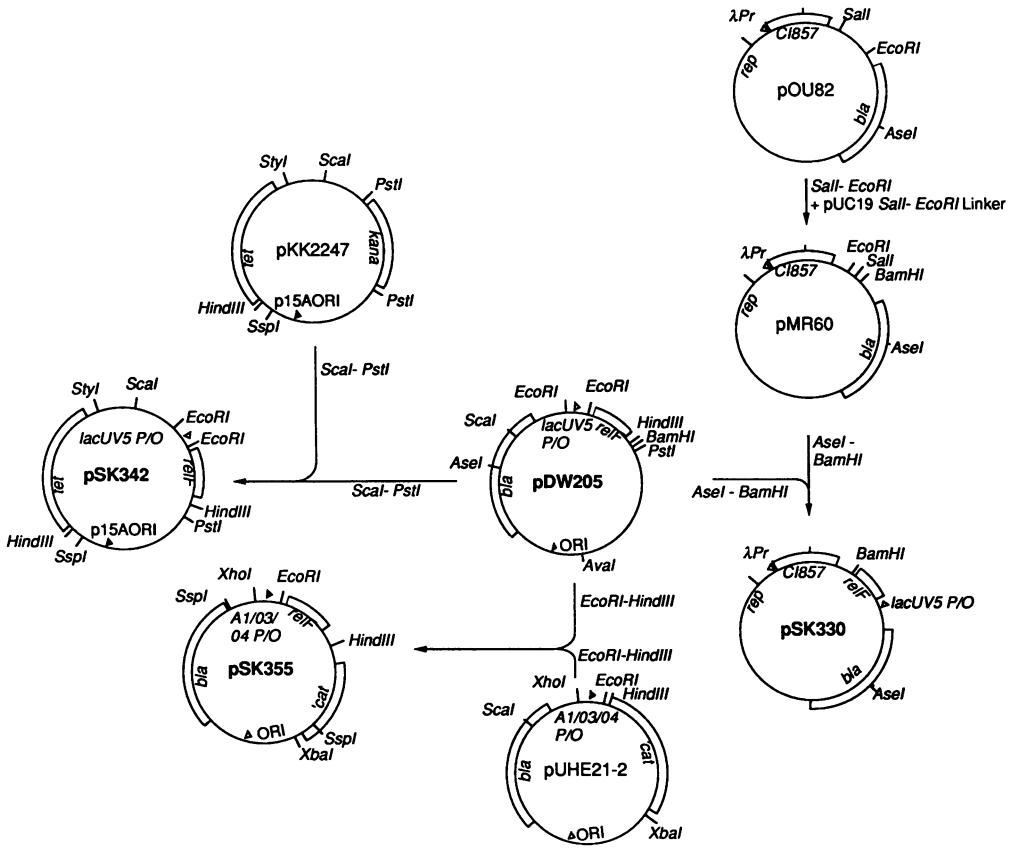

FIG. 2. Construction of plasmids used in the experiments.

was transformed with plasmid pR2172, which carries $lacI^{q1}$ to repress the suicide function, as in the designed host cell. From this strain the plasmids were transferred to *E. coli* XAC and BD76, which are *lacI* and $lacI^{+}$, respectively. The results show that the pBR322 cointegrate was efficiently transferred (Table 7). The cointegrate with the containment vector was transferred several orders of magnitude less efficiently. This is true even if the recipient has a wild-type *lacI* repressor gene (strain BD76).

In all three types of transfer experiments the fraction of cells escaping the suicide was comparable to the fraction of cells surviving IPTG induction, as presented above. Thus, it seemed that the mutation rate was also the limit in the containment of plasmids. From several clones of cells that had received the contained plasmid and survived in different plasmid transformation experiments, plasmid DNA was isolated and used to transform strain NF1815. The transformation frequency confirmed that the plasmids had mutant suicide functions (data not shown). From the equal survival rates in the transductional and conjugational mobilization experiments, we assume survival to be caused by mutant plasmids in these experiments as well.

Improvement of efficiency of suicide function. The use of a tightly controlled promoter to reduce selection of mutants was described above. However, it is also desirable to reduce the mutation rate itself. This reduction could be achieved by a duplication of the complete suicide function. A suicide plasmid, pSK342, compatible with the previously used pSK355 was constructed. BD3364 cells harboring these two suicide plasmids were tested in the IPTG plate assay with one modification. The chromogenic beta-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as well as tetracycline was included in the plates. Cells that have lost the suicide system but maintained the normal *lac* control form blue colonies on these plates. A different class of cells that survive on IPTG plates consists of mutants unable to recognize the *lac* inducer. The latter type of cells, superrepressor mutants, form white colonies on plates with X-Gal and IPTG because they are unable to induce the chromosomal *lacZ*. The Luria-Delbrück experiment was repeated by using the modified assay, but in 20 cultures of 10^7 cells each, no cells with mutant suicide function were found, suggesting a rate of inactivation of the suicide functions of $<5 \times 10^{-9}$ per cell per generation (the superrepressor mutation occurred at a frequency of 10^{-8} per cell per generation).

DISCUSSION

The experiments presented here all point to one conclusion. Cells equipped with the model suicide system survive conditional suicide because of mutations in the suicide function. Variation in other parameters as shown in Table 3 does not significantly affect the fraction of surviving cells. The fraction of surviving cells, however, varies from exper-

TABLE 3. Assay to determine fraction of cells surviving suicide induction under different conditions

Plasmid[a]	Medium[b]	Gene dosage per chromosome equivalent	Temp (°C)	CFU		
				−IPTG (*N*)	+IPTG (*M*)	*M/N*
205	NY	10–20	37	2×10^7	1×10^3	5×10^{-5}
205	Succinate	10–20	37	1×10^7	7×10^2	6×10^{-5}
205	NY	10–20	16[c]	6×10^7	6×10^4	4×10^{-3}
205	NY	10–20	20[c]	6×10^7	2×10^5	3×10^{-3}
205	NY	10–20	30[c]	1×10^8	4×10^5	4×10^{-3}
205	NY	10–20	37[c]	6×10^7	6×10^5	1×10^{-2}
330	NY	1–2[d]	30[e]	5×10^6	1×10^3	3×10^{-4}
330	NY	5–10[d]	39[e]	5×10^6	3×10^2	7×10^{-5}
330	NY	>100	42[e]	1×10^4[f]	2×10^2	2×10^{-2}

[a] Plasmids: 205 = pDW205; 330 = pSK330. pSK330 is a replication runaway plasmid carrying the same suicide function as pDW205. In a control experiment the replication runaway plasmid pMR60 without the suicide function was plated at the same temperatures to ensure that these temperatures are not lethal to cells harboring the replication runaway plasmid.
[b] Succinate stands for minimal medium with succinate as the carbon source.
[c] The culture was propagated, plated, and incubated at the stated temperature.
[d] Copy numbers estimated from MICs of ampicillin.
[e] The culture was propagated at 30°C and then plated and incubated at the stated temperature.
[f] Due to the increase in plasmid copy number to more than 100 after plating, the repression of the suicide function is no longer efficient.

iment to experiment, because of the variation in time point of the first mutation and because of different conditions of mutant selection (with inadequately repressed suicide functions, there is mutant selection in stationary phase [data not shown]). These factors can contribute significantly to the fraction of surviving cells (Table 3). The results presented in Tables 3 and 5 through 7 in each case represent one of several similar experiments. Within each such experiment in which only one parameter is varied, the different cultures were all inoculated from the same culture, thus probably containing the same number of mutant cells at the start of the experiment minimizing the internal variation.

A single copy of plasmid pSK330 at 30°C is sufficient to bring down the number of survivors of the suicide condition to an extent that only cells containing mutant plasmids survive. On this plasmid, the *relF* gene is transcribed from the *lacL8UV5* promoter, which, compared with other commonly used promoters, including the synthetic A1 promoter (15), is not very strong. These results suggest that the gene dosage and transcription rate of the suicide gene do not limit the suicidal effect in any of the experiments of this report. The same conclusion is true for low growth rate caused by poor medium (succinate minimal) or low temperature (16°C).

Cells resistant to activation of the suicide function arise at a much lower rate when the suicide function is duplicated. This rate, which we measured in Luria-Delbrück experiments, then reached an unsurpassed low level: $<5 \times 10^{-9}$ per cell per generation. The mutation rate calculated per gene copy per generation is approximately 1 order of magnitude higher (see Appendix). The reduction in the number of survivors may, however, be diminished by two factors. (i) The superrepressor mutation sets the lower limit to the inactivation rate in containment systems that rely on induction of the suicide function within the original host cell. (Plasmid containment systems are not subject to this limitation.) (ii) Homologous recombination and gene conversion between the two suicide functions may reduce the advantage

TABLE 4. Growth rates of cells with suicide plasmids in different media

Plasmid	Strain	Medium[a]	Growth rate (h^{-1})[b]	% Disadvantage compared with plasmid:
pKCL11	NF1830	NY + ampicillin	0.98	
pDW205	NF1830	NY + ampicillin	0.90	9, pKCL11(NF1830)
pKCL11	NF1830	ABTG + ampicillin	0.36	
pDW205	NF1830	ABTG + ampicillin	0.25	30, pKCL11(NF1830)
pMR60	NF1830	NY + ampicillin (30°C)	0.72	
pSK330	NF1830	NY + ampicillin (30°C)	0.68	6, pMR60(NF1830)
pMR60	NF1830	ABTG + ampicillin (30°C)	0.23	
pSK330	NF1830	ABTG + ampicillin (30°C)	0.14	40, pMR60(NF1830)
pUHE21	NF1830	ABTG + ampicillin	0.34	
pSK355	NF1830	ABTG + ampicillin	0.33	<5, pUHE21(NF1830)

[a] ABTG, AB minimal salts medium plus thiamine plus 0.2% glycerol.
[b] Growth rate is μ of equation 1 in the Appendix. The uncertainty in determination of growth rate is approximately 4%.

TABLE 5. Plasmid transformation experiments[a]

Strain	No. of transformants/μg of plasmid DNA from donor:	
	pDW205	pBR322
MC1000	6.0×10^1	2.8×10^6
BD3346	1.0×10^6	2.6×10^6

[a] Transformation of plasmids to wild-type cells (MC1000) and the designated host cells (BD3346).

TABLE 6. Plasmid transduction experiment[a]

Characteristic selected for	No. of transductants/infected cell from donor with:	
	pDW205	pBR322
Plasmid-carried Amp^r	1×10^{-6}	3×10^{-2}
Chromosomal Kan^r	2×10^{-5}	3×10^{-5}

[a] Transduction of plasmids by T4GT7. The recipient strain was MC1000. We used transduction of a chromosomal marker (Kan^r) as an internal control.

TABLE 7. Plasmid mobilization experiments with strain S17.1[a]

Recipient strain	No. of conjugants/donor cell from donor with:	
	pDW205::pBOE93	pBR322::pBOE93
XAC	5×10^{-5}[b]	$>10^{-1}$
BD76	5×10^{-5}[b]	$>10^{-1}$
BD3346	$>10^{-1}$	$>10^{-1}$

[a] Transfer of fused plasmids by conjugation. BD3380 = S17.1(pR2172, pDW205::pBOE93); BD3379 = S17.1(pBR322::pBOE93). In all experiments, a donor/recipient ratio (counted at the time of plating) of <1 was used to ensure that the recipient cell density was not the limiting factor. As a control experiment, BD3380 and BD3379 were mated with the designated host strain BD3346 ($lacI^{q1}$) to show that the transfer rates to this strain were similar.

[b] There was some fluctuation from experiment to experiment (see Discussion); an approximate value is given.

obtained from a duplicated suicide system. Use of the *recA* mutation as in our experiments eliminates this problem. Another solution is to use nonidentical suicide functions between which homologous recombination cannot take place.

There is some evidence that highly transcribed genes are subject to higher mutation rates than those repressed (12), especially in stationary phase. Under normal circumstances, our suicide genes are repressed. Upon the induction of the suicide genes, the majority of cells die instantly (13). A scenario in which a cell that is not instantly killed might escape suicide by mutation of the suicide function seems unlikely because such a mutation would be recessive and mutant plasmids would have to segregate and fully express the mutant suicide gene before survival.

The experiments discussed here indicate that, once the mutation problems have been solved, a plasmid efficiently contained within its designed host can be constructed. The application of our results to containment systems for practical use would be facilitated by the cloning of several suicide functions within the same plasmid or on separate locations of the host chromosome. In both cases a repressor gene should be located on the host chromosome. In the case of containing genes cloned on a plasmid, plasmid loss would be of no concern because it would mean loss of the cloned gene as well. Consequently, antibiotic selection of plasmids should not be necessary and has only been used in the present study to allow the detection of events much more rare than plasmid loss. In the case of containing host cells as a whole, cloning of the suicide genes on the chromosome could be one way to avoid loss of suicide function. Containment systems for practical use based on these principles will be described elsewhere along with experiments to prove that the effect of these systems is bacteriocidal and not bacteriostatic.

APPENDIX

This Appendix shows a Luria-Delbrück type of analysis of the accumulation of mutants in multicopy plasmid systems in which mutants gain a growth advantage. A similar analysis for chromosomal mutations has been undertaken by Stewart et al. (25).

The increase in the number of mutants in a growing culture is described by the equations

$$\frac{dM}{dt} = N \cdot R + \mu_m M, \quad \frac{dN}{dt} = \mu N \tag{1}$$

where M is the number of mutants; N is the number of normal cells; μ_m and μ are the growth constants for mutant and normal cells, respectively; and R is the mutation rate.

The coupled differential equations 1 can be solved by using standard methods whereby the solution to the differential equations can be expressed as

$$M = M_0 \exp(\mu_m t) + \frac{RN_0}{(\mu_m - \mu)} [\exp(\mu_m t) - \exp(\mu t)]$$

Expressing the fraction of mutant to normal cells

$$\boxed{\frac{M}{N} = \frac{M_0}{N_0} \exp[(\mu_m - \mu)t] + \frac{R}{(\mu_m - \mu)} \{\exp[(\mu_m - \mu)t] - 1\}} \tag{2}$$

If the growth constants of mutant and normal cells are equal, or the difference is neglected, solution 2 is not valid. Instead, the solution to 1 becomes

$$\boxed{M = M_0 \exp(\mu t) + RNt}$$

The mutation rate per generation, R_G, is related to the mutation rate, R, per time unit by

$$R_G = \frac{R \ln 2}{\mu}$$

The mutation rate determined in one experiment can only be extrapolated to another experiment when it is known whether the mutation frequency is fixed with respect to replication cycle or with respect to time unit. The choice depends on which kind of mutation is predominant under the given circumstances.

Formula 2 can be used to predict enrichment of mutants in growing cultures, if the growth retardation of nonmutated cells is known. Some examples are given below for a mutation rate of 10^{-6} per generation. For the safety evaluation of containment systems, it would be relevant to ask what the fraction of mutant cells would be 50 generations after the first mutation occurred (an extreme situation). With no growth advantage of the mutant the enrichment would be 50-fold. At 5, 10, and 20% growth advantage for the mutant, the enrichment according to equation 2 would amount to approximately a factor of 150, 500, and 8,000, respectively. Thus, we can conclude that the enrichment will be without practical importance as long as the growth retardation is <5%.

Segregation lag. If the mutation studied is a recessive mutation of a gene on a multicopy number plasmid, then there is a segregation

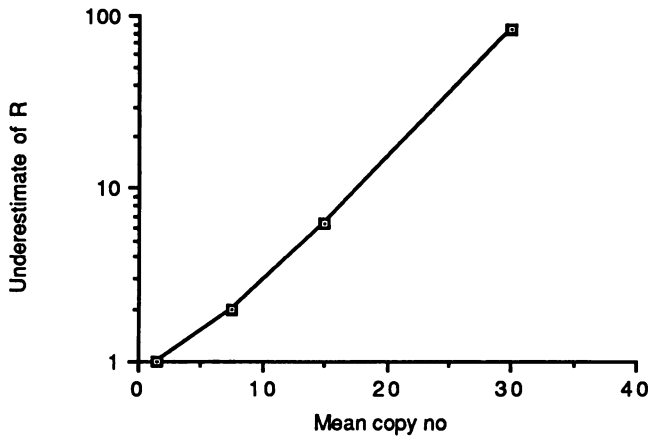

FIG. A1. Factor of underestimation of the mutation rate per gene copy in a Luria-Delbrück experiment as a function of mean copy number of gene.

lag before the mutation is scored in a cell in which all plasmids harbor the mutation. In the Luria-Delbrück assay used in this report, the fraction of cultures in which no mutant cells are scored is quantitated. Because of the segregation lag there may be hidden mutations at the time of the assay, and consequently the mutation rate per gene may be underestimated in this kind of experiment. An analysis of this problem involves analysis of the separate Poisson distributions of mutations generated at each generation of the experiment as well as the iterative binomial distributions describing the segregation of each mutant plasmid. Iterative problems of this type are best solved numerically by using computer algorithms. Luria-Delbrück experiments as presented in this paper were simulated by using a random-number generator to supply the parameters of the distributions described above. Random segregation and equal replication rates of mutant and normal plasmids were assumed. The underestimation of the mutation rate from these approximative simulations is shown in Fig. A1. The underestimation is slightly dependent on the fraction of cultures with no mutants. The results shown in Fig. A1 are based on simulations in which the fraction is close to 0.5. On a plasmid with a mean copy number per cell of approximately 20 at the time of plating in a Luria-Delbrück experiment, the mutation rate will be underestimated by approximately 1 order of magnitude.

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