APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1993, p. 1361-1366
0099-2240/93/051361-06$02.00/0
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Vol. 59, No. 5

Conditional Suicide System of *Escherichia coli* Released into Soil That Uses the *Bacillus subtilis sacB* Gene

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Received 27 July 1992/Accepted 22 February 1993

The *sacB* gene from *Bacillus subtilis* confers sucrose sensitivity upon gram-negative bacteria. The gene was investigated for use as a potential conditional suicide system for *Escherichia coli* released into soil. To ensure against the loss of the cell death function encoded under nonselective conditions, the *nptI–sacR-B* suicide cassette was inserted into the *E. coli* chromosome by using a circular nonreplicative integration vector. Stability studies yielded no loss of the suicide cassette in the integrated *E. coli* EL1026 strain. *sacB* induction in the absence of a selective pressure resulted in a lysis efficiency of up to 99.9%. The microcosm experiments confirmed the ability of the suicide cassette to limit the growth and reduce the survival of *E. coli* strains released into soil. Sucrose addition to sterile soil resulted in a 10^{-3}-fold reduction of the final *E. coli* population density. *sacB* induction prevented the proliferation and triggered the rapid disappearance of *E. coli* from natural soil. Mutation to sucrose tolerance occurred at a frequency of 10^{-5}, making *E. coli* EL1026 a potential counterselectable donor strain for gene transfer studies. Specificity and potential adaptability to a wide range of gram-negative bacteria are additional conveniences of this conditional suicide system for the containment and counterselection of engineered microorganisms.

Bacterial strains with various promising applications either for industrial production or for environmental use have been designed by recombinant DNA techniques. However, questions about the consequences of introducing such strains into various ecosystems have been raised, leading to an increased need to assess the possible effects of their release into soil (18, 22). In some cases, genetically engineered microorganisms were described as perturbing indigenous microbial populations and their processes in soil (1, 8, 25). The possibility of reducing or eliminating bacterial populations newly introduced into ecosystems once their function is completed or in case of inadvertent release is a main component of the mechanism for containing genetically engineered microorganisms (6, 24).

Other concerns are the possible transfer of DNA of the native microflora to new hosts after a bacterial release and the effects of such transfers. Evidence of the occurrence of gene transfer to the indigenous bacteria of soil has been obtained (10, 20). Detection of genetic exchange to the resident organisms requires an efficient method of counterselecting the donor strain (20). Bacteria that are unable to express the selectable marker or that have a short-term survival or phage-sensitive microorganisms have been used as counterselectable parents (10, 20, 23). However, these techniques are limited to a narrow range of specific microorganisms.

The use of suicide systems, which allows for the efficient monitoring of conditional lethality in different microorganisms, offers an attractive counterselection method. The induction of a suicide gene in situ and in vitro would presumably allow for the specific lysis of the released bacteria. Systems responsible for conditional lysis have been described previously (2, 5). In those systems, killing is achieved by inducing a cell death function encoded by either the *hok* or the *gef* gene located on suicide plasmids (2, 5). However, the development of resistant populations and the use of a selective pressure to ensure against the loss of the vector make those suicide systems unreliable under environmental conditions.

We investigated an approach which combines the use of the suicide cassette *nptI–sacR-B* (17) and its chromosomal integration for host maintenance of cell death functions. The *nptI–sacR-B* cassette confers sucrose sensitivity upon gram-negative bacteria. The activity of levansucrase, an enzyme encoded by the *Bacillus subtilis sacB* gene, which is inducible in the presence of sucrose, results in the synthesis of lethal amounts of levan and the accumulation of levan in the periplasm, causing cell lysis (9). We developed this procedure using as the host the genetically engineered strain *Escherichia coli* EL1003 (12). The effectiveness of the suicide system was tested under in vitro conditions and in soil microcosms.

MATERIALS AND METHODS

Bacterial strain and media. Plasmid-free *E. coli* EL1003 (12) carries chromosome-borne resistance to kanamycin (500 μg/ml) and neomycin (500 μg/ml) on the *nptII* gene. Cultures were grown at 37°C in Luria broth (LB) (14) supplemented with appropriate antibiotics (Sigma Chemical Co., St. Louis, Mo.) for the selection of recombinants. Antibiotic concentrations were as follows: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml.

Blomfield broth (BB), consisting of LB lacking sodium chloride, was used to screen sucrose sensitivity in *E. coli* recombinants. The isolates were spread inoculated onto BB agar (1.5%; wt/vol) plates supplemented with sucrose (6%; wt/vol), and the plates were incubated overnight at 30°C (3). Media were supplemented with appropriate antibiotics to

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ensure vector maintenance when plasmid-encoded sucrose sensitivity was tested.

Soil and microcosms. The microcosms used in the present study consisted of 15-ml polypropylene tubes (Falcon) filled with 5 g (dry weight) of a silt loam soil (16). Soil characteristics were as follows: 32.2% sand, 31.4% clay, 36.4% silt with a pH (1:2 in water) of 6.2, 2.64% organic carbon, a C/N ratio of 7.6, and a water-holding capacity of 40%. After sampling, moist field soil was passed through a 2-mm-pore-size sieve and was kept at 4°C. Nonsterile soil was preincubated at 28°C for 1 week before use to allow the indigenous microflora to reach an equilibrium state, avoiding a thermic artifact at time zero of the experiment. The air-dried soil was sterilized by exposing it to 25 kGy of gamma irradiation by using a ^{60}Co radiation source. Soil samples were assayed for sterility by plating them onto nutrient agar (Biomérieux, Charbonnières, France). Incubation at 28°C took place in a moisture-tight chamber, and humidity was controlled.

Molecular techniques. Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, total DNA extraction, and agarose gel electrophoresis (14). Restriction DNA fragments were isolated from agarose gels by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.), as recommended by the manufacturer. Southern hybridization and DNA labelling were carried out by using the ELC gene detection kit (Amersham International, Amersham, England). Transformations were performed by electroporation by the procedure described by Dower et al. (7).

Integration procedure. To avoid the loss of plasmid-encoded traits in the absence of selective pressure, the *nptI–sacR-B* suicide cassette was integrated into the *E. coli* chromosome. The integration transformation was based on the use of a nonreplicative circular vector bearing a selectable antibiotic resistance marker and the suicide cassette located within a chromosomal sequence used to target the integration by homologous recombination (Fig. 1). The chromosomal integration of the cassette can result from (i) a single crossover event, leading to the integration of the complete vector, and (ii) a further recombination event between duplicate sequences of the target, leading either to the complete excision of the vector (Fig. 1, part 2B) or to a partial excision of the antibiotic gene marker (Fig. 1, part 2A). In this case, the resulting structure of the integrated cassette is equivalent to those obtained by a gene exchange procedure. The nonreplicating vector encoding antibiotic resistance allows for the positive selection of the transformants containing both the integrated vector and the nonselectable allele encoding sucrose sensitivity. Since one copy of the chloramphenicol acetyltransferase-encoding gene (*cat*) (4) enables the efficient expression of chloramphenicol resistance, this marker was chosen for the selection of vector integrates (15). Having been cloned and sequenced (unpublished data), the threonine dehydrogenase-encoding gene (*tdh*) was selected as the integration site.

Plasmid and probe constructs. Vector pKGR (Fig. 1), a pBluescript II KS (+) derivative (Stratagene, La Jolla, Calif.), was designed to contain an excisable and nonreplicative fragment that included the *nptI–sacR-B* cassette flanked by *tdh* homologous regions (1.2 and 0.4 kb) and *cat* (unpublished data).

The 1.8-kb *Bam*HI-*Hin*dIII restriction fragment of pUM24 (17), which does not contain IS*1* sequences homologous to *E. coli* DNA (21), was used as the *sacR-B* probe. After purification from agarose gels, the fragment was labelled as described above.

Chromosomal integration of the *nptI–sacR-B* cassette by using a circularized nonreplicating DNA fragment. Digestion of pKGR with *Bam*HI and *Bgl*II produced a 6.3-kb fragment containing the cassette flanked by sequences homologous to *E. coli* chromosomal DNA, an adjacent *cat* gene, and a 2.9-kb DNA fragment coding for ampicillin resistance and replication functions (Fig. 1). The 6.3-kb fragment was purified from an agarose gel and was self-ligated to form closed circles. This circularized fragment was used to transform *E. coli* EL1003 by selecting for a single homologous recombination event that confers resistance to chloramphenicol. Chloramphenicol-resistant transformants were screened for ampicillin and sucrose sensitivities. Small-scale DNA preparations were made to check for the absence of plasmids. Total DNA was digested with *Eco*RV, and Southern blots were hybridized with the *sacR-B* probe to confirm the chromosomal integration of the suicide cassette. Vector-integrated strain EL1026 was selected for further studies.

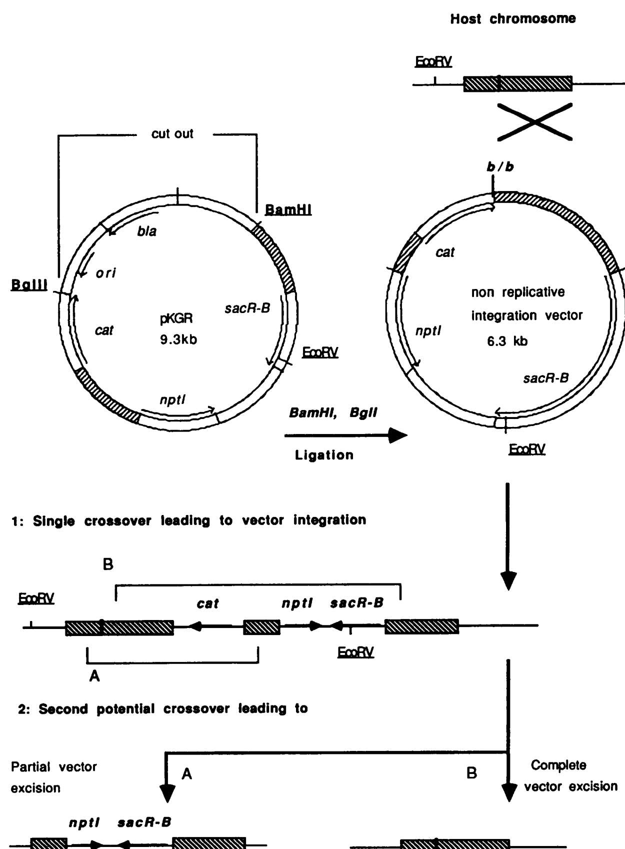

FIG. 1. Schematic representation of the mechanisms leading to the integration of the *nptI–sacR-B* cassette from pKGR into the *tdh* target region (hatched boxes) of the *E. coli* chromosome.

Stability of the integrated structure. To assess the stability of sucrose sensitivity and chloramphenicol resistance phenotypes under nonselective growth conditions, vector DNA deletion was favored, as follows. A single colony of the EL1026 strain was grown overnight in 2 ml of LB medium supplemented with chloramphenicol. The culture was diluted 1:100 into a second 2 ml of LB lacking antibiotic to allow vector excision, and the mixture was incubated overnight. Six additional cycles of growth were carried out under similar conditions, and appropriate dilutions of the culture were spread inoculated onto LB agar supplemented or not

with chloramphenicol. The colonies that grew on nonselective medium were isolated and were further screened for sucrose sensitivity and chloramphenicol resistance to determine the stable inheritance of the vector-encoded phenotypes.

Mutation rate of the killing system. To determine the frequency of reversion to sucrose tolerance of strain EL1026, it was grown in BB medium supplemented with chloramphenicol and kanamycin until the culture reached 5 $\times$ 10^6 cells per ml. Appropriate dilutions were spread inoculated onto BB agar supplemented or not with sucrose. Plates were incubated for 1 day at 37°C before counting.

***sacR-B* induction under nonselective growth conditions.** To examine the kinetics of *sacR-B* induction and its effectiveness as a suicide system in the absence of selection, sucrose was added to *E. coli* cells in broth lacking antibiotic. Overnight cultures were diluted 1:100 in 50 ml of BB and cells were grown at 37°C until the early log phase, reaching 5 $\times$ 10^6 CFU/ml. Induction of *sacR-B* was performed by adding sucrose (6%; wt/vol) to a 20-ml culture sample. The sucrose solution was replaced with a similar volume of sterile water in control cultures. Incubation was carried out at 37°C with shaking, and growth was monitored spectrophotometrically at 600 nm.

***sacR-B* induction in soil microcosms.** To examine *sacR-B* induction in soil microcosms, *E. coli* strains were grown to the mid-log phase at 37°C in LB supplemented with appropriate antibiotics, washed twice, and resuspended in sterile saline solution to the desired cell concentration. Soil microcosms were inoculated with a volume of suspension adjusted to obtain a final moisture content equivalent to field capacity. To study bacterial population dynamics after amending the soil with sucrose (5%; wt/dry wt), 500 μl of a sterile sucrose solution (500 mg/ml) was added to sterile and nonsterile soil microcosms. In control experiments, the sucrose solution was replaced with the same volume of sterile water. Three soil microcosms for each treatment were periodically used to determine bacterial population densities.

Microorganisms were extracted by blending a 5-g (dry weight) soil sample with 50 ml of sterile water for 2 min in a mixer (Waring blender; Eberbach Corporation). The soil suspension was then serially diluted in sterile water and the bacterial populations were enumerated by the plate count method (three plates per dilution level). Selective enumeration of *E. coli* cells from natural soil was performed on LB agar supplemented with cycloheximide (200 μg/ml), kanamycin (500 μg/ml), and neomycin (500 μg/ml), with a threshold of 10^2 CFU/g of dry soil. LB agar was used to count the *E. coli* cells extracted from sterile soil. Plates were incubated for 1 day at 37°C. Screening for chloramphenicol resistance and sucrose sensitivity in the *E. coli* EL1026 cells recovered from soil was performed to determine the stability of the integrated structure under environmental conditions. Plating onto nutrient agar supplied with cycloheximide (200 μg/ml) was used to determine the indigenous bacterial numbers. Viable counts were performed after 2 days of incubation at 28°C.

Data were plotted as the log of cell number per gram of dry soil against the incubation time (in days).

RESULTS

Chromosomal integration of the *nptI–sacR-B* cassette. As expected from selection for the integration of the nonreplicating vector via a single crossover event (Fig. 1), chloramphenicol-resistant transformants of *E. coli* EL1003 exhibiting ampicillin and sucrose sensitivities were recovered. In such transformants, the isolation of a circular plasmid was attempted by alkaline lysis. No plasmid DNA was visible in agarose gels after staining with ethidium bromide. The chromosomal integration of the vector was confirmed by Southern hybridization analysis (data not shown).

Stability of the integrated structure and mutation rate to sucrose tolerance. The number of chloramphenicol-resistant CFU determined on media with and without selection for chloramphenicol was similar after plating *E. coli* EL1026 subcultured for 200 generations without antibiotic pressure. All the colonies tested (n = 600) isolated from nonselective medium exhibited sucrose sensitivity and chloramphenicol resistance, indicating that the inheritance of the chromosomal insert was stable under nonselective growth conditions.

We compared the frequency of reversion to sucrose resistance on selective and nonselective media. The number of CFU determined on sucrose agar supplemented or not with chloramphenicol (i.e., sucrose-tolerant mutants) was similar after plating the EL1026 strain grown without antibiotic pressure. All the colonies isolated from nonselective sucrose agar plates exhibited sucrose resistance in the presence of chloramphenicol (data not shown). This demonstrated that the sucrose tolerance phenotype, which occurred at a frequency of 10^{-5}, did not result from vector excision.

A similar experiment was conducted with strain EL1003 (pKGR); while all the screened isolates were growth inhibited onto sucrose agar supplemented with chloramphenicol, they demonstrated sucrose tolerance under nonselective conditions, indicating that antibiotic pressure was required for the maintenance of the plasmid-encoded cell death function (data not shown).

***sacR-B* induction in the absence of a selective pressure.** The growth kinetics of *E. coli* EL1026 in the absence and presence of sucrose are plotted in Fig. 2B. In comparison with control experiments (Fig. 2A), the growth of the vector integrate (Fig. 2B) was inhibited 2 h after the addition of sucrose, demonstrating the induction of *sacR-B*. Whereas control cells continued to grow exponentially, induced cultures of *E. coli* EL1026 demonstrated bacterial lysis, as indicated by declining optical densities. Because no increase in the optical density occurred in the induced culture at the end of the time period of the experiment (Fig. 2B), samples of the final cultures were spread inoculated onto LB agar plates to determine the proportion of remaining viable cells. Of an initial inoculum of 5 $\times$ 10^6 CFU/ml, 1 $\times$ 10^3 CFU/ml survived 10 h after the addition of sucrose. This can be expressed as a suicide efficiency of up to 99.9% achieved in the absence of antibiotic selection. Of the *E. coli* cells that survived *sacR-B* induction, 96% of the tested isolates were chloramphenicol resistant, indicating that the chromosomal insert was retained despite the lethal pressure.

***sacR-B* induction in soil microcosms.** To assess the ability of the suicide cassette to contain strains released into the environment, the effect of *sacR-B* induction was examined in soil microcosms. After 24 h, sterile soil to which sucrose was added resulted in a 10^3-fold reduction of the *E. coli* EL1026 population density (Fig. 3A, curve b) compared with that in unamended soil controls (Fig. 3A, curve a). The *E. coli* cell numbers remained constant at 4.5 $\times$ 10^4 CFU/g 24 h after sucrose addition. Concurrently, the percentage of sucrose-sensitive cells, as determined by replica plating of colonies isolated at random from nonselective plates, showed a sine

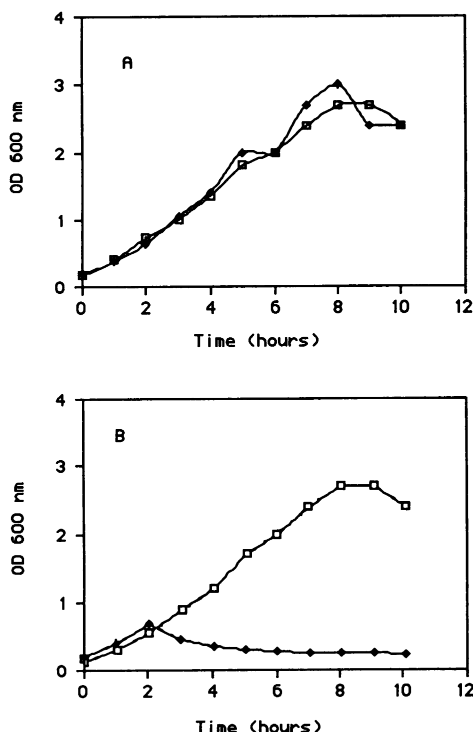

FIG. 2. Kinetics of *sacB* induction in the absence of selective pressure. *E. coli* growth was measured as the optical density at 600 nm (OD_{600}) at 37°C in BB supplemented (full symbols) or not supplemented (open symbols) with sucrose. (A) *E. coli* EL1003; (B) *E. coli* EL1026.

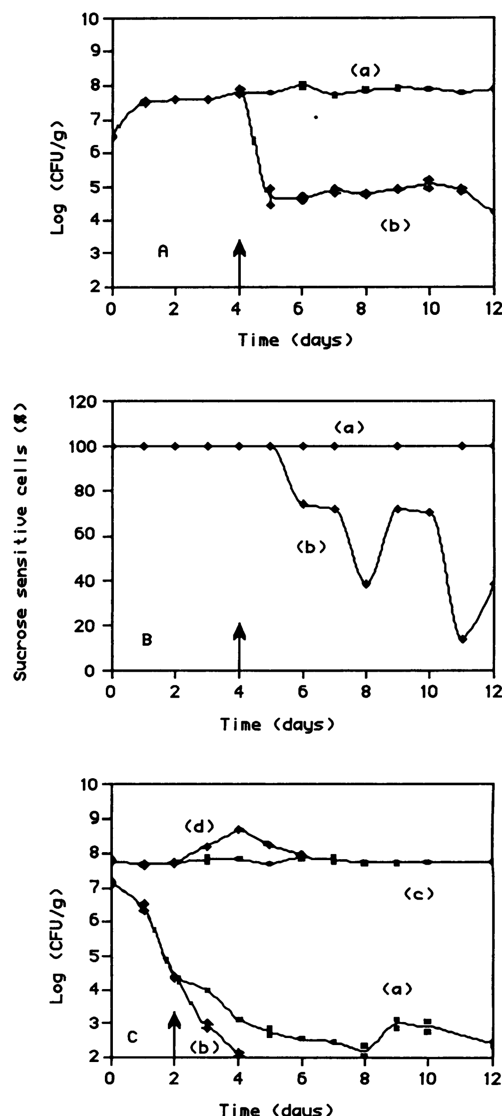

FIG. 3. Survival of *E. coli* EL1026 in sterile soil (A) and natural soil (C) in the absence (a) or presence (b) of sucrose and of indigenous bacteria in the absence (c) or presence (d) of sucrose. (B) The percentage of cells demonstrating sucrose sensitivity after isolation from sterile soil amended (b) or not amended (a) with sucrose. Arrows indicate the time of soil sucrose amendments. Curves connect the logs of the means of triplicate experiments. Error bars represent the standard deviation of the mean and are included within the symbol when not shown.

pattern throughout the time period of the experiment (Fig. 3B, curve b). Despite the appearance of sucrose-tolerant cells, there was no evidence of growth of either sucrose-sensitive or -resistant populations in sucrose-amended soils (Fig. 3A, curve b). To check the growth ability of the cells that survived sucrose addition, isolates recovered from sterile soil after 8 days of incubation were grown in liquid broth in the absence and presence of sucrose. The sucrose-tolerant population showed an exponential multiplication in both cases, indicating that these mutants were not impaired in their growth ability (data not shown). The sucrose-sensitive cells displayed the same growth patterns as the isolates recovered from unamended soil (data not shown) and the original parent strain EL1003. In unamended sterile microcosms, 100% of the tested colonies isolated from nonselective agar exhibited chloramphenicol resistance and sucrose sensitivity, demonstrating the stability of the vector integrate under environmental conditions (Fig. 3B, curve a). The addition of sucrose to sterile soil did not affect the population density of the isogenic parent *E. coli* EL1003 (Fig. 4A), indicating that the added substrate did not act as an abiotic factor on the survival of bacteria in the soil.

In nonsterile soil, sucrose was added 2 days after the inoculation of *E. coli* EL1026. The *E. coli* density decreased from 2×10^4 CFU/g to below the threshold of detection after 48 h (Fig. 3C, curve b). Concurrently, the uninduced population maintained a 10-fold higher density than the induced population and remained constant at about 5×10^2 CFU/g throughout the experiment (Fig. 3C, curve a). All tested isolates recovered from natural soil that was amended or not with sucrose exhibited chloramphenicol resistance and sucrose sensitivity (data not shown). Compared with the unamended control (Fig. 3C, curve c), sucrose addition to natural soil led to a fivefold increase in the indigenous bacterial density after 48 h, which decreased back to its original level of 5×10^7 CFU/g by day 7 (Fig. 3C, curve d). Sucrose addition to natural soil increased the viability of the sucrose-resistant isogenic parent strain *E. coli* EL1003 in comparison with its viability in unamended microcosms; its population density kept constant at 10^5 CFU/g of soil until day 5, remaining present at a level of 2 log units higher than that in unamended controls (Fig. 4B, curves a and b). Concurrently, the indigenous bacteria showed a fivefold

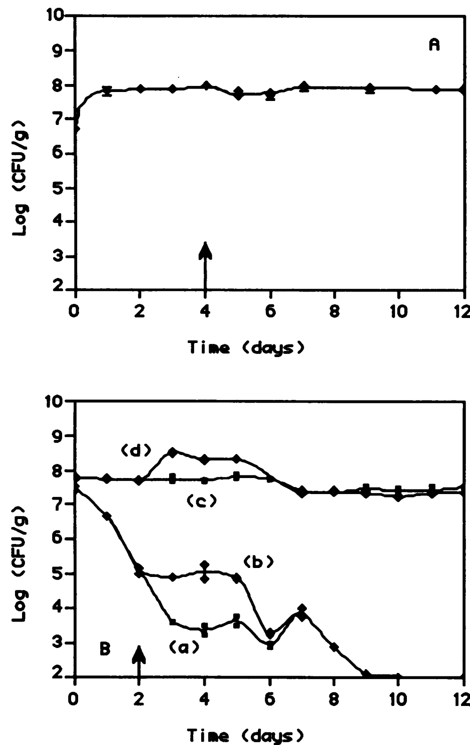

FIG. 4. Survival of *E. coli* EL1003 in sterile soil (A) and natural soil (B) in the absence (a) or presence (b) of sucrose and of indigenous bacteria in the absence (c) or presence (d) of sucrose. Arrows indicate the time of soil sucrose amendments. Curves connect the logs of the means of triplicate experiments. Error bars represent the standard deviation of the mean and are included within the symbol when not shown.

increase in number 24 h after sucrose addition and maintained this density for the subsequent 48 h (Fig. 4B, curve d).

DISCUSSION

In previously described conditional suicide systems for *E. coli*, the genes encoding the cell-killing function were carried on multicopy plasmids such as pUC19 derivatives (2). However, the selective pressure required for their stable maintenance in the host forbids their use under environmental conditions. Although several copies of integrated genes may allow for a more efficient expression, insertion of the suicide system into the chromosome could improve its stability outside batch processes. To avoid the described failures of *hok* and *gef* gene containments, the *nptI–sacR-B* suicide cassette was chosen since resistance to kanamycin offered an easy means of selecting for sucrose sensitivity. Repeated attempts to integrate the cassette into the *E. coli* chromosome by using either mini-mu duction (12) or transformation of *recBC sbcBC* or *recD* mutants by linearized DNA (11, 19) failed to produce recombinants (data not shown).

Increased integration efficiency was achieved by using positive selection for chloramphenicol resistance encoded by a nonreplicating circular vector. The procedure proved to be efficient for the chromosomal integration of the suicide cassette via a single recombination event, resulting in sucrose-sensitive chloramphenicol-resistant transformants. Despite repeated attempts to obtain the cassette insertion via replacement recombination, chloramphenicol-sensitive clones from which vector DNA was deleted by a second crossover event could not be isolated. Such a result can be explained by assuming that the vector can be excised at a low frequency; otherwise, selection for excised plasmid integrates by using temperature-dependent replicons has been reported to be unreliable (3).

Stability studies yielded no loss of either chloramphenicol resistance or sucrose sensitivity markers in the vector integrate *E. coli* EL1026. These results are consistent with previous studies reporting the stability of nonreplicating vectors integrated into the *E. coli* or *Lactococcus lactis* chromosome via a single crossover event (13, 15). In agreement with the data reported by Blomfield et al. (3), mutation to sucrose tolerance occurred at a frequency of 10^{-5}, implying that *E. coli* EL1026 is a reliable counterselectable parental strain for gene transfer studies (20). The lack of growth of the induced cultures indicates that the efficiency of suicide system is not affected by selection for sucrose-tolerant mutants.

The microcosm experiments confirmed the ability of the *sacR-B* suicide cassette to restrict the growth and survival of *E. coli* strains introduced into soil (Fig. 3A and B). Although the isolates recovered from sucrose-amended soil were not impaired in their growth ability, renewed soil colonization was not observed (Fig. 3A, curve b). The *E. coli* cells that survived sucrose addition might be space or energy limited, preventing them from multiplying. This hypothesis can be strengthened by the fact that sucrose addition into sterile soil 24 h after the inoculation of *E. coli* did not prevent the residual population from increasing (data not shown). Alternatively, the sine curve kinetics of the percentage of sucrose-sensitive cells suggest a competitive disadvantage for the sucrose-tolerant mutants (Fig. 3B, curve b).

The growth of the indigenous microflora at the expense of sucrose did not impair *sacR-B* induction in natural soil. The *E. coli* EL1026 population decreased below the detection threshold 48 h after the addition of sucrose (Fig. 3C, curve b). This was not the result of the multiplication of the indigenous bacteria, since the isogenic parent did not exhibit a decrease in number but a better overall survival under similar conditions (Fig. 4B, curve b). Because of the inability of *E. coli* EL1003 to use sucrose as a carbon source, increased viable counts of this organism are likely to occur at the expense of the substrates provided via the metabolism of the indigenous microorganisms. The comparison of the population dynamics of the isogenic counterparts in sucrose-amended soil demonstrates that not only did *sacR-B* induction prevent the proliferation of *E. coli* cells but it also triggered their rapid death in natural soil. Additionally, it indicates that the insertion of the suicide assette into the chromosome provided neither an advantag nor a disadvantage to the host cells for survival in soil the absence of sucrose (Fig. 3C, curve a; Fig. 4B, curve).

The efficiency of the *sacR-B* condition killing system in triggering in situ specific bacterial lysis ma es it a very useful tool for ecological studies. The low expensive, the nontoxic effector, and the specificity and adaptability to a wide range of gram-negative bacteria are additional conveniences of this conditional killing system for containing and counterselecting engineered microorganisms.

ACKNOWLEDGMENTS

We are grateful to the Eurolysine laboratory members for their kindness, assistance, and encouragement. We thank very much M. Bocarra, who kindly provided plasmid pUM24.

This work was supported by a BDI grant from CNRS and Eurolysine Co.

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