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Maintenance and killing efficiency of conditional lethal constructs in *Pseudomonas putida*

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SUMMARY

Conditional lethal (suicidal) genetic constructs were designed and employed in strains of Pseudomonads as models for containment of genetically-engineerd microbes that may be deliberately released into the environment. A strain of *Pseudomonas putida* was formed with a suicide vector designated pBAP24*h* that was constructed by cloning the host killing gene (*hok*) into the RSF1010 plasmid pVD*tac*24 and placing it under the control of the *tac* promoter. After *hok* induction in *P. putida* only 40% of surviving cells continued to bear the *hok* sequences within 4 h of induction; in contrast, 100% of the cells in uninduced controls bore *hok*. A few survivors that demonstrated resistance to *hok*-induced killing developed in *P. putida*, which may have been due to a mutation or physiological adaptation that rendered the membrane 'resistant' to *hok*. Conditional lethal strains of *P. putida* also were formed by inserting *gef* (a chromosomal homolog of *hok*) under the control of the *tac* promoter into the chromosome using a transposon. Constructs with chromosomal *gef*, as well as an RK2-derived plasmid construct containing *gef*, were only marginally more stable than the *hok* constructs; they were effective in killing *P. putida* when induced and within 2 h post-induction killing from either *gef* construct resulted in a 10^3–10^5-fold reduction in viable cell count compared to uninduced controls.

INTRODUCTION

The *parB* locus, which is responsible for partitioning of R1 plasmids in *Escherichia coli* by post-segregational killing of plasmid-free cells, contains host killing (*hok*) and suppressor of killing (*sok*) genes [11,12]. A chromosomal gene (*relF*) from *Escherichia coli* has been identified that was homologous (≥60%) to R1 *hok* gene [12,18]. A second chromosomal locus *gef* (gene expression fatal or gene family) from *E. coli* has also been detected through its homology to R1 *hok* [18]. Overexpression of the R1 *hok* or chromosomal *relF* genes can cause breakdown of the transmembrane potential and concurrent drop in oxygen consumption followed by the death of the cells [12] and *gef* presumably has the same effect.

The lethal function of the R1 *hok* gene has been considered by several groups as the basis for constructing a conditional suicide system that could be used for the containment of genetically engineered microorganisms deliberately released into the environment [6,16,17]. Conditional lethal plasmid constructs containing *hok* (suicide vectors) have been tested in model systems, primarily *E. coli*. While effective in large part in containing the plasmid constructs, the suicide systems have not been fail-safe and *hok* resistance has developed in survivors [6,13].

Here we describe the construction and stability of model conditionally lethal systems containing R1 *hok* or the chromosomally-derived *hok* homolog, *gef*, in low or high copy number, *E. coli–Pseudomonas* plasmid vectors or as a chromosomal insertion, and examine the effectiveness of such systems upon induced death (suicide) in *Pseudomonas* sp. Our aim was to compare the efficiency and maintenance of such systems in a candidate organism for deliberate release into the environment, such as *P. putida*, with results obtained previously for *E. coli* using similar constructs. Given the previous observation that survivors of *hok* induction were often due to loss or debilitation of the plasmid bearing the lethal construct [6] we also examined whether greater stability and efficiency of killing could be achieved by chromosomal insertion of the conditional lethal construct.

MATERIAL AND METHODS

Bacterial strains, plasmids, and growth media

Plasmid pPR633 (pBR322 replicon, Amp^r, Lac^-, $ParB^+$) was used as the source of the *hok* gene [11]. Since the *lac* promoter does not function efficiently in

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pseudomonads [1,3,4] and since the *trp* repression system apparently is not tight enough in *P. putida* [16], we used the plasmid vector pVD*tac*24 which contains the hybrid *trp-lac* promoter, *tac* [4], for constructing conditionally lethal constructs for use in pseudomonads. The promoter *tac* functions well in pseudomonads [1,4] and was used to construct pBAP24*h*. The vector pVD*tac*24 used in the construction of pBAP24*h* also contains $lacI^q$, which is required to suppress the expression of the *hok* gene [6]. Plasmid pVD*tac* 24 (Amp^r, *lacZ'*, $lacI^q$, mob, *nic*, *oriV*, *tac*) contains multiple cloning sites within the *lacZ'* gene, $lacI^q$, and a gene coding for carbenicillin and ampicillin resistance [8]. pVD*tac*24, which belongs to the IncQ incompatibility group and contains the *mob* gene for mobilization from one host to another, is a high copy number broad host range vector [8]; it was originally derived from the RSF1010 replicon and should be maintained in pseudomonads [3]. Plasmid pSUP202 (a mobilizable pBR322 replicon) [19] was used as a vector for plasmid construction as was plasmid pCB303 (low copy-number, broad-host-range RK2 replicon, Tc^r, *lacZ'*) [5].

The following bacterial strains were used: *E. coli* HB101 (*recA*13, *hsdS*20, *ara*-14, *proA*2, *rpsL*20, *supE*44), *E. coli* JM83 (*ara*, Δ*lac* – *pro*, *rpsL*, *thi*, φ80d*lacZ*M15), *P. putida* mt – 2 (cure), *P. putida* KT2440 (from K. Timmis). *E. coli* strains were maintained on TYE agar [9] and grown at 37 °C. *P. putida* mt – 2 (cure) was grown at 28 °C on *Pseudomonas* isolation agar (Difco Laboratories) and *P. putida* 2440 was grown in LB broth [14].

Enzymes and chemicals

The sources of enzymes and other chemicals were as follows: restriction endonucleases (*Xba*I, *Eco*RI, *Bam*HI, *Acc*II, *Sal*I, *Pst*I) and nick translation kit (Amersham Corp., Arlington Heights, IL); DNA polymerase I (Klenow fragment), T4 DNA ligase, and dNTPs (U.S. Biochemical, Cleveland, OH); [α-^{32}P]dATP (>600 Ci/mmol) (New England Nuclear Corp., Boston, MA); chloramphenicol, ampicillin, carbenicillin, 2,4,5-T, IPTG, dithiothreitol, and ATP (Sigma Chemical Co., St. Louis, MO); lambda DNA digested with *Hin*dIII, and 1-kb size ladder for use as size standard in gel electrophoresis (Bethesda Research Laboratories, Inc., Gaithersburg, MD); agarose and polyacrylamide (International Biochemicals Inc.); and Seaplaque low melt agarose (FMC Corp. Marine Colloids Div., Rockland, MD).

Isolation and purification of plasmid DNAs

For plasmid DNA extractions bacterial strains were grown in 2 × YT broth [9]. Plasmid DNAs were released from bacterial cells by alkaline lysis of the cells in the presence of SDS [2]. Plasmid DNAs were isolated and purified by cesium chloride ethidium bromide density gradient centrifugation [2]. The purified plasmids were subjected to restriction digestion following procedures supplied by the manufacturer for each enzyme used. The restriction enzyme digested DNAs were analyzed by agarose gel electrophoresis run in 1% agarose with 1 × TAE buffer [2] at 5.5 V/cm for 3–4 h or by polyacrylamide gel electrophoresis (run in 5% polyacrylamide gels) with 1 × TBE buffer [14] at 5.0 V/cm for 1.5–2 h.

Construction of hok-*containing plasmid vector*

Plasmid pPR633 was digested with *Acc*II restriction enzyme to generate a series of blunt end fragments (Fig. 1A). Fragments in the range of 400–600 bp, which would include the 493-bp fragment containing the *hok* gene, were isolated from 0.8% SeaPlaque low melt agarose. The vector pVD*tac*24 (an RS1010 replicon derivative) was digested with *Xba*I and the ends were filled in with the Klenow fragment of DNA polymerase I. The treated vector was then ligated to the *Acc*II fragments from pPR633 using T4 DNA ligase and the ligation mixture was used to transform *E. coli* JM83 competent cells according to the procedure described by Bej et al. [6]. Ampicillin resistant colonies containing the newly formed plasmid (pBAP24*h*) were detected by replica plating onto TYE agar containing 30 μg IPTG and 40 μg ampicillin per ml.

Construction of gef-*containing vector for chromosomal insertion*

Two conditional lethal constructs were produced using the *gef* gene fused to the *tac* promoter. A mobilizable pBR322 derivative, pSUP202, was used and transposon Tn*5* was transposed into the ampicillin resistance gene of this plasmid (Fig. 1B). The $lacI^q$ gene was isolated as an *Eco*R1-*Sca*I fragment to which a *Sal*I linker was added at the *Eco*R1 end. This *Sal*I-*Sca*I fragment was then cloned into the *Sal*I-*Sma*I restricted region of the Tn*5*. A fragment containing the *tac-gef* fusion was subsequently ligated into the unique *Bam*HI site of Tn*5*, to yield the new transposon Tn*950*. The plasmid containing Tn*950* — designated pSM950 — was used for chromosomal insertion of *gef*.

Construction of gef-*containing plasmid vector*

The *Bgl*II fragment from Tn*950* containing the *tac-gef* fusion was ligated into the *Bam*HI site of the broad-host-range vector pCB303 to form a conditional-lethal (suicide) plasmid vector designated pSM970 (Fig. 1C).

Transfer of hok *and* gef *containing plasmids into pseudomonads*

The conditional lethal plasmids containing *hok* or *gef* were transferred to *P. putida* by transformation following the method described by Mercer and Loutit [15]. The cells

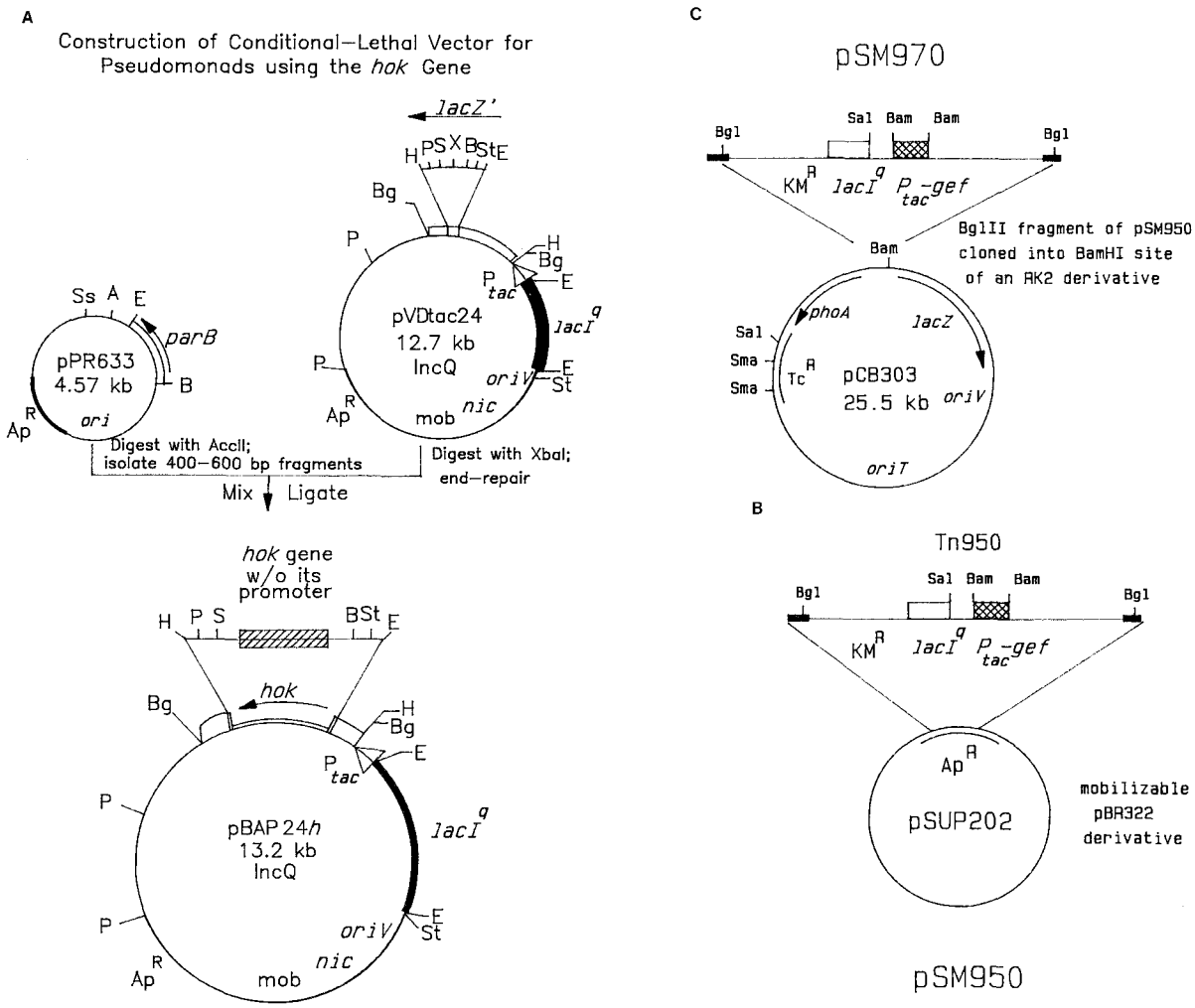

Fig. 1. Design of suicide systems. Conditions were described in the text. Restriction endonuclease sites are as follows: A, *Acc*II; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI, H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Ss, *Ssp*I; St, *Sst*I; X, *Xba*I; Bgl, *Bgl*II; Bam, *Bam*HI; Sal, *Sal*I; Sma, *Sma*I. Ap^R, resistance to ampicillin; P_{tac}, *tac* promoter; *lacZ*, the gene coding for β-galactosidase, *ori*, origin of replication; bp, base pairs; kb, kilobase pairs; Tc^R, resistance to tetracycline; $lacI^q$, gene which overproduces the lacI repressor. A prime denotes a gene not completely present or interrupted by other DNA sequences. (A) Construction of pBAP24*h*; (B) construction of pSM950/Tn950; and (C) construction of pSM970.

were grown for the expression of the transformed DNAs at 30 °C for 90 min. A 0.2-ml aliquot was then spread onto a selective medium containing 0.5 mg/ml carbenicillin and grown at 30 °C for 36 h.

Attempts were made to transfer plasmid pBAP24*h* into *P. cepacia* AC1100, *P. aeruginosa* PAO, and *P. putida* mt – 2 by triparental mating [7], using *E. coli* HB101 carrying helper plasmid pRK2013 [10] and *E. coli* JM83 (pBAP24*h*) on non-selective medium.

The presence of *hok* gene sequences in the clones was determined by colony hybridization and Southern blot analysis following the procedure of Ausubel et al. [2]. As a probe, a 480-bp *Bam*HI-*Eco*RI fragment from pPR633 containing *hok* gene was nick translated using [α-^{32}P]dCTP and a kit (Amersham, Arlington Heights, IL). For colony hybridizations, 1.2-μm nylon membranes (ICN Biomedical, Costa Mesa, CA) were used and colonies were lysed and further treated according to procedures recommended by Ausubel et al. [2]. Hybridization was visualized by autoradiography on Kodak XAR-5 film at −70 °C for 0.3–10 h.

Transfer of gef *into the chromosome of* P. putida *by triparental mating*

In order to transfer *gef* from pSM950 into the chromosome of *P. putida* 2440, donor strain *E. coli* S17.1 [19]

was used, which harbors plasmid RP4 integrated into the chromosome. Triparental matings were carried out as described [19] at 30 °C overnight on agar plates. Following matings, the pseudomonad mating cultures were transferred to selective agar media containing appropriate antibiotics. Plasmid pSM950 is a mobilizable, pBR322 derivative, and as such, is not capable of stable replication in pseudomonads. Therefore, isolates in which Tn*950* had transposed from pSM950 to the *P. putida* 2440 chromosome could be selected as kanamycin-resistant colonies (Fig. 1B). This chromosomally-based system was maintained in strains both in the presence and absence of kanamycin selection for at least five passages in liquid or solid media.

Induction of hok *constructs*

The kinetics of *hok* induction in *P. putida* mt – 2, carrying pBAP24*h* plasmid was determined by adding IPTG when the cultures reached 10^7–10^8 CFU/ml. Controls without IPTG addition were also run. Bacterial strains were grown in 2 × YT broth [9] for these *hok* induction studies. Cultures were grown to approximately 10^8 cells/ml at 30 °C, and IPTG was added to a final concentration of 0.1 mM to replicate cultures to induce *hok* expression. Samples were collected immediately after IPTG addition (0 time) and hourly for the next 5 h. The A_{450} of the samples was determined as a measure of growth. Cells were grown at 30 °C with or without 500 μg per ml carbenicillin (CB) and serial dilutions of the samples were plated onto TYE agar containing 500 μg per ml CB to determine numbers of CFUs (viable cells). For *E. coli*, serial dilutions were plated onto TYE agar containing 40 μg ampicillin per ml. *Pseudomonas* transformants were maintained on *Pseudomonas* isolation agar containing 1000 μg carbenicillin per ml. Colony hybridization was used to identify colonies containing the *hok* gene.

Induction of gef *constructs*

For *P. putida* strains bearing either Tn*950* or pSM970, strains were grown in LB broth containing 200 μg kanamycin per ml. At approximately 10^7 CFUs per ml the cultures were split and to half of the culture IPTG was added to a final concentration of 1 mM to induce *gef* expression. The other half of each culture was grown without IPTG for comparison. Samples were collected hourly for 5 h and serial dilutions were plated onto IPTG-containing TYE agar to detect survivors.

RESULTS

Viable counts of *P. putida* (pBAP24*h*) showed an increase in cell number for the uninduced culture, whereas the induced culture (starting at 10^8 CFU/ml) showed a decrease of 2 log in the number of cells between 1 h and 3 h following *hok* induction — after which cell number increased (Fig. 2); the outcome was an overall decline of only approximately 0.5 log. Colony hybridization analyses showed a decrease in the number of cells containing pBAP24*h* — from 97 to 38% 4 h after *hok* induction in the absence of carbenicillin and from 96% prior to *hok* induc-

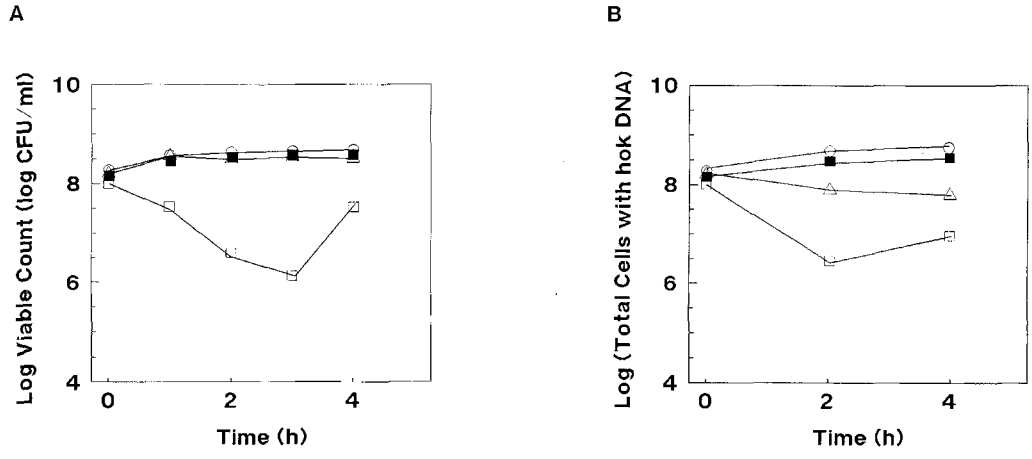

Fig. 2. Effect of *hok* induction on *P. putida* mt – 2 (pBAP24*h*). (A) Cells were grown in 2 × YT broth at 30 °C with or without drug (500 μg carbenicillin per ml) to early log phase before they were allowed to continue growth in the presence or absence of IPTG (final concentration 0.1 mM). (B) The number of cells carrying *hok* sequences was determined by colony hybridization using as probe the ^{32}P-labelled 480-bp *Bam*HI-*Eco*RI fragment from pPR633 containing the *hok* gene. For viable cell count and colony hybridization experiments samples were plated onto agar containing 500 μg carbenicillin per ml. Cells grown without drug, ○, – IPTG; Δ, + IPTG. Cells grown with drug, ■, – IPTG, □, + IPTG.

tion to 24% 4 h after induction in the presence of carbenicillin. These results indicate that *hok* insertion resulted in selective survival of *hok*-free cells and that *hok* induction caused cell death of *P. putida*, but that subsequently surviving cells lacking *hok* began to multiply, so that total numbers of cells containing *hok* sequences eventually increased (Fig. 2B). For the uninduced culture, colony hybridization showed 100% hybridization with the *hok* gene, indicating that *hok* induction was necessary for selection against pBAP24*h*-containing cells.

If 'efficiency of killing' is defined as the fold reduction in viable cell count (compared to starting numbers or uninduced cultures), then the efficiency of *hok*-induced killing in *P. putida* (pBAP24*h*) was lower than in *E. coli* (pBAP24*h*). Total viable counts of *E. coli* JM83 with pBAP24*h* showed a decrease on selective media between 1 and 3 h of *hok* induction. Cells containing *hok* declined from 98% at the time of induction to 1% 4 h after induction (Fig. 3). All surviving *E. coli* cells had complete or partial *hok* gene as evidenced by colony hybridizations using the *hok* gene as a probe (Fig. 3). The plasmids from survivors which hybridized to the *hok* gene contained intact pBAP24*h* as determined by restriction analysis. When fresh JM83 cells were transformed with such plasmids, they again responded to induction with IPTG and showed a similar killing pattern as seen previously (Fig. 3). Also, pBAP24*h*-containing survivors of induction, tested by plating on agar containing 40 μg ampicillin per ml and again subjected to induction, displayed the original killing pattern seen in Fig. 3.

Randomly-selected *E. coli* survivors from the selective media had a major deletion compared to pBAP24*h*. Plasmids isolated from such survivors were approximately 4.0 kb compared to 13.2 kb for pBAP24*h*. Such deletion-derivatives of pBAP24*h* were found only in *E. coli* induced in the presence of ampicillin. No deletion-derivatives were isolated from the pseudomonad strains upon induction with simultaneous selection for the plasmid. Southern blot DNA-DNA hybridizations confirmed the presence of at least part of the *hok* gene in the deleted plasmids. *E. coli* cells carrying the deleted plasmids failed to die upon induction.

A *gef*-bearing plasmid (pSM970) alternate suicide vector was tested. Induction of *gef* in *P. putida* containing pSM970 produced a 50-fold reduction in viable cell count within 2 h. This was a 10^3-fold reduction relative to the uninduced cultures which continued to grow. Only 10–50 *gef* resistant survivors per ml were observed at 90 min post-*gef*-induction. Subsequently the number of *gef*-resistant survivors, however, increased, reaching 300 per ml induced culture by 3 h post-induction.

For *P. putida* 2440 (Tn*950*), which has *gef* in the chromosome, *gef* induction by addition of IPTG to growth cultures produced a 10^3-fold reduction in viable cell count within 2 h. This was a 10^5-fold reduction relative to the uninduced cultures. Less than 10 survivors per ml of induced culture were observed for samples within the first 90 min of induction. After 90 min the number of survivors of *gef* induction increased so that by 4 h post-induction there were approximately 1000 survivors per ml induced culture.

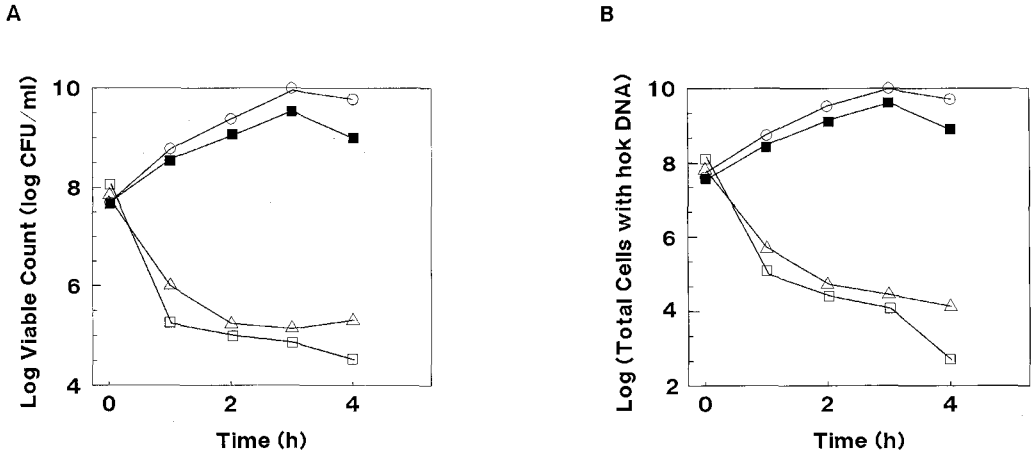

Fig. 3. Effect of *hok* induction on *E. coli* JM83 (pBAP24*h*). All conditions were as in Fig. 2 except that cultures were grown at 37 °C and viable counts and colony hybridizations were performed on agar containing 40 μg ampicillin per ml. Also, when cultures were grown in the presence of drug prior to induction, 40 μg ampicillin per ml replaced carbenicillin. For cultures grown without drug, ○, − IPTG; □, + IPTG; for cultures grown with ampicillin, ■, − IPTG; Δ, + IPTG.

DISCUSSION

The suicide vector pBAP24*h*, which was originally constructed and isolated from *E. coli* JM83 transformants, was transferred into *P. putida* mt – 2 via transformation. The efficiency of transformation of *P. putida* was low, ca. 3–5 transformants per μg pBAP24*h* DNA. Molin et al. (1987) previously reported that transfer of a *hok*-bearing plasmid into *P. putida* occurred at low efficiencies and suggested that one reason might be that incomplete repression of *hok* could have resulted in killing of transformed cells receiving *hok*. They did not examine the kinetics of *hok* induced killing or the stabilities of the conditional lethal *P. putida* constructs as has been done in the current study. We found that, although pBAP24*h* was maintained in *E. coli* grown without selection (100% of colonies hybridized to the *hok* gene probe), pBAP24*h* was lost from *P. putida* after repeated transfers on non-selective media (only 14% of colonies hybridized to the *hok* gene probe after 6–10 transfers), indicating instability of the suicide vector plasmid in pseudomonads under non-selective conditions. In contrast, on selective media, the pBAP24*h* plasmid was maintained in *P. putida* in the absence of *hok* induction but was rapidly lost from the population following *hok* induction as evidenced by colony hybridization with the gene probe for *hok*.

Hok induction in the pseudomonads resulted in a lower efficiency of killing than in *E. coli*. This does not necessarily mean that the action of *hok* is less efficient in pseudomonads than in *E. coli*, but may reflect the fact that pBAP24*h* was less stably maintained in *P. putida* than in *E. coli* as evidenced by the greater proportion of cells lacking the suicide vector in *P. putida* than in *E. coli* following *hok* induction. While the loss of the suicide vector from pseudomonads represents a failure to function adequately as a containment system for the target strain, if recombinant DNA was the same plasmid as the suicide vector, *hok* induction would have resulted in the elimination of almost all cells containing the recombinant DNA.

Although the progenitor plasmid, pVD*tac*24 was successfully transferred into pseudomonads by triparental mating at high efficiency, we repeatedly failed to transfer pBAP24*h* into the *Pseudomonas* species used in this study by this method. The failure to transfer pBAP24*h* from *E. coli* to *P. putida*, *P. aeruginosa* and *P. cepacia* by triparental mating could have been due to rapid cell death following the entrance of pBAP24*h*. Tests with *P. cepacia* AC1100 containing *hok* introduced by transformation indicate that *hok* efficiently kills that strain following induction (results not shown).

The killing provided by *gef* from the Tn*950* chromosomal construct was rapid and efficient in *P. putida*. Nevertheless, like all other suicide vector systems so far examined as models for the containment of genetically engineered microorganisms, under selective pressure to maintain the introduced systems, survivors of *gef*-induction appeared by 90 min post-induction. Since the Tn*950* system was chromosomally located, one would expect less survivors than in the plasmid-borne constructs, where plasmid loss is a factor. This prediction was borne out at 90 min post induction. Surprisingly, however, by 4 h post-induction, the frequency of survivors for the Tn*950* system was equivalent if not greater than that observed for pSM970. Thus, for purposes of containing recombinant bacteria designed for deliberate environmental release, chromosomal placement of *gef* as a conditional lethal (suicide) system does not appear to provide a fail-safe or even more protective system than plasmid suicide vectors.

Knudsen and Karlström [13] investigated factors in *E. coli* which reduced survival after induction of the *E. coli relF* gene, which is homologous to *hok* and *gef*. They found that the use of more tightly-controlled promoters to repress the suicide gene improved the efficiency of their system by preventing selection of cells mutated in the killing function. An additional improvement was observed when the conditional lethal system was introduced into the test organism in multiple copies; in such cases mutation rates were reduced to 'acceptable levels' [13] $< 5 \times 10^{-9}$ per cell per generation. Our results suggest that an additional factor in containment using such conditional lethal constructs is the maintenance of the kill gene in the host bacteria. Improved stability via chromosomal insertion alone proved inadequate as a fail-safe system. However, the numbers of survivors might be reduced still further via gene replacement methods using a cloned gene from *P. putida* rather than a transposon to introduce the suicide system into the chromosome. A combined approach linking improved stability, tightly-controlled promoters, and multiple redundant suicide systems, must be examined if an adequate system that can provide a measure of containment for deliberately released genetically-engineered microorganisms is to be developed.

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