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# Characterization of Cell Lysis in *Pseudomonas putida* Induced upon Expression of Heterologous Killing Genes

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**Active biological containment systems are based on the controlled expression of killing genes. These systems are of interest for the *Pseudomonadaceae* because of the potential applications of these microbes as bioremediation agents and biopesticides. The physiological effects that lead to cell death upon the induction of expression of two different heterologous killing genes in nonpathogenic *Pseudomonas putida* KT2440 derivatives have been analyzed. *P. putida* CMC4 and CMC12 carry in their chromosomes a fusion of the $P_{A1-04/03}$ promoter to the *Escherichia coli gef* gene and the ϕX174 lysis gene *E*, respectively. Expression of the killing genes is controlled by the LacI protein, whose expression is initiated from the XylS-dependent Pm promoter. Under induced conditions, killing of *P. putida* CMC12 cells mediated by ϕX174 lysis protein E was faster than that observed for *P. putida* CMC4, for which the Gef protein was the killing agent. In both cases, cell death occurred as a result of impaired respiration, altered membrane permeability, and the release of some cytoplasmic contents to the extracellular medium.**

Active biological containment (ABC) systems have been envisaged as a way to control the survival of genetically modified microorganisms and the putative consequences of their introduction into the environment (Fig. 1) (for reviews, see references 20 and 26). ABC systems are based on the use of genes that encode killing proteins regulated by a control element that activates (or derepresses) the killing function under defined environmental conditions (1, 21).

The development of ABC systems for the *Pseudomonadaceae* is of interest because of the potential applications of these microbes under field conditions. The so-called fluorescent *Pseudomonas* group includes strains whose biochemical, physiological, and genetic properties have been well characterized (7, 27, 35). A number of genetic tools have made it possible to design recombinant derivatives of this group of bacteria for the biological control of pests (4, 24), the promotion of plant growth (13, 17, 18), and the detoxification of polluted sites (8, 27, 35).

*Pseudomonas putida* KT2440 is a DNA restriction-modification system-negative strain derived from the soil bacterium *P. putida* mt-2, the natural host for the archetypal TOL plasmid pWW0 (39). Strain KT2440 has been shown to be a nonaggressive soil rhizosphere colonizer (22, 25, 28). In addition, this strain stably maintains and expresses heterologous genes, including catabolic segments for the expansion of its metabolic versatility and killing genes of interest for biological containment (5, 21, 26–28). The genes encoding killing functions successfully used in *P. putida* were those that encode lysis proteins (1, 11, 14, 30), nucleases (3), and streptavidin (34). In previous studies, we demonstrated that the regulatory gene expression system of the TOL plasmid *meta*-pathway for the metabolism of alkylbenzoates could be combined with the *gef* gene of *Escherichia coli*, which encodes a porin-like protein, in such a way that cell killing became a consequence of the absence of the substrate (and inducer) 3-methylbenzoate both under laboratory conditions (1, 11, 30, 31) and in soil microcosms (11, 30). We also showed that a *P. putida* strain carrying an ABC system on the host chromosome functioned as expected under field conditions (23). A modified version of this system based on lysis gene *E* of bacteriophage ϕX174 has also been constructed (30). However, the above studies have not dealt in detail with the physiological phenomena that lead to cell death in *P. putida* upon induction of the expression of killing genes in this heterologous host. In *E. coli*, the natural host for ϕX174 phage protein E-mediated cell lysis, killing occurs as the consequence of the formation of a transmembrane tunnel made of E protein; the tunnel fuses the outer and inner membranes and allows the escape of cytoplasmic material (36–38). In *E. coli*, the Gef protein forms dimers that are anchored in the cytoplasmic membrane and lead to the collapse of the cell membrane potential (reviewed in reference 20).

In this study, we show that once the Gef protein or the ϕX174 E protein is expressed in *P. putida*, cell death occurs as a consequence of the formation of membrane holes, which impair respiration, alter membrane permeability, and release cell material.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The *P. putida* strains used or constructed in this study are derivatives of *P. putida* KT2440 (6). Their relevant characteristics are given in Table 1. *P. putida* EEZ29 (31), CMC4 (23), and EEZ15K-3 (29) were described before; these three strains bear the archetypal TOL plasmid pWW0, which confers on them the ability to grow on 3-meth-

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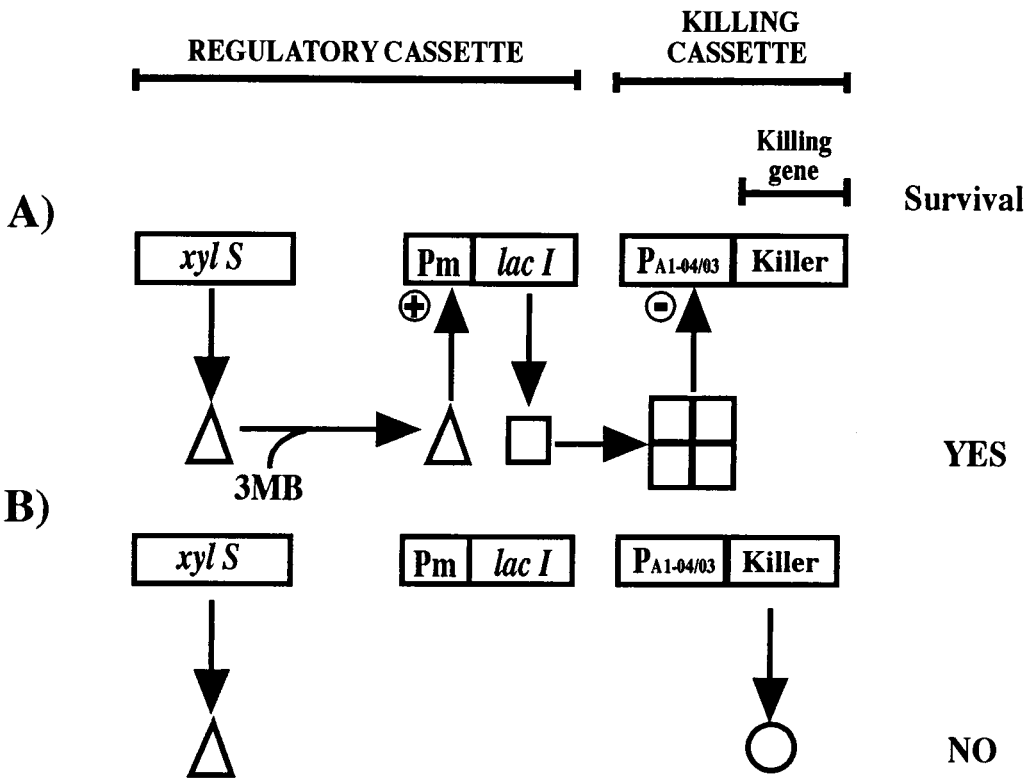


FIG. 1. Detail of the biological containment system for alkylbenzoates. This model consisted of the Pm promoter, which drives the transcription of the *meta*-cleavage pathway of the TOL plasmid, and the *xylS* gene, which encodes the sensor protein that interacts with alkylbenzoates and stimulates transcription from Pm. In the containment system, the *lacI* gene, coding for the LacI repressor protein, was cloned downstream from Pm. The lethal element consisted of the $P_{A1-04/03}$ promoter fused to the *gef* gene of *E. coli* or ϕX174 gene *E*, each of which codes for pore-forming proteins. The system was shown to perform as follows. In the presence of 3-methylbenzoate, the XylS protein became active and stimulated the synthesis of the LacI protein, which in turn prevented the expression of the killing gene; concomitantly, degradation of the alkylaromatic compound took place. Once the compound was exhausted, the XylS protein became inactive, the LacI protein was not made any longer, and expression from $P_{A1-04/03}$ led to the synthesis of Gef or E protein, which in turn collapsed the cell membrane potential and led to the death of the cell.

TABLE 1. Strains and plasmids used in this study[a]

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *Escherichia coli* | | |
| Mv1190λ*pir* | F′ (*traD36 proAB*$^+$ *lacZ*Δ*M15 lacI*$^q$) *thi* Δ(*lac-proAB*) Tn*10*λ*pir* | 9 |
| JM109 | F′ (*traD36 proAB*$^+$ *lacZ*Δ*M15 lacI*$^q$) *thi* Δ(*lac-proAB*) *recA1 gyrA96* | 19 |
| *Pseudomonas putida* | | |
| CMC4 | (pWW0) Km$^r$ 3MB$^+$ mini-Tn5-Km (*xylS* Pm::*lacI* $P_{A1-04/03}$::*gef*) | 23 |
| CMC12 | (pWW0) Km$^r$ Tel$^r$ 3MB$^+$ EEZ29::mini-Tn5-Tel ($P_{A1-04/03}$::gene *E*) | This study |
| CMC13 | (pWW0) Tel$^r$ Km$^r$ 3MB$^+$ EEZ29::mini-Tn5-Tel | This study |
| EEZ15K-3 | (pWW0) Km$^r$ 3MB$^+$ mini-Tn5-Km | 29 |
| EEZ29 | (pWW0) pCC102 Km$^r$ 3MB$^+$ | 31 |
| Plasmids | | |
| pCC102 | Km$^r$ *xylS2* Pm::*lacI* | 1 |
| pJMSB4 | Ap$^r$ Tel$^r$ mini-Tn5-Tel R6K *oriV mob*$^+$ | 33 |
| pMCC26 | pUHE24-1 derivative; Ap$^r$ Cm$^s$ | This study |
| pMCC27 | Ap$^r$ $P_{A1-04/03}$::gene *E* | This study |
| pMCC30 | Ap$^r$ $P_{A1-04/03}$::gene *E* fragment of pMCC27 inserted between *Sal*I and *Hin*dIII sites of pUC18Not | This study |
| pMCC31 | Ap$^r$ Tel$^r$ mini-Tn5 $P_{A1-04/03}$::gene *E* R6K *oriV mob*$^+$ | This study |
| pRK600 | Cm$^r$ ColE1 *oriV* RK2 *mob*$^+$ *tra*$^+$ | 12 |
| pUC18Not | Ap$^r$ | 2, 9 |
| pUHE24-1 | Ap$^r$ Cm$^r$ ColE1 *oriV* | 16 |
| pWW0 | IncP9 *mob*$^+$ *tra*$^+$ 3MB$^+$ | 39 |

[a] Km$^r$, Ap$^r$, Cm$^r$, and Tel$^r$ indicate resistance to kanamycin, ampicillin, chloramphenicol, and potassium tellurite, respectively; Cm$^s$ indicates sensitivity to chloramphenicol; 3MB$^+$ indicates the ability to grow on 3-methylbenzoate. $P_{A1-04/03}$ is a synthetic lactose promoter (16).

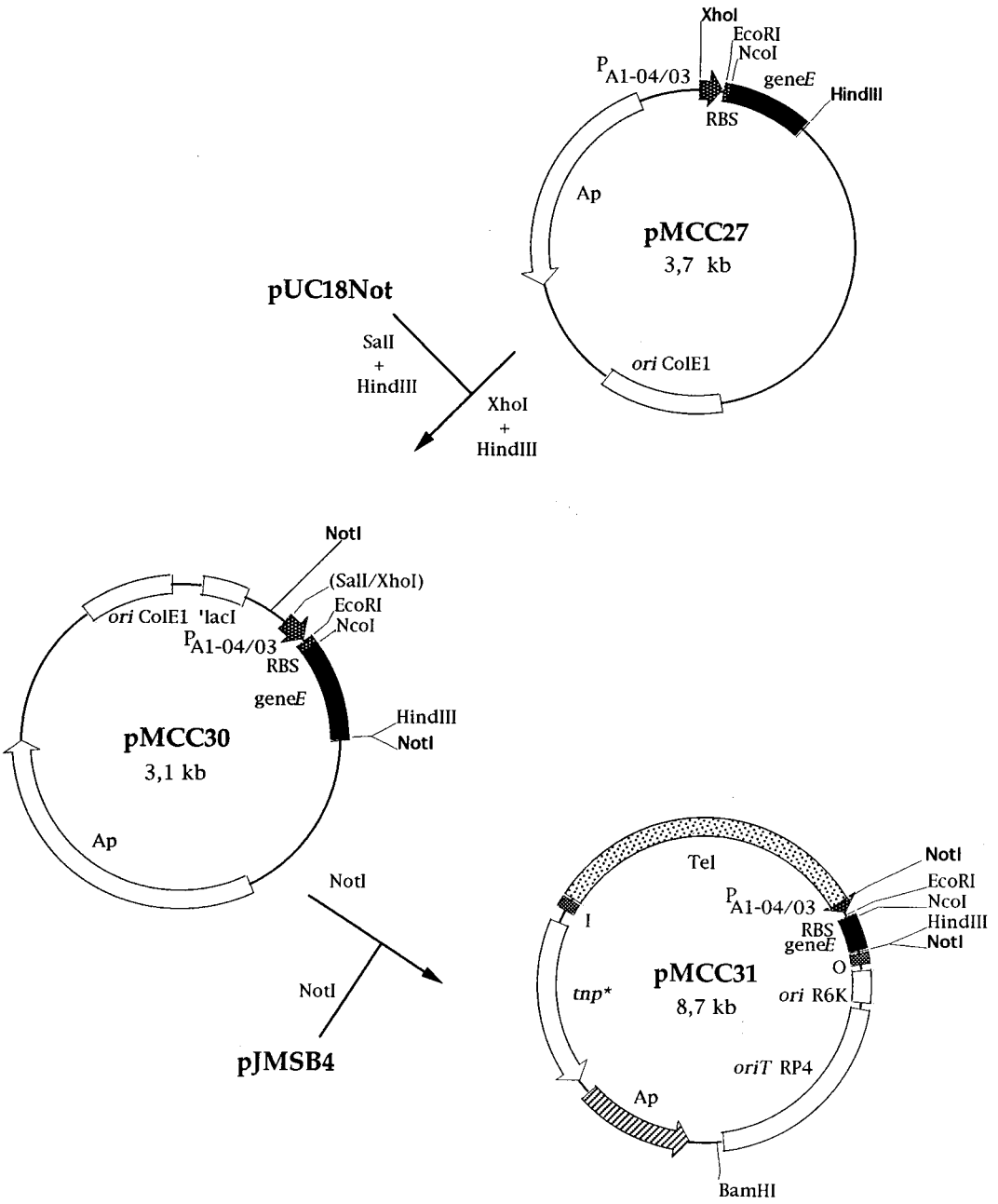


FIG. 2. Construction of a Tn5-based insertion delivery plasmid containing an inducible cell lysis system based on lysis gene *E* from bacteriophage ϕX174. Restriction sites relevant for the constructions are shown. Plasmid pUC18Not and the pUT-based plasmid pJMSB4 have been described elsewhere (9, 33). Abbreviations: gene*E*, lysis gene *E* of bacteriophage ϕX174; RBS, ribosome binding site from the *E. coli* expression plasmid pUHE24-1 (16); $P_{A1\text{-}04/03}$, synthetic lactose promoter from plasmid pUHE24-1; ori, origin of replication; *ori* R6K, origin of replication dependent on the Pir protein; *ori* ColE1, origin of replication from plasmid ColE1; *oriT* RP4, origin of transfer; *tnp**, transposase; *′*lacI, gene encoding the repressor protein for the *lac* operon.

ylbenzoate. Strains that were constructed in the course of this study are described below. *P. putida* strains were grown with shaking at 30°C in modified M9 minimal medium (1) with 28 mM glucose or 5 to 15 mM 3-methylbenzoate as the sole carbon source.

In cloning experiments, *E. coli* Mv1190λ*pir* was used to replicate the pMCC plasmids (Table 1). These plasmids are based on the R6K plasmid origin of replication, which is not recognized in *P. putida* strains, and behaves as a suicide replicon. *E. coli* JM109 was used to maintain other plasmids or in cloning experiments with vectors that did not require the Pir protein for replication. *E. coli* strains were grown at 37°C in LB medium (19). The plasmids used in this work are listed in Table 1.

Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 30; and kanamycin, 50. Potassium tellurite was used at 5 to 30 μg per ml.

**Construction of the killing cassette bearing the $P_{A1\text{-}04/03}$::gene *E* fusion.** Plasmid pUHE24-1 was described before (16). It carries ampicillin and chloramphenicol resistance and exhibits two *Nco*I sites. One of them lies 3′ with respect to the synthetic isopropyl-β-D-galactopyranoside (IPTG)-inducible promoter $P_{A1\text{-}04/03}$, and the other lies at the chloramphenicol resistance gene. To ensure that the plasmid contained only the *Nco*I site 3′ with respect to the synthetic $P_{A1\text{-}04/03}$ promoter, the plasmid was partially digested with *Nco*I and treated with the Klenow enzyme and the four deoxynucleoside triphosphates to fill in the sticky *Nco*I ends. $Ap^r$ clones were selected after ligation and transformation. A $Cm^s$ clone was selected, and the plasmid that it bore was called pMCC26. We

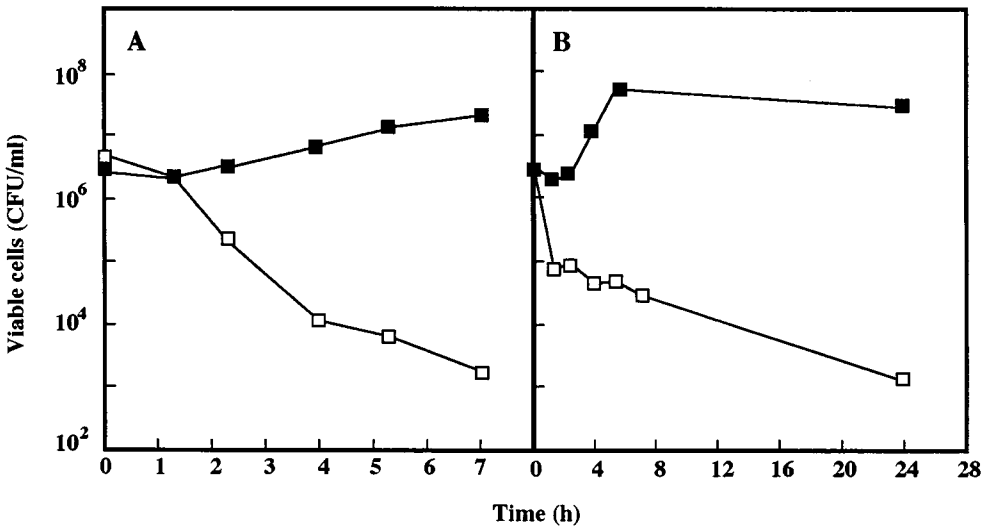


FIG. 3. Cell viability of *P. putida* strains after induction of the expression of killing genes. Cells of *P. putida* CMC4 ($P_{A1\text{-}04/03}$::*gef*) (A) and CMC12 ($P_{A1\text{-}04/03}$::gene *E*) (B) growing exponentially in M9 minimal medium with glucose and 3-methylbenzoate were transferred at time zero to medium containing glucose and either 3-methylbenzoate (closed symbols) or IPTG (open symbols). At the indicated times, viable cells were counted on LB medium plates containing 3-methylbenzoate and the appropriate antibiotics.

confirmed that the single *Nco*I site remaining in this plasmid was located 3′ with respect to the synthetic $P_{A1\text{-}04/03}$ promoter.

ϕX174 gene *E* was amplified by the PCR method with phage DNA as a template. The oligonucleotides used for amplification (5′-GTTTCTGGCCATG GTACGCTGGACTTTGTG-3′ and 5′-TCATTATCTTAAGCTTACGTTTTT TACCTTTAGA-3′) were partly complementary to the ends of gene *E* and were designed so that *Nco*I and *Hin*dIII sites would be generated near the ends of the amplified DNA fragment. The amplified gene *E* DNA was cleaved with *Nco*I and *Hin*dIII and cloned into pMCC26 cut with *Nco*I and *Hin*dIII, so that the amplified promoterless ϕX174 gene *E* was read from the synthetic $P_{A1\text{-}04/03}$ promoter. The resulting plasmid was called pMCC27 (Fig. 2). A 418-bp region from pMCC27 containing the $P_{A1\text{-}04/03}$::gene *E* fusion was isolated after digestion with *Xho*I and *Hin*dIII and cloned in pUC18Not digested with *Sal*I and *Hin*dIII. The resulting plasmid, pMCC30, was selected after transformation of the ligation mixture into *E. coli* JM109 (Fig. 2). Plasmid pMCC30 was digested with *Not*I, and a 430-bp fragment containing the $P_{A1\text{-}04/03}$::gene *E* fusion was cloned into the unique *Not*I site of pJMSB4 and transformed into *E. coli* Mv1190λ*pir*. The resulting plasmid carried a $P_{A1\text{-}04/03}$::gene *E* fusion and the tellurite-resistant determinant within mini-Tn5. This plasmid was called pMCC31 (Fig. 2).

**Triparental matings.** Triparental matings were performed as described by Herrero et al. (9). Equal numbers (about $10^8$ cells) of the recipient strain *P. putida* EEZ29, the donor strain *E. coli* Mv1190λ*pir*(pMCC31) or *E. coli* Mv1190λ*pir*(pJMSB4), and the helper strain *E. coli* HB101(pRK600) were mixed and deposited on a nitrocellulose filter placed on the surface of an LB medium plate supplemented with 5 mM 3-methylbenzoate. Appropriate controls with unmixed cells, otherwise treated identically to the mixture, were always included. *P. putida* transconjugants were selected on M9 minimal medium plates containing kanamycin and tellurite and supplemented with 3-methylbenzoate as the sole carbon source. One random transconjugant of *P. putida* EEZ29 that had received the minitransposon mini-Tn5-Tel from pJSB4 was selected and called CMC13; another random transconjugant that had received mini-Tn5-Tel $P_{A1\text{-}04/03}$::gene *E* from pCMC31 was selected and called CMC12.

**Tests of killing efficiency.** Killing efficiency was tested with liquid medium. Bacteria were grown in M9 minimal medium containing glucose and 3-methylbenzoate and supplemented with the appropriate antibiotics. Cells in the early exponential phase were harvested by centrifugation (12,000 × *g*, 15 min), washed twice in M9 minimal medium without a C source, and resuspended in M9 minimal medium with glucose. The sample was divided in half. To one half, 5 mM 3-methylbenzoate was added; 1 mM IPTG was added to the other half. All samples were then incubated at 30°C with shaking. For determination of viable counts, triplicate samples from a series of dilutions of the cultures were plated on LB medium plates containing 5 mM 3-methylbenzoate and the appropriate antibiotics.

**Transmission electron microscopy.** *P. putida* cells were harvested by centrifugation, immediately fixed with 2% (vol/vol) glutaraldehyde–1% (vol/vol) formaldehyde in cacodylate buffer, postfixed with osmium tetroxide in the presence of 2% (wt/vol) potassium ferrocyanide, and embedded in Eponate 12. Thin sections were poststained with uranyl acetate and lead citrate and examined in a Zeiss transmission electron microscope at an accelerating voltage of 75 kV.

**High-resolution scanning electron microscopy.** Scanning electron micrographs were taken with a Hitachi S-800 field-emission scanning electron microscope. The cells were fixed and prepared for electron microscopy essentially as described previously (38).

**Protein analysis.** Proteins in culture supernatants of *P. putida* were analyzed as follows. Whole cells were removed by centrifugation at 12,000 × *g* for 2 min, and the supernatant was concentrated by precipitation with 10% trichloroacetic acid. Cells were then resuspended in Laemmli buffer and analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels with the discontinuous buffer system of Laemmli (15). After electrophoresis, the gels were silver stained (32).

**Rubidium efflux.** *P. putida* cells were grown in 30 ml of M9 minimal medium containing 15 mM 3-methylbenzoate as the sole carbon source and supplemented with the appropriate antibiotics and 1 mCi of $^{86}$RbCl (1 mCi/mmol). Cells were harvested by centrifugation (12,000 × *g*, 10 min), washed in M9 minimal medium without a C source, and resuspended in the same minimal medium with either 15 mM 3-methylbenzoate or 28 mM glucose plus 5 mM IPTG. The amount of $^{86}$RbCl retained intracellularly by the cells was measured

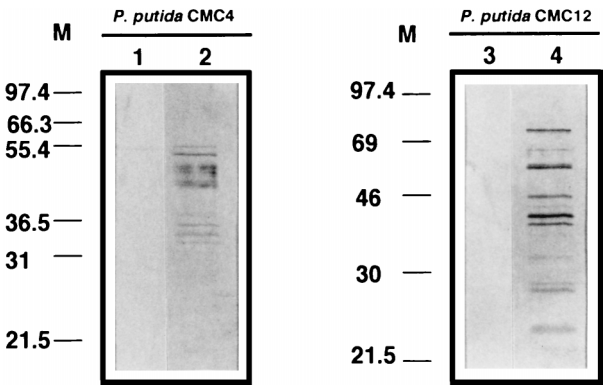


FIG. 4. Protein release from *P. putida* CMC4 ($P_{A1\text{-}04/03}$::*gef*) and CMC12 ($P_{A1\text{-}04/03}$::gene *E*) after induction of the expression of killing genes. *P. putida* cells growing exponentially in M9 minimal medium with glucose and 3-methylbenzoate were harvested by centrifugation, washed twice in M9 minimal medium without a C source, and resuspended in M9 minimal medium with glucose and 1 mM IPTG. Samples were taken at 0 h (lanes 1 and 3) and 6 h after induction (lanes 2 and 4). The released proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Numbers to the left of each panel indicate molecular masses (M) in kilodaltons.

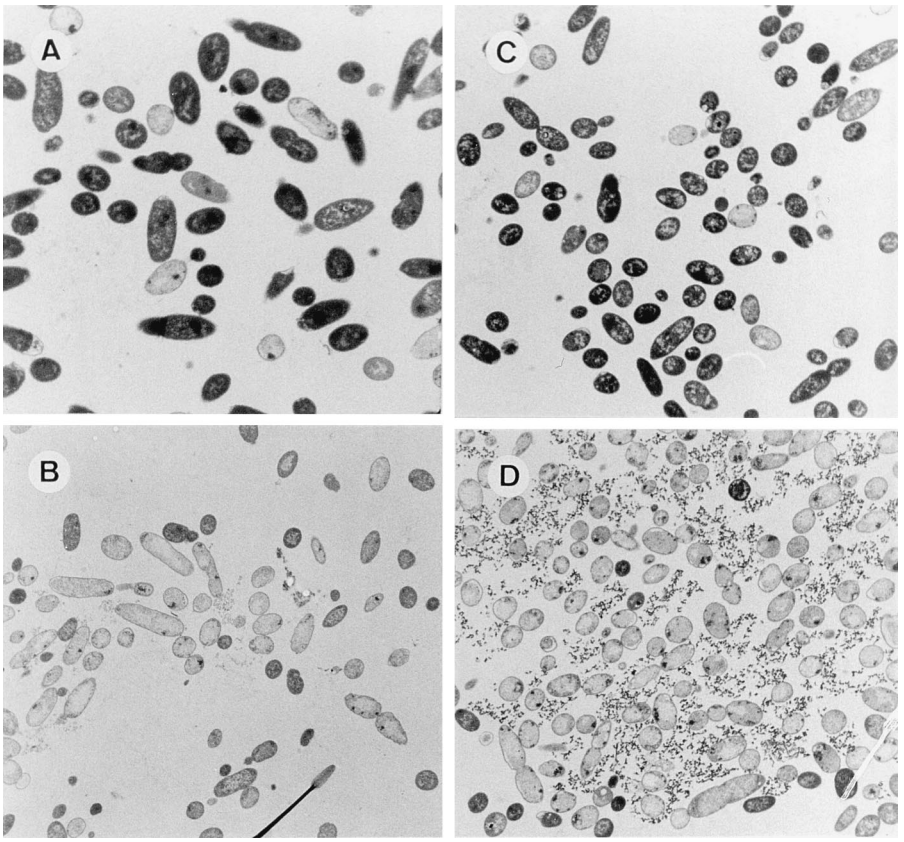


FIG. 5. Transmission electron micrographs of ultrathin sections of *P. putida* CMC4 (A and B) and *P. putida* CMC12 (C and D) cells. A and C, noninduced cells growing in M9 minimal medium with glucose and 3-methylbenzoate. B and D, Induced cells 3 h after transfer to M9 minimal medium with glucose and IPTG. Magnification, ×4,000.

by harvesting 200-μl aliquots of the culture by filtration. The pellets were resuspended in 200 μl of M9 minimal medium and mixed with 500 μl of scintillation liquid, and emission was counted with a Packard scintillation counter.

**Oxygen uptake assays.** Oxygen consumption rates of whole cells of *P. putida* were determined with a polarographic Clark oxygen electrode. A 0.1-ml aliquot of a *P. putida* culture was transferred to 1 ml of fresh medium kept at 30°C in the chamber of the oxygen electrode. The rate of oxygen consumption was then recorded for 5 to 10 min.

## RESULTS AND DISCUSSION

**Loss of viability of *P. putida* strains that express heterologous killing genes.** Two different killing genes were incorporated separately into the chromosome of *P. putida* KT2440 cells. *P. putida* CMC4 carries mini-Tn5-Km with a $P_{A1\text{-}04/03}$::*gef* fusion integrated in the host chromosome (23). *P. putida* CMC12 carries mini-Tn5-Tel with a $P_{A1\text{-}04/03}$::gene *E* fusion on the chromosome (this study). To control expression of the killing genes, the *lacI* gene, encoding the LacI repressor, was expressed from the Pm promoter for the *meta*-cleavage pathway of the *P. putida* TOL plasmid pWW0, whose expression is in turn controlled by the *xylS* gene (10, 30). In the presence of XylS effectors, such as 3-methylbenzoate, expression of the killing proteins is prevented and the strains survive. However, in the absence of effectors and in the presence of IPTG, cell growth is rapidly arrested as a consequence of expression of the lethal proteins from the $P_{A1\text{-}04/03}$ promoter (1, 11, 30). Such is not the case with the control strains *P. putida* EEZ15-K3 and CMC13, which do not bear the killing genes. (Note that in this series of assays, IPTG was added to rapidly titrate out the LacI protein in the cells).

In order to analyze whether cell growth arrest in cells bearing the containment system was the result of a loss of cell viability, we counted viable cells after induction of the system by transferring cells to a culture medium without 3-methylbenzoate and with IPTG. Cells of *P. putida* EEZ15-K3, CMC4, CMC13, and CMC12 growing exponentially (about $10^6$ to $10^7$ CFU/ml) in M9 minimal medium with glucose and 3-methylbenzoate were harvested by centrifugation, washed with 50 mM phosphate buffer, and then resuspended at the same cell density in M9 minimal medium containing glucose and either 3-methylbenzoate or IPTG. The number of cells of the two control strains increased with time regardless of the growth medium (data not shown). In contrast, the number of CFU of CMC4 and CMC12 per milliliter increased with time in medium with 3-methylbenzoate (Fig. 3) but not in the presence of IPTG. After an initial lag, the number of viable cells decreased in both strains. One hour after induction, about 33 and 4% of the initial cells were viable in cultures of *P. putida* CMC4 and CMC12, respectively (Fig. 3). The initial lag probably represents the time required for LacI turnover and synthesis and accumulation of the killer proteins. The fact that ϕX174 lysis protein E-mediated killing of *P. putida* CMC12 cells was faster than that of *P. putida* CMC4 cells, which expressed the *gef* gene, might indicate that the critical concentration of protein E needed to trigger killing is lower than that of the Gef protein or that lysis protein E is more efficient than the Gef protein in provoking cell death when the cells are growing exponentially. We assumed that there were equal levels of expression of the killing genes in both strains, because the two killing genes used

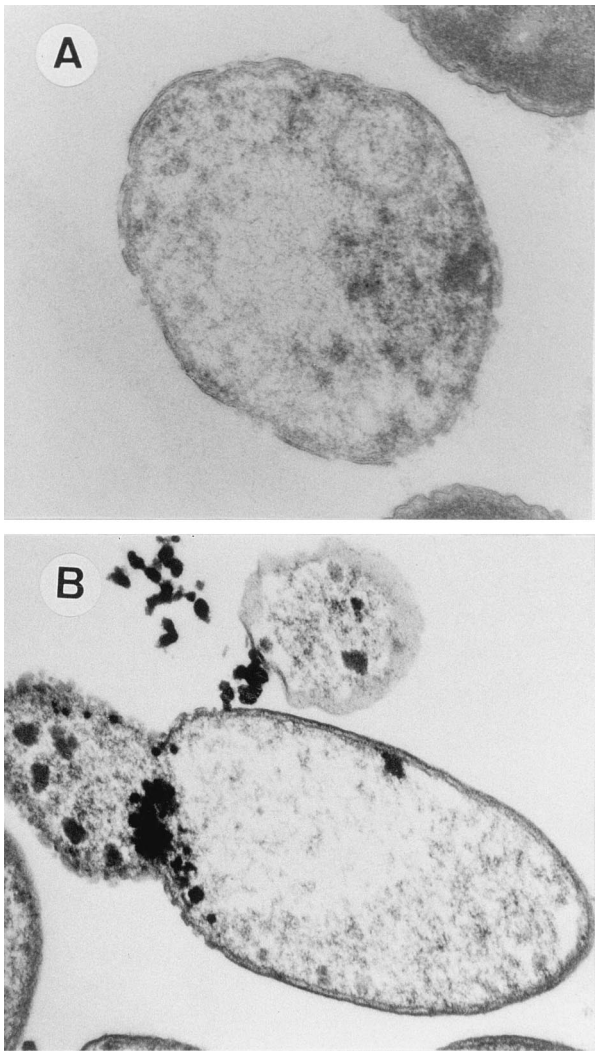


FIG. 6. Transmission electron micrographs of ultrathin sections of lysed *P. putida* CMC4 (A) and *P. putida* CMC12 (B) cells after the expression of killing genes. Magnification, ×40,000.

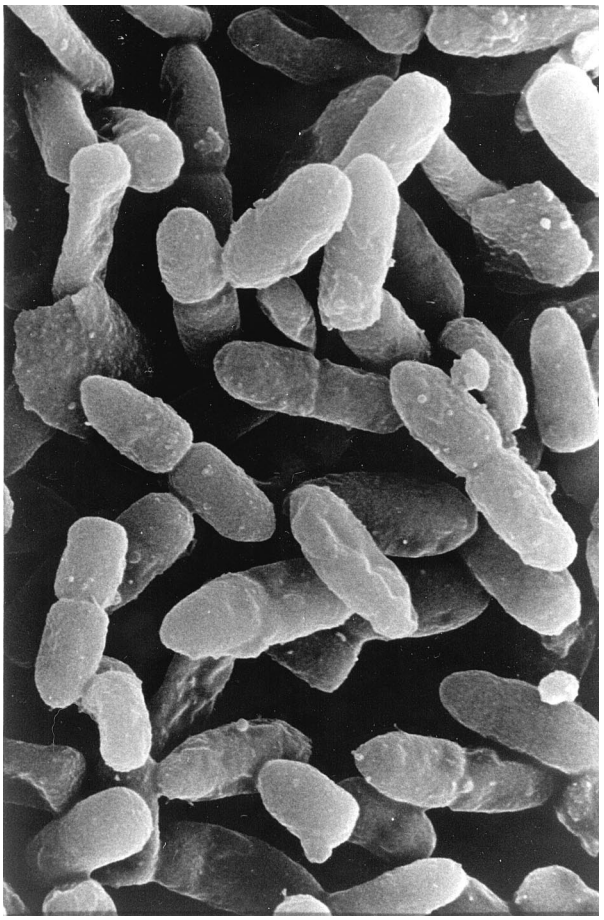

FIG. 7. Scanning electron micrograph of lysed *P. putida* CMC12 cells after the expression of gene *E* from bacteriophage ϕX174. Magnification, ×3,500.

in this study were expressed from the same promoter and the respective fusions were located on the host chromosomes. Nonetheless, prolonged incubation of CMC4 and CMC12 with IPTG led to a steady reduction in cell viability. The number of viable cells was on the order of 0.01% the initial number for strain CMC12 7 h after induction of the system, and the number was even lower for strain CMC4. Prolonged incubation led to a further decrease in cell viability in both strains, although in some cases killing-resistant mutants appeared.

**Physiological consequences of the expression of killing genes.** As stated in the introduction, the cytoplasmic membrane is the ultimate target of both ϕX174 lysis protein E and Gef in *E. coli*. The insertion of these proteins in the cell membrane of this microorganism leads to cell death, most likely via an alteration of cell membrane permeability.

To test the physiological consequences of the expression of the heterologous *E. coli* proteins in *P. putida*, we first monitored oxygen consumption in *P. putida* cells expressing each of these two killing proteins. At 30 min after induction of expression of the killing proteins, the rate of oxygen consumption by *P. putida* CMC12 and CMC4 (about 0.2 ± 0.1 μmol of $O_2$/mg of cell protein per min) was about 8% that in noninduced, control cultures kept in the presence of 3-methylbenzoate (3.0 ± 0.4 μmol of $O_2$/mg of cell protein per min). The rate of respiration in the control strains was not significantly affected by the removal of 3-methylbenzoate or the presence of IPTG and was about 3.5 ± 0.5 μmol of $O_2$/mg of protein per min. These results suggest that the cytoplasmic membrane of *P. putida* is indeed the target of Gef and ϕX174 lysis protein E and that the expression of these proteins leads to alterations in cell respiration.

This hypothesis is also supported by the observation that induction of the synthesis of these killing proteins in *P. putida* CMC4 and CMC12 led to a rapid loss of $K^+$ ions from the cytoplasm. To model $K^+$ loss, cells were preloaded with $^{86}Rb^+$ as described in Materials and Methods and then transferred to culture medium with or without 3-methylbenzoate. We found less retention of $^{86}Rb^+$ ions in the cytoplasm of cells incubated without 3-methylbenzoate (less than 3% the loaded $^{86}Rb^+$) than in that of cells kept in culture medium containing 3-methylbenzoate (about 20 to 30% the loaded $^{86}Rb^+$). Control cells in culture medium with or without 3-methylbenzoate retained similar amounts of loaded $^{86}Rb^+$, which were in the same range as those retained by strains bearing the containment system and kept in culture medium with 3-methylbenzoate.

The release of cellular material due to Gef- and ϕX174 lysis protein E-mediated membrane damage was investigated. We used SDS-polyacrylamide gel electrophoresis to analyze the

release of proteins to the culture supernatants of *P. putida* EEZ15-K3, CMC4, CMC13, and CMC12 in cultures with and without IPTG. No proteins could be detected in culture supernatants of control strains EEZ15-K3 and CMC13 during the 6-h experiment regardless of the growth medium or in culture supernatants of CMC4 and CMC13 grown with 3-methylbenzoate but without IPTG (data not shown). However, in culture supernatants of CMC4 and CMC12 incubated in the absence of 3-methylbenzoate but in the presence of IPTG, proteins were detected 90 min after the addition of IPTG (data not shown). In both cases, the total amounts of proteins detected increased with time, as deduced by the number and density of the bands in the gels (Fig. 4). These results indicate that lysis of *P. putida* cells occurs after the expression of Gef and ϕX174 lysis protein E. The differences in the patterns of proteins released (Fig. 4) from each of the two *P. putida* strains after the induction of killing suggest that Gef-mediated lysis and ϕX174 protein E-mediated lysis may occur through different mechanisms.

**Ultrastructure of *P. putida* bacteria that express killing genes.** The ultrastructure of *P. putida* EEZ15-K3, CMC4, CMC13, and CMC12 cells was analyzed before and after transfer to 3-methylbenzoate-free medium. The ultrastructure of the control strain was not significantly affected by the culture medium; almost 100% of the cells appeared electron dense when examined by transmission electron microscopy (data not shown). In contrast, significant differences were observed in CMC4 and CMC12, depending on the culture medium. Before the induction of the killing genes, more than 92% of the cells (counted in six different field exposures) of these two strains were electron dense. These cells exhibited well-defined outer and inner membranes and showed the typical rod morphology of *Pseudomonas* (Fig. 5A and C). Induction of the expression of Gef or ϕX174 lysis protein E was typically followed by a change in the appearance of the cells; in both cases, the cells became almost completely transparent (Fig. 5B and D). The occurrence of ghost cells is evidence that cytoplasmic material has been lost, as discussed above. Three hours after transfer to medium lacking 3-methylbenzoate, 80% of the cells (counted in six different field exposures) were lysed and appeared as ghosts (Fig. 5B and D).

*P. putida* CMC4 cells expressing the *gef* gene showed numerous holes in the cell envelope (Fig. 6A). In *P. putida* CMC12, which expressed gene *E*, the holes appeared to be grouped together instead of distributed along the cell envelope. Cytoplasmic material was observed leaking out through these holes (Fig. 6B). Lysed cells of *P. putida* CMC12 were also observed by high-resolution scanning electron microscopy. Bleb-like structures could be seen emanating from some of the cells (Fig. 7). It is well known that in *E. coli*, protein E forms a unique transmembrane hole through which cytoplasmic material is released to the external medium (37, 38). The protein may act in a similar way in *P. putida*.

The results presented in this study indicate that the cytoplasmic membrane is the target of Gef and ϕX174 lysis protein E in *P. putida*. After the expression of the genes for these two *E. coli* killing proteins in *P. putida* cells, the cell envelope of the heterologous host loses its integrity, numerous holes appear, and the cytoplasmic content is released to the extracellular medium; as a consequence, cell death occurs. Comparative studies on the efficiency of host killing by these two genes under environmental conditions should provide new insights into the potential of these genes for the biological containment of *P. putida* strains.

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