APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2001, p. 5384–5391 Vol. 67, No. 12
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.12.5384–5391.2001

Requirement of DNA Repair Mechanisms for Survival of *Burkholderia cepacia* G4 upon Degradation of Trichloroethylene

CHRIS M. YEAGER,[1] PETER J. BOTTOMLEY,[1,2] AND DANIEL J. ARP[1,3]*

Molecular and Cellular Biology Program,[1] Department of Microbiology and Crop and Soil Sciences,[2] and Department of Botany and Plant Pathology,[3] Oregon State University, Corvallis, Oregon 97331-2902

Received 22 June 2001/Accepted 17 September 2001

A Tn*5*-based mutagenesis strategy was used to generate a collection of trichloroethylene (TCE)-sensitive (TCS) mutants in order to identify repair systems or protective mechanisms that shield *Burkholderia cepacia* G4 from the toxic effects associated with TCE oxidation. Single Tn*5* insertion sites were mapped within open reading frames putatively encoding enzymes involved in DNA repair (UvrB, RuvB, RecA, and RecG) in 7 of the 11 TCS strains obtained (4 of the TCS strains had a single Tn*5* insertion within a *uvrB* homolog). The data revealed that the *uvrB*-disrupted strains were exceptionally susceptible to killing by TCE oxidation, followed by the *recA* strain, while the *ruvB* and *recG* strains were just slightly more sensitive to TCE than the wild type. The *uvrB* and *recA* strains were also extremely sensitive to UV light and, to a lesser extent, to exposure to mitomycin C and H_2O_2. The data from this study establishes that there is a link between DNA repair and the ability of *B. cepacia* G4 cells to survive following TCE transformation. A possible role for nucleotide excision repair and recombination repair activities in TCE-damaged cells is discussed.

Trichloroethylene (TCE), a suspected human carcinogen (17), has been used extensively as a metal degreaser, fumigant, and solvent for dry cleaning and in other commercial applications. Because of its widespread use and persistence, TCE is one the most commonly detected organic pollutants at hazardous waste sites and in municipal groundwater supplies in the United States (24, 48). Although it has not been demonstrated that microorganisms can utilize TCE as a growth-supporting substrate under aerobic conditions, a number of bacteria are able to degrade TCE cometabolically; in this process nonspecific oxygenases catalyze the initial transformation (1, 8, 44).

The practicality of utilizing bacteria to degrade TCE via aerobic cometabolism has been questioned, however, due to the cytotoxicity that is almost universally associated with this process. Loss of TCE-degradative activity is often observed with whole cells during TCE transformation (34, 36, 43, 45, 49), and each of the TCE-degrading enzymes that have been purified to homogeneity and examined to date (toluene dioxygenase, toluene 2-monooxygenase, and soluble methane monooxygenase) exhibits turnover-dependent inactivation upon TCE oxidation (12, 22, 33). Additionally, TCE degradation can result in injuries that adversely affect more basic cellular functions, such as general respiratory activity and cell viability (4, 16, 43, 49). Although the exact nature of the destructive species remains unknown, it has been proposed that acyl chlorides, generated from hydrolysis or rearrangement of TCE epoxide (monooxygenase-catalyzed reactions) or TCE-dioxetane (dioxygenase-catalyzed reactions), cause damage by alkylating various cellular constituents (12, 22, 33, 43, 46).

Since cellular toxicity can potentially limit the sustainability of TCE biodegradation under aerobic conditions, a concerted effort has been directed towards identifying strains that resist inactivation during TCE transformation. Initial observations suggested that the toluene-oxidizing bacterium *Burkholderia cepacia* G4 was such an organism (10, 11, 20). Since then, this strain (or derivatives of it) has become one of the best-known and most-studied microorganisms in terms of TCE bioremediation. However, results of recent studies indicate that *B. cepacia* G4 is indeed susceptible to cellular damage as a result of TCE degradation. Newman and Wackett (33) demonstrated that purified toluene 2-monooxygenase, which catalyzes TCE oxidation in *B. cepacia* G4, is inactivated during TCE oxidation in vitro. In another study, a fourfold increase in the maintenance energy requirement of *B. cepacia* G4 cells was observed when they were cultivated in a toluene-fed batch reactor and exposed to TCE under nongrowth conditions (28). The authors speculated that maintenance energy and growth conditions may play a role in influencing the extent to which *B. cepacia* G4 cells can repair damage incurred during TCE degradation, thus influencing the ultimate sustainability of TCE degradation. Finally, in a previous study we directly demonstrated that rapid rates of TCE degradation severely compromised the culturability and general respiratory activity of *B. cepacia* G4 cells, while inactivation of toluene 2-monooxygenase proceeds at a relatively slow pace in vivo (49).

The available data suggested to us that although *B. cepacia* G4 is indeed susceptible to toxic effects upon TCE oxidation, it is also likely to possess protective mechanisms and/or repair systems that influence the extent to which TCE degradation ultimately damages the cell. Without such repair or protective mechanisms or under physiological conditions that prevent them from functioning fully, cells would be particularly sensitive to TCE-mediated injury. In the present study we utilized transposon insertion mutagenesis to identify and characterize mutants of *B. cepacia* G4 that were ultrasusceptible to TCE-mediated cytotoxicity.

* Corresponding author. Mailing address: Department of Botany and Plant Pathology, 2082 Cordley, Oregon State University, Corvallis, OR 97331-2902. Phone: (541) 737-1294. Fax: (541) 737-5310. E-mail: arpd@bcc.orst.edu.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. cepacia* G4 was provided by Malcolm Shields (University of West Florida, Pensacola). All strains were maintained on minimal medium agar plates containing 20 mM sodium lactate (49). To obtain cells for experimental assays, liquid cultures were grown overnight at 30°C with shaking in sealed serum vials (160 ml) containing minimal media (60 ml) with 20 mM sodium lactate. Alternatively, cells were grown on toluene (initial aqueous phase concentration, 1.0 mM) by adding 9 μl of toluene twice sequentially, as previously described (49). To collect cells, cultures were centrifuged (8,000 × *g* for 10 min), and the cells were rinsed twice with 50 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.0) (phosphate buffer) and resuspended in fresh phosphate buffer to obtain a concentrated cell suspension. The cell suspension was stored at room temperature for 1 h or less before use.

Transposon mutagenesis. Tn*5*-OT182 was introduced into *B. cepacia* G4 via conjugal transfer using an adaptation of a filter-mating technique (6). *Escherichia coli* S17-1 (= ATCC 47055) was grown overnight in 5 ml of Luria-Bertani (LB) medium containing tetracycline (15 μg/ml) and ampicillin (100 μg/ml). *B. cepacia* G4 was grown similarly without antibiotics. A portion of each culture (100 μl) was added to 3 ml of a sterile $MgSO_4$ solution, and the preparation was thoroughly mixed and filtered through a single 0.45-μm-pore-size membrane filter disk (Millipore type HA). The filter disks obtained were subsequently placed upright on LB medium plates containing 10 mM $MgSO_4$ and incubated at 37°C for 12 to 14 h. The filters were then placed in sterile 15-ml culture tubes containing 0.85% NaCl, and cells were washed from the filters by vortexing. Aliquots (100 μl) of each resulting cell suspension were spread onto minimal medium plates containing sodium lactate (20 mM) and tetracycline (15 μg/ml). Transconjugants of *B. cepacia* G4 were identified following incubation at 30°C for 72 to 96 h and were obtained at a frequency of 2.3×10^{-5} transconjugant per initial donor cell. Wild-type *B. cepacia* G4 is sensitive to tetracycline, and *E. coli* S17-1 cannot utilize lactate as a growth-supporting substrate. Control assays were performed by using *B. cepacia* G4 or *E. coli* S17-1 alone, and no colonies were observed on the selective plates.

Screening for TCS mutants. Tn*5* insertion mutants of *B. cepacia* G4 were replica plated onto two sets of minimal medium plates, which were then incubated at 30°C for 4 to 5 days in sealed, 1-gal polyethylene jars containing toluene vapors. To select for TCE-sensitive (TCS) mutants, one of the replica plate sets was incubated in the presence of both toluene and TCE vapors. Toluene vapors were supplied by adding toluene (150 μl) to a Durham tube, plugging the tube with cotton, and placing the tube in an empty petri dish at the bottom of the polyethylene jar. TCE vapors were supplied by including neat TCE (30 μl) in the Durham tube containing toluene. During the incubation period, the colony size of each mutant strain grown on toluene alone was monitored periodically by visual inspection and was compared to that of the corresponding strain grown on toluene in the presence of TCE. Growth of all strains was impaired in the presence of TCE, but against this background the inhibitory effect of TCE was exaggerated in certain strains, which were classified as TCS. All strains identified as TCS were subjected to two more rounds of screening (as described above) to confirm the TCS phenotype.

Cloning and sequencing DNA flanking the Tn*5* inserts. Chromosomal DNA from TCS strains were isolated by using a standard protocol (38). Self-cloning of DNA flanking the Tn*5* insert in the TCS mutants was accomplished by the method of Merriman and Lamont (30). Approximately 4 to 5 μg of chromosomal DNA was digested with either *Eco*RI, *Xho*I, *Sal*I, *Cla*I, *Hin*dIII, *Bam*HI, or *Nhe*I, heated at 75°C for 20 min to inactivate the endonuclease, and precipitated with sodium acetate and absolute ethanol. The DNA pellets were washed in 70% ethanol, air dried, and resuspended in 30 μl of distilled H_2O. Twenty-five microliters of each DNA suspension was self-ligated overnight at 12°C in a 50-μl reaction mixture containing 1 U of ligase (Promega, Madison, Wis.). A portion (5 μl) of the ligated DNA was transformed into competent DH5α cells (Gibco BRL, Rockville, Md.) as described by the supplier, and Ap^r Tc^r transformants were selected for isolation of plasmids. Plasmids were prepared as previously described (21) and were purified with the Concert Rapid PCR purification system from Gibco BRL prior to sequencing.

Automated DNA sequencing was performed by the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. Primers OT182-RT and OT182-LT were used to sequence the cloned DNA immediately flanking the Tn*5* insertion site, as described by DeShazer et al. (6). OT182-RT and OT182-LT were synthesized by Gemini Biotech, Ltd. (Alachua, Fla.). A primer walking strategy was used to determine the entire coding sequence of each gene that was disrupted by Tn*5* insertion in the TCS mutants. Both strands were sequenced 80 to 90% of the time. All sequences had at least 2× coverage (average, about 3.5× coverage); each sequence alignment was examined by eye, and ambiguities were resolved by further sequencing. The synthetic primers used to prime these reactions were obtained from Sigma Genosys (The Woodlands, Tex.).

Recovery of growth by *B. cepacia* G4 cells exposed to TCE. Toluene-grown cells (1 mg of protein) were added to sealed serum vials (10 ml) containing phosphate buffer with 250 μM TCE (final reaction volume, 1 ml). The reaction vials were incubated at 30°C with shaking (150 rpm). At selected time points, samples (50 μl) were removed from the TCE reaction mixtures and added to sterile glass serum vials (160 ml) containing minimal medium (60 ml) with 20 mM sodium lactate. The inoculated vials were then incubated at 30°C with shaking, and 1-ml portions were removed periodically to monitor the optical density at 600 nm (OD_{600}) of each culture.

Chemical and UV sensitivity assays. Toluene-grown cells of wild-type *B. cepacia* G4 and the TCS mutants were exposed to TCE, UV light, mitomycin C, or hydrogen peroxide. In chemical exposure experiments cells (1 mg of total cell protein) were added to sealed serum vials (10 ml) containing phosphate buffer and either TCE (250 μM), mitomycin C (0.25, 1.0, 5.0, or 25 mg/ml), or H_2O_2 (0.1, 0.5, or 2 mM). The final reaction volumes were 1 ml for the TCE and H_2O_2 treatments and 2 ml for the mitomycin C treatments. The reaction vials were incubated at 30°C with shaking for 30 min for the mitomycin C and H_2O_2 treatments and for 15, 30, or 60 min for the TCE treatments. The viabilities of chemically treated cells were determined by plating appropriate dilutions onto R2A agar plates (Difco, Sparks, Md.). We previously observed that addition of catalase or sodium pyruvate to the surfaces of LB agar plates increased the culturability of TCE-treated cells of *B. cepacia* G4 by as much as 100-fold (49), and our unpublished results indicated that R2A agar plates (which contain sodium pyruvate) act similarly. It is thought that catalase and pyruvate increase the numbers of physically or chemically injured bacteria counted on agar plates by preventing the accumulation of hydrogen peroxide in and/or around injured cells. Therefore, R2A agar plates were used to enumerate chemically challenged and UV light-challenged cells. For UV light exposure cells were diluted and spread onto R2A agar plates, and the surface of each plate was exposed to 3, 6, 12, or 30 J of UV light per m^2 with a Fotodyne Foto/Prep II transilluminator which produced 312-nm UV light. UV light exposure intensities were determined with a Spectroline DM-254N UV meter. Cells were exposed to UV light under subdued overhead lighting conditions, and the R2A agar plates were incubated at 30°C in the dark.

Analytical and other methods. The aqueous concentrations of toluene and TCE in liquid-gas systems at 30°C were calculated with dimensionless Henry's constants of 0.343 (50) and 0.494 (15), respectively. Toluene (99.8% pure) and TCE (>99% pure) were obtained from Aldrich (Milwaukee, Wis.). The protein concentrations of cell suspensions were determined by measuring the OD_{600} of appropriate dilutions of the cells and applying an appropriate conversion factor (suspensions of *B. cepacia* G4 cells with an OD_{600} of 1.0 contain 0.2 mg of total cell protein ml^{-1}). Protein concentrations were determined with the biuret assay (14) following cell solubilization in 3 M NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard. The dry weights of culture samples were determined by resuspending cells in distilled H_2O in preweighed Eppendorf tubes, drying the preparations for 2 days at 55°C, and weighing the cell pellets. It was determined that 2.1 mg (dry weight) of *B. cepacia* G4 cells contained approximately 1.0 mg of protein.

Hydrocarbons were analyzed with a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame ionization detector and a stainless steel column (0.3 by 61 cm) packed with Porapak Q 80-100 mesh (Alltech, Deerfield, Ill.). To detect ethylene, a column temperature of 100°C was utilized, and for TCE the column temperature was 155°C. The injector and detector temperatures were set at 200°C for all analyses. Hydrocarbons were quantified by comparing peak heights to standard curves constructed by using known amounts of authentic compounds.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in GenBank under accession numbers AY036066 (*recA*), AY036067 (*ruvB*), AY036068 (*uvrB*), and AY036069 (*recG*).

RESULTS

Isolation and genetic characterization of TCS mutants of *B. cepacia* G4. To identify genetic loci that are involved in mediating the toxic effects associated with TCE oxidation in *B. cepacia* G4, a Tn*5* mutagenesis strategy was employed. *B. cepacia* G4 cells were randomly mutagenized with Tn*5*-OT182, and mutants were screened for TCE sensitivity by monitoring

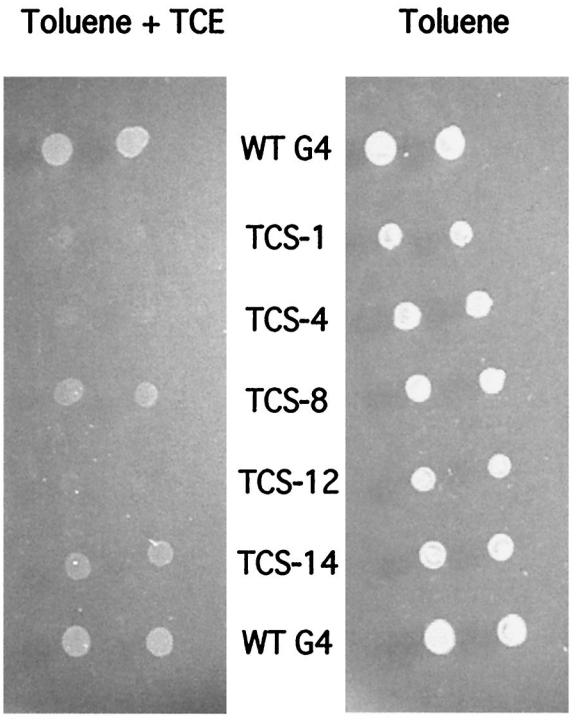

FIG. 1. Colony growth of wild-type *B. cepacia* G4 (WT G4) and select TCS mutants on minimal medium agar plates with toluene vapors with and without TCE vapors. Colonies were spotted in duplicate on each plate.

colony growth on minimal medium agar plates in the presence of toluene vapors supplied as a source of carbon and energy with or without TCE (Fig. 1). Approximately 4,500 Tn*5* insertion strains were initially screened for sensitivity to TCE. All mutants whose growth was inhibited compared to the growth of their peers in the presence of TCE were screened twice more to confirm the phenotype. Following the third round of screening, 20 TCS mutants were identified. To ensure that the Tn*5*-OT182 cassette had been inserted into the chromosome of each of the TCS mutants and that it had not been inserted into multiple sites, genomic DNA was isolated from each mutant and digested with *Eco*RI. A Southern blot analysis was performed by using the DNA fragments from each mutant and a radiolabeled portion of pOT182 as the probe. Chromosomal fragments from nine of the mutants did not hybridize with the probe. Further characterization of these mutants was not pursued. A single radiolabeled band was observed in lanes containing DNA from the other 11 mutants (data not shown), indicating that each of these mutants contained a single copy of Tn*5*-OT182 inserted into its chromosome.

DNA flanking the Tn*5*-OT182 insertion site in each mutant was isolated by self-cloning and was subsequently sequenced (30). The readable sequences from each mutant were approximately 600 bp long. A Blastx search performed with these sequences revealed that the Tn*5* insertion site in 7 of the 11 TCS mutants occurred in genetic loci predicted to code for proteins involved in DNA repair (Table 1). These proteins, UvrB, RuvB, RecA, and RecG, are involved in several distinct DNA repair systems, including nucleotide excision repair

TABLE 1. Blastx analysis of DNA sequences flanking the Tn*5*-OT182 insertion sites in TCS mutants of *B. cepacia* G4

TCS mutant	Homologous sequence	Function of putative gene product
TCS-1	*uvrB*	Subunit B of (A)BC excinuclease: NER
TCS-3	*uvrB*	Subunit B of (A)BC excinuclease: NER
TCS-4	*uvrB*	Subunit B of (A)BC excinuclease: NER
TCS-8	*ruvB*	Holliday junction DNA helicase: genetic recombination and recombination repair
TCS-12	*recA*	Genetic recombination and recombination repair, regulation of the SOS response, stress response regulator
TCS-13	*uvrB*	Subunit B of (A)BC excinuclease: NER
TCS-14	*recG*	Holliday junction DNA helicase: genetic recombination and recombination repair

(NER), recombination repair, and the SOS response system. Four TCS mutants had a Tn*5* insertion in a putative *uvrB* gene. Sequence analysis revealed that the Tn*5* insertions in the putative *uvrB* gene occurred at separate sites in a 770-bp section of the gene and that there was at least 65 bp between insertion sites (Fig. 2). The sequencing data, coupled with further Southern analysis results (data not shown), indicated that the Tn*5* insertions occurred in the same *uvrB* gene copy in TCS-1, TCS-3, TCS-4, and TCS-13. The other four TCS mutants had insertion sites in genes associated with carbon metabolism. Because preliminary experiments indicated that the TCS mutants with Tn*5* insertions in genes encoding putative DNA repair enzymes were the mutants most sensitive to TCE damage and because we wanted to limit the scope of this study, further analysis was limited to these strains.

By selectively inactivating toluene 2-monooxygenase with alkynes, we previously showed that the sensitivity of wild-type *B. cepacia* G4 to TCE is turnover dependent (49, 50). Similarly, the sensitivity of TCS-1 and TCS-12 to TCE was insignificant when 2-hexyne was included in the incubation mixtures (data not shown; other TCS mutants were not tested).

Sequence analysis of the putative *uvrB*, *ruvB*, *recA*, and *recG* coding regions from *B. cepacia* G4. Further sequencing of the DNA fragments self-cloned from TCS-4, TCS-8, TCS-12, and TCS-14 was performed to characterize the coding regions of the Tn*5*-interrupted genes (Fig. 2). In each case a single open reading frame (ORF) was identified. Analysis of the DNA fragment from TCS-8 revealed an ORF that putatively encoded a 356-amino-acid protein. The amino acid sequence was very similar to the amino acid sequences of RuvB proteins from a number of bacteria, including *Neisseria meningitidis* (76% identity), *Vibrio cholerae* (72% identity), *Pseudomonas aeruginosa* (70% identity), and *E. coli* (70% identity). It is interesting that a DNA sequence homologous to the *ruvA* gene was not found immediately upstream of the putative *ruvB* gene in *B. cepacia* G4, since bacterial *ruvA* and *ruvB* genes are almost always found together in an operon (40). A partial ORF was identified from the DNA fragments self-cloned from TCS-14. The complete sequence of the ORF was not determined (the 3′-terminal region was not sequenced) due to the small size of the cloned fragments. The partial ORF was determined to encode 654 amino acids, and amino acids 37 to 654 exhibited significant similarity with the amino acids of RecG proteins from *N. meningitidis* (56% identity), *P. aeruginosa* (57% iden-

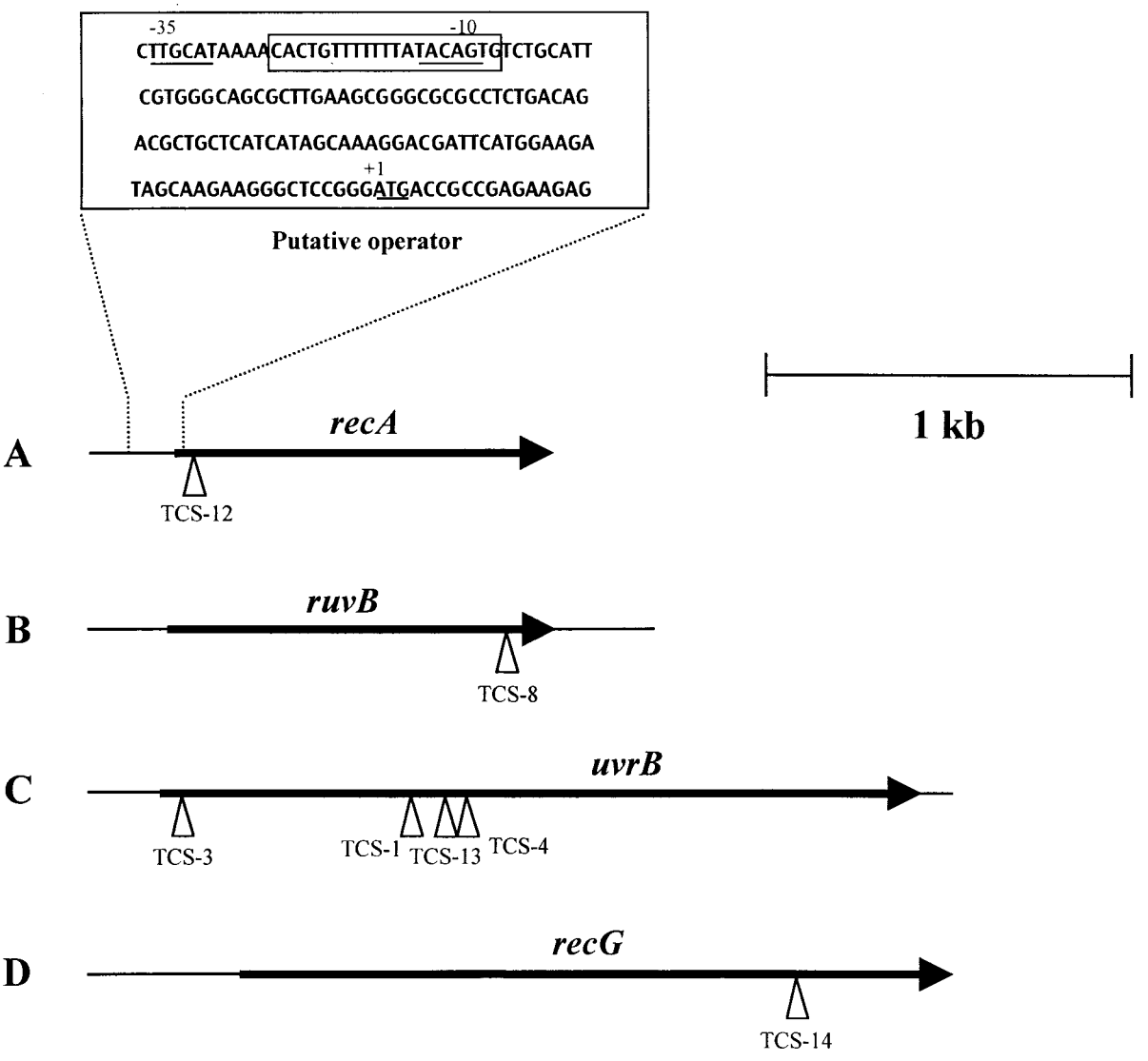

FIG. 2. Schematic maps of the DNA fragments self-cloned from the TCS mutants. The open triangles indicate the locations of the Tn*5*-OT182 insertions in the TCS mutants. The *recA*, *ruvB*, *uvrB*, and *recG* homologs are indicated by thick lines, and the directions of transcription are indicated by arrowheads. The nucleotide sequence of the *recA* upstream region is shown in the inset, and the putative transcriptional start site (+1) and −35 and −10 promoter sequences are underlined. The putative LexA binding region (SOS box) is enclosed in a box.

tity), and *E. coli* (54% identity), whose lengths range from 680 to 693 amino acids. The ORF disrupted by the Tn*5* insertion in strain TCS-4 putatively encoded a 697-amino-acid protein. The first 18 amino acids encoded by the ORF did not exhibit significant similarity with any proteins in the Blastx database, but the remainder of the amino acid sequence was 68, 67, and 65% identical to the sequences of UvrB proteins from *N. meningitidis*, *P. aeruginosa*, and *E. coli*, respectively.

The sequence obtained from the TCS-12 DNA fragment revealed that there was a single ORF consisting of 1,041 bp. The amino acid sequence encoded by this ORF was found to be 100% identical to the sequence of the RecA protein from several strains of *Burkholderia vietnamiensis*, and it exhibited 97 to 98% identity with RecA proteins from multiple strains of *B. cepacia*. The sequence upstream of the *recA* start codon was very similar to that previously described for *Pseudomonas cepacia* (32). A putative SOS box (a palindromic sequence that binds the LexA repressor protein of the SOS response system) was identified 100 bp upstream of the start codon, overlapping with a potential −10 promoter consensus sequence (Fig. 2A). Analysis of the upstream regions (200 to 400 bp) of the putative *ruvB*, *uvrB*, and *recG* genes from *B. cepacia* G4 did not reveal the presence of sequences resembling the SOS box (CTG-N_{10}-CAG).

Recently, Mahenthiralingam et al. (26) found that DNA sequence analysis of *recA* genes from members of the *B. cepacia* complex provided a way to taxonomically classify these organisms. The DNA sequence of the entire *recA* homolog from *B. cepacia* G4 exhibits 99% identity with *recA* sequences from known *B. vietnamiensis* strains and 93 to 94% identity with *recA* sequences from various *B. cepacia* strains. In addition, phylogenetic analysis of the 16S rRNA gene sequence

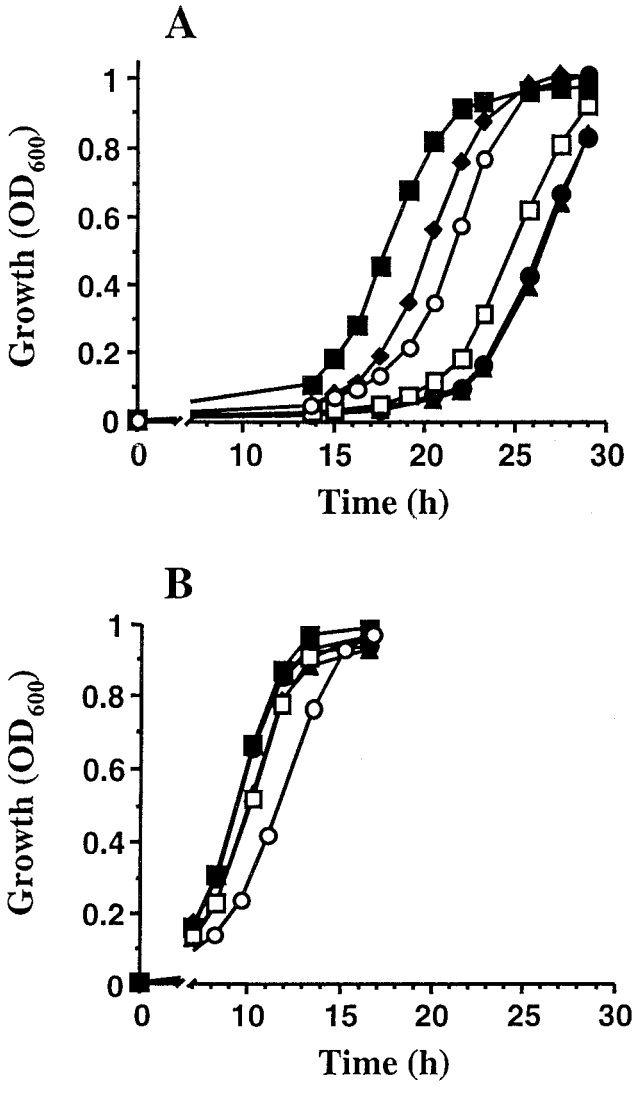

FIG. 3. Time courses for recovery of growth by TCE-treated cells of wild-type *B. cepacia* G4 (■), TCS-1 (*uvrB*) (●), TCS-4 (*uvrB*) (▲), TCS-8 (*ruvB*) (♦), TCS-12 (*recA*) (□), and TCS-14 (*recG*) (○). Cells were grown overnight with toluene vapors and incubated with (A) or without (B) TCE for 30 min. Portions of each cell suspension were then added to vials containing minimal medium with 20 mM sodium lactate, and culture growth (at 30°C with shaking) was monitored by determining OD_{600}.

from *B. cepacia* G4 obtained from the Ribosomal Database Project (accession no. L28675) (27) also suggested that this organism is more closely related to the *B. vietnamiensis* group (data not shown).

Growth characteristics of TCS strains following TCE exposure. We found previously that monitoring the growth (OD_{600}) of TCE-treated cells in liquid medium provides a consistent method for assessing general cellular damage incurred by cells that have oxidized TCE (49). Figure 3B shows typical growth curves of wild-type *B. cepacia* G4 and select TCS cultures when sodium lactate was supplied as the growth substrate. With the exception of TCS-14, the growth curves of the TCS strains were similar to that of wild-type *B. cepacia* G4 in the absence of TCE treatment. TCS-14 cells tended to clump during growth on lactate until the cultures reached OD_{600} of approximately 0.4 to 0.5. Cell suspensions of each strain that had been exposed to TCE exhibited markedly longer lag periods prior to the onset of exponential growth (recovery time), but once exponential growth was observed, there were no obvious differences in the growth rates of the TCE-treated and nontreated cell suspensions (Fig. 3A). Relative to the lag times of wild-type *B. cepacia* G4, the lag times were extended by approximately 8.0, 8.0, 2.3, 6.5, and 3.5 h in TCS-1, TCS-4, TCS-8, TCS-12, and TCS-14 cultures, respectively. Similar results were obtained when the TCE recovery experiment was repeated, and the lag times observed for each strain differed by 1 h or less from the lag times given above. The growth curves of TCE-treated TCS-1 and TCS-4 cultures were almost identical. In fact, the phenotypes of the *uvrB* mutants, TCS-1, TCS-3, TCS-4, and TCS-13, were essentially indistinguishable from one another when they were determined throughout this study. Each of the TCS mutants degraded TCE at a rate similar to the rate observed for wild-type *B. cepacia* G4 (14 nmol min^{-1} mg of total cell $protein^{-1}$) during the TCE exposure period.

Sensitivity to TCE, mitomycin C, hydrogen peroxide, and UV light. Since the TCS strains examined were presumably deficient in DNA repair activity, we examined the survival of select TCS strains following exposure to several known DNA-damaging agents or TCE (Fig. 4). TCS-1 cells showed the most sensitivity to each of the agents tested, usually followed by TCS-12 and then TCS-8 and TCS-14. When challenged with TCE or UV light, TCS-1 and TCS-12 were much more sensitive than wild-type *B. cepacia* G4 (trends that were observed when these experiments were repeated), whereas the responses of TCS-1 and TCS-12 to H_2O_2 or mitomycin C were far less exaggerated compared to the responses of wild-type cells. It is also interesting that most wild-type cells (96%) remained culturable following 15 min of TCE exposure, whereas only 3 and 9% of TCS-1 and TCS-12 cells, respectively, remained culturable. With the exception of H_2O_2, TCS-8 and TCS-14 were slightly more susceptible to killing by the agents tested.

DISCUSSION

In mammals, TCE transformation is catalyzed by cytochrome P450-dependent monooxygenases, and the genetic toxicity associated with the reaction has been studied extensively (9). From these studies it is known that [^{14}C]TCE metabolites bind to DNA in vitro (2, 3, 7, 31). There is also some evidence that covalent modification of DNA by [^{14}C]TCE metabolites occurs in vivo; however, the data are somewhat questionable, particularly because of the interference caused by the metabolic incorporation of C_1 products formed during [^{14}C]TCE oxidation (3, 9). There is also limited evidence that TCE metabolites bind irreversibly to DNA in prokaryotes. Wackett and Householder (46) observed radiolabel incorporation into the DNA fraction of *Pseudomonas putida* F1 cells upon [^{14}C]TCE oxidation. In the present study, genetic and physiological characterization of TCS mutants of *B. cepacia* G4 revealed that functional DNA repair mechanisms play a role in the survival of TCE-damaged cells of this organism. Furthermore, the genetic identity of each TCS mutant, in conjunction with data

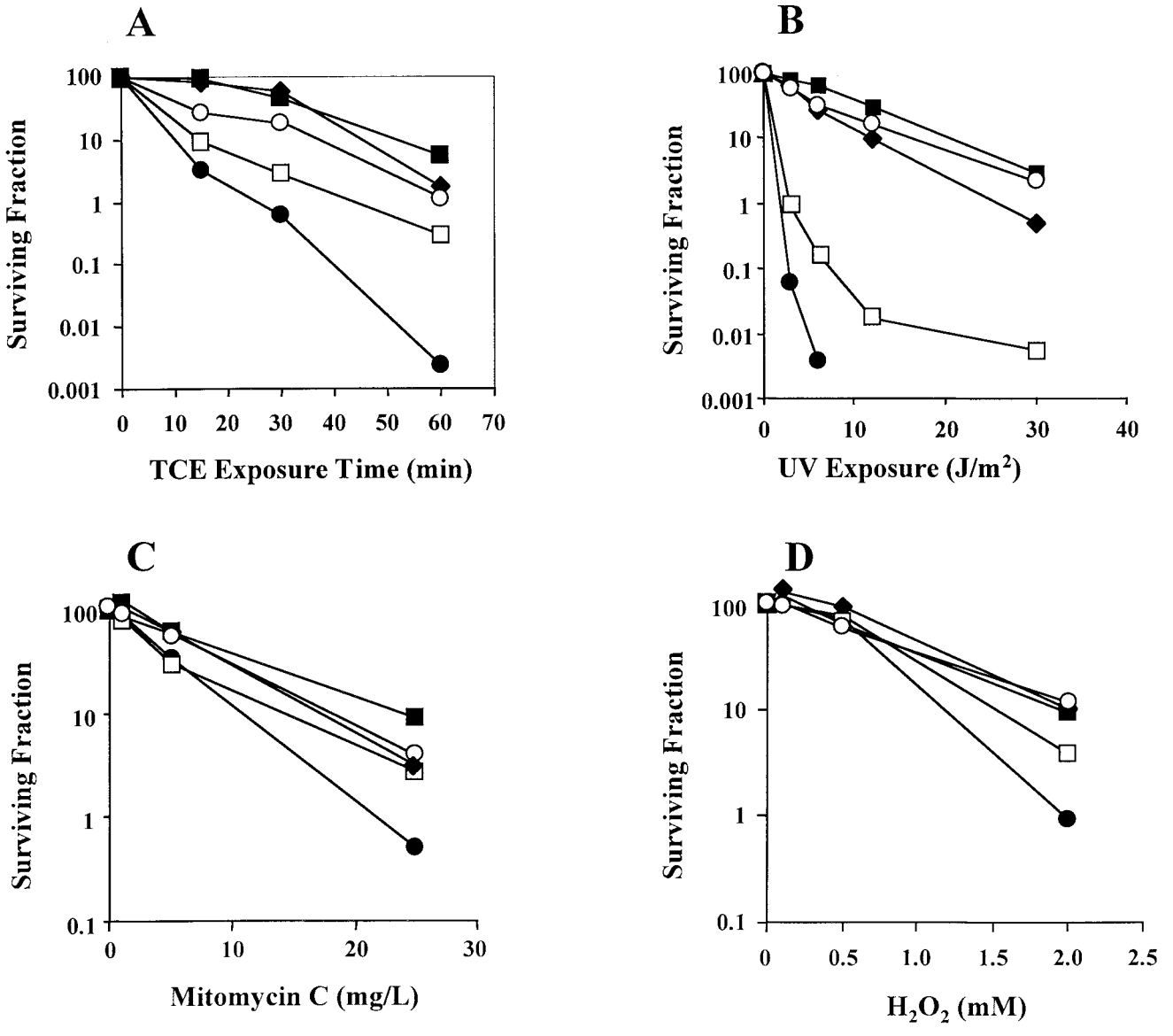

FIG. 4. Survival of wild-type *B. cepacia* G4 (■), TCS-1 (*uvrB*) (●), TCS-8 (*ruvB*) (♦), TCS-12 (*recA*) (□), and TCS-14 (*recG*) (○) following exposure to TCE (A), UV light (B), mitomycin C (C), or H_2O_2 (D). Toluene-grown cells were exposed to each agent as described in Materials and Methods, diluted in phosphate buffer, and spread on R2A agar plates. After 3 days of incubation at 30°C, the number of colonies per plate was determined and compared to the number of colonies on control plates containing untreated cells to determine the surviving fraction.

obtained from the survival assays, provides insight into the DNA repair mechanisms involved and leads us to speculate that potentially lethal DNA adducts are formed during TCE degradation in *B. cepacia* G4.

In *E. coli*, cells require NER working in combination with homologous recombination repair enzymes to fully recover from damage caused by many types of DNA lesions (13, 39). When damage to DNA overwhelms the excision repair capacity, DNA replication enzymes can stall at the blocking lesions. In this situation, either the replisome can wait for the arresting lesion to be repaired via NER or it can bypass the damage and restart downstream, leaving single-stranded gaps of approximately 1,500 nucleotides. The gaps are subsequently filled by recombinational exchange with an undamaged sister duplex, a process termed daughter strand gap repair. Reassembly, maintenance, and reinitiation of the replication fork are thought to require the strand exchange proteins RecA, RecF, RecO, and RecR, and in daughter strand gap repair, RecG and/or RuvABC is required to promote branch migration and resolution of the Holliday junction recombination intermediates (13, 19, 35). RecG and RuvABC are thought to have partially overlapping functions (40). Indeed, *ruv* or *recG* single mutants are usually only modestly susceptible to UV light and other DNA-damaging agents (as TCS-8 and TCS-14 were in the present study), while *ruv recG* double mutants are far more sensitive to such treatments (23).

The preponderance of TCS mutants containing a Tn*5* insertion in homologs of genes encoding enzymes involved in daughter strand gap repair (*ruvB*, *recG*, and *recA*), reinitiation of the replication fork (*recA*), and NER *(uvrB*) provides evidence which suggests that *B. cepacia* G4 cells utilize NER in conjunction with daughter strand gap repair to recover from damage accumulated during TCE oxidation. The extreme sensitivities of the *uvrB* mutants (TCS-1, TCS-3, TCS-4, and TCS-13) to both TCE and UV light exposure further underscore the importance of a functional NER system for recovery of *B. cepacia* G4 cells from these treatments. These observations imply that DNA adducts accumulate in vivo during TCE transformation by *B. cepacia* G4.

We identified an SOS consensus region upstream of a *recA* coding region in *B. cepacia* G4, yet found no evidence of the SOS consensus sequence upstream of the *uvrB*, *recG*, or *ruvB* homologs in this organism. These observations suggest that RecA does not directly regulate transcription of the *uvrB*, *recG*, and *ruvB* homologs in *B. cepacia* G4. Likewise, the *uvrB* gene of *P. aeruginosa* is not DNA damage inducible, nor is an SOS consensus region found upstream of its promoter (37). Although other DNA repair enzymes may be part of the SOS regulon in *B. cepacia* G4, it is likely that RecA plays a more pivotal role as a recombinase in protecting the cells from TCE-related damage.

Although TCS-4 and TCS-12 were each more susceptible to mitomycin C and H_2O_2 than wild-type cells of *B. cepacia* G4 were (there was up to a 1-order-of-magnitude difference in survival), we found that these strains were much more sensitive to TCE exposure and UV light exposure. NER mechanisms are not normally required to repair H_2O_2-induced DNA damage in bacteria; thus, it is not surprising that cells of TCS-4 are less susceptible to damage from H_2O_2 than to damage from UV light. Additionally, microbial resistance to H_2O_2 is a complex phenotype that depends on the action of numerous proteins, including antioxidant enzymes, DNA binding proteins, DNA repair enzymes, and free-radical-scavenging agents (5, 29); thus, the loss of RecA activity could certainly be masked by the actions of other defense systems. Indeed, under high-cell-density conditions (such as those utilized in our experiments) catalase often acts as the first line of defense for bacteria against H_2O_2 stress (25).

The weak responses of TCS-12 and TCS-4 cells to mitomycin C compared to their responses to TCE and UV light are more difficult to reconcile, since mitomycin C induces bulky DNA lesions, particularly DNA cross-links (42). Damage caused by mitomycin C in *B. cepacia* G4 could conceivably be constrained by some other factor, such as alternative DNA repair mechanisms, uptake into the cell, efflux pumps, etc., any of which could mask the *uvrB* or *recA* phenotypes. In fact, resistance to mitomycin C has been linked to drug export systems in both *E. coli* and *Streptomyces lavendulae* (41, 47).

A connection between DNA repair mechanisms and the degradation of another chlorinated aliphatic hydrocarbon, dichloromethane, has been established previously. It was recently discovered that DNA polymerase I is essential for growth of *Methylobacterium dichloromethanicum* DM4 with dichloromethane (18). DNA polymerase I exhibits polymerase, 5′-3′ exonuclease, and 3′-5′ exonuclease activities, and as a DNA repair enzyme one of its primary functions is to fill the gaps formed during excision repair processes. Kayser et al. suggested that DNA polymerase I could allow *M. dichloromethanicum* DM4 cells to grow on dichloromethane by aiding in the removal of DNA adducts formed between DNA and *S*-chloromethylglutathione, a proposed intermediate in dichloromethane conversion to formaldehyde (18).

Our results suggest that the recovery characteristics of TCE-damaged *B. cepacia* G4 cells depend on NER and also on recombination repair enzymes. In a previous study, we observed that *B. cepacia* G4 cells can accumulate a certain amount of damage during TCE oxidation (i.e., there is a toxicity threshold) before cell culturability is affected significantly (49). A similar phenomenon has been reported for *Methylosinus trichosporium* Ob3B during TCE oxidation (4). In the present study, the culturability of wild-type *B. cepacia* G4 on R2A agar plates did not decrease during a 15-min exposure to TCE, yet cell suspensions of TCS-1 and TCS-12 lost 97 and 90% of their culturable members, respectively, during a similar treatment. It is possible that the toxicity threshold observed in *B. cepacia* G4 upon TCE transformation marks the point in time at which DNA damage overwhelms the functional capacity of the NER and recombination repair systems of this organism. In light of these observations, it seems plausible that the ultimate TCE- or chlorinated aliphatic hydrocarbon-degrading potential of a given microorganism under aerobic conditions may be partially dependent on the efficiency of its DNA repair systems. The possibilities definitely warrant further exploration.

ACKNOWLEDGMENT

Funding for this study was provided by the office of Research and Development, U. S. Environmental Protection Agency, under agreement PR-0345 through the Western Region Hazardous Substance Research Center.

REFERENCES

1. **Arp, D. J.** 1995. Understanding the diversity of trichloroethylene co-oxidations. Curr. Opin. Biotechnol. **6:**352–358.
2. **Banerjee, S., and B. L. Van Duuren.** 1978. Covalent binding of the carcinogen trichloroethylene to hepatic microsomal proteins and to exogenous DNA *in vitro*. Cancer Res. **38:**776–780.
3. **Bergman, K.** 1983. Interactions of trichloroethylene with DNA *in vitro* and with RNA and DNA of various mouse tissues *in vivo*. Arch. Toxicol. **54:**181–193.
4. **Chu, K. H., and L. Alvarez-Cohen.** 1999. Evaluation of toxic effects of aeration and trichloroethylene oxidation on methanotrophic bacteria grown with different nitrogen sources. Appl. Environ. Microbiol. **65:**766–772.
5. **Demple, B., and L. Harrison.** 1994. Repair of oxidative damage to DNA: enzymology and biology. Annu. Rev. Biochem. **63:**915–948.
6. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.
7. **Di Renzo, A. B., A. J. Gandolfi, and I. G. Sipes.** 1982. Microsomal bioactivation and covalent binding of aliphatic halides to DNA. Toxicol. Lett. **11:**243–252.
8. **Ensley, B. D.** 1991. Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. **45:**283–299.
9. **Fahrig, R., S. Madle, and H. Baumann.** 1995. Genetic toxicology of trichloroethylene (TCE). Mutat. Res. **340:**1–36.
10. **Folsom, B. R., and P. J. Chapman.** 1991. Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. Appl. Environ. Microbiol. **57:**1602–1608.
11. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.
12. **Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb.** 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry **29:**6419–6427.

13. **Friedberg, E. C., C. W. Graham, and W. Siede.** 1995. DNA repair and mutagenesis. ASM Press, Washington, D.C.
14. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. **177:**751–766.
15. **Gossett, J. M.** 1987. Measurement of Henry's law constants for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21:**202–208.
16. **Heald, S., and R. O. Jenkins.** 1994. Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. **60:**4634–4637.
17. **Institute, N. C.** 1976. Carcinogenesis bioassay of trichloroethylene CAS no. 79-01-6. U.S. Department of Health, Education, and Welfare publication (NIH) 76-802. U.S. Department of Health, Education, and Welfare, Washington, D.C.
18. **Kayser, M. F., M. T. Stumpp, and S. Vuilleumier.** 2000. DNA polymerase I is essential for growth of *Methylobacterium dichloromethanicum* DM4 with dichloromethane. J. Bacteriol. **182:**5433–5439.
19. **Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer.** 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol. Rev. **58:**401–465.
20. **Landa, A. S., E. M. Sipkema, J. Weijma, A. A. Beenackers, J. Dolfing, and D. B. Janssen.** 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl. Environ. Microbiol. **60:**3368–3374.
21. **Lee, S., and S. Rasheed.** 1990. A simple procedure for maximum yield of high-quality plasmid DNA. BioTechniques **9:**676–679.
22. **Li, S., and L. P. Wackett.** 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. **185:**443–451.
23. **Lloyd, R. G.** 1991. Conjugational recombination in resolvase-deficient *ruvC* mutants of *Escherichia coli* K-12 depends on *recG*. J. Bacteriol. **173:**5414–5418.
24. **Love, O. T., Jr., and R. G. Eilers.** 1982. Treatment of drinking water containing trichloroethylene and related industrial solvents. J. Am. Water Works Assoc. **74:**413–425.
25. **Ma, M., and J. W. Eaton.** 1992. Multicellular oxidant defense in unicellular organisms. Proc. Natl. Acad. Sci. USA **89:**7924–7928.
26. **Mahenthiralingam, E., J. Bischof, S. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay, and P. Vandamme.** 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. J. Clin. Microbiol. **38:**3165–3173.
27. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
28. **Mars, A. E., J. Houwing, J. Dolfing, and D. B. Janssen.** 1996. Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Appl. Environ. Microbiol. **62:**886–891.
29. **Martinez, A., and R. Kolter.** 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bacteriol. **179:**5188–5194.
30. **Merriman, T. R., and I. L. Lamont.** 1993. Construction and use of a self-cloning promoter probe vector for gram-negative bacteria. Gene **126:**17–23.
31. **Miller, R. E., and F. P. Guengerich.** 1983. Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. Cancer Res. **43:**1145–1152.
32. **Nakazawa, T., M. Kimoto, and M. Abe.** 1990. Cloning, sequencing, and transcriptional analysis of the *recA* gene of *Pseudomonas cepacia*. Gene **94:**83–88.
33. **Newman, L. M., and L. P. Wackett.** 1997. Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J. Bacteriol. **179:**90–96.
34. **Oldenhuis, R., J. Y. J. Oedzes, J. van der Waarde, and D. B. Janssen.** 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. **57:**7–14.
35. **Pandya, G. A., I.-Y. Yang, A. P. Grollman, and M. Moriya.** 2000. *Escherichia coli* responses to a single DNA adduct. J. Bacteriol. **182:**6598–6604.
36. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*-cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. **57:**2986–2994.
37. **Rivera, E., L. Vila, and J. Barbe.** 1996. The *uvrB* gene of *Pseudomonas aeruginosa* is not DNA damage inducible. J. Bacteriol. **178:**5550–5554.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sancar, A.** 1996. DNA excision repair. Annu. Rev. Biochem. **65:**43–81.
40. **Sharples, G. J., S. M. Ingleston, and R. G. Lloyd.** 1999. Holliday junction processing in bacteria: insights from the evolutionary conservation of RuvABC, RecG, and RusA. J. Bacteriol. **181:**5543–5550.
41. **Sheldon, P. J., Y. Mao, M. He, and D. H. Sherman.** 1999. Mitomycin resistance in *Streptomyces lavendulae* includes a novel drug-binding-protein-dependent export system. J. Bacteriol. **181:**2507–2512.
42. **Tomasz, M., and Y. Palom.** 1997. The mitomycin bioreductive antitumor agents: cross-linking and alkylation as the molecular basis of their activity. Pharmacol. Ther. **76:**73–87.
43. **van Hylckama Vlieg, J. E. T., W. De Koning, and D. B. Janssen.** 1997. Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63:**4961–4964.
44. **Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson.** 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55:**2960–2964.
45. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.
46. **Wackett, L. P., and S. R. Householder.** 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. **55:**2723–2725.
47. **Wei, Y., A. C. Vollmer, and R. A. LaRossa.** 2001. In vivo titration of mitomycin C action by four *Escherichia coli* genomic regions on multicopy plasmids. J. Bacteriol. **183:**2259–2264.
48. **Westrick, J. J., J. W. Mello, and R. F. Thomas.** 1984. The groundwater supply survey. J. Am. Water Works Assoc. **5:**52–59.
49. **Yeager, C. M., P. J. Bottomley, and D. J. Arp.** 2001. Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67:**2107–2115.
50. **Yeager, C. M., P. J. Bottomley, D. J. Arp, and M. R. Hyman.** 1999. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. Appl. Environ. Microbiol. **65:**632–639.