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Isolation and Characterization of an *s*-Ethyl-*N*,*N*-Dipropylthiocarbamate-Degrading *Arthrobacter* Strain and Evidence for Plasmid-Associated *s*-Ethyl-*N*,*N*-Dipropylthiocarbamate Degradation†

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***Arthrobacter* sp. strain TE1 isolated from *s*-ethyl-*N*,*N*-dipropylthiocarbamate (EPTC)-exposed soil degraded this herbicide effectively and could grow on EPTC as the sole carbon source. TE1 harboured four plasmids of 65.5, 60, 50.5, and 2.5 megadaltons. Spontaneous mutants unable to degrade EPTC arose at a high frequency, and this was further increased by treatment of the culture with acridine orange or incubation at high temperature. All EPTC degradation-deficient (E^-) mutants lacked the 50.5-megadalton plasmid. This plasmid could be transferred from TE1 to E^- mutants by conjugation, resulting in the restoration of EPTC-degrading ability to the mutants.**

It has been shown by several workers (8, 12, 15, 16, 21, 22) that microorganisms are mainly responsible for the degradation of the herbicide *s*-ethyl-*N*,*N*-dipropylthiocarbamate (EPTC) in natural environments. Similar to the phenomenon observed with some other herbicides, the rate of decomposition of EPTC has been shown to be accelerated in soils with a history of previous herbicide application (16, 20, 30). Lee (15) isolated EPTC-degrading bacterial species from such soil by an enrichment technique. The bacterial isolates, however, lost their ability to decompose EPTC after prolonged storage. He speculated that this loss of EPTC-degradative ability may be due to the loss of some indigenous plasmid in the bacteria similar to the reported loss of plasmid-associated 2,4-D-degradative ability in bacteria (5).

We report here the isolation and characterization of an *Arthrobacter* strain which degrades EPTC efficiently. Furthermore, we demonstrate that this degradative capability of the isolate is associated with one of the indigenous plasmids harbored by this bacterium.

(Reports of these observations were presented at the annual meeting of the Canadian Society of Microbiologists [C.S.M.] and at a Joint Symposium of the C.S.M. and the American Society for Microbiology, June 1986.)

MATERIALS AND METHODS

Bacterial strains. Bacterial strains, isolated, used, or derived, and their plasmids and relevant phenotypes are shown in Table 1.

Spontaneous gentamicin-resistant (Gen^r) and streptomycin-resistant (Str^r) mutants were isolated by plating nutrient broth-grown cells on nutrient agar supplemented with 30 μg of gentamicin or 50 μg of streptomycin per ml. Rifampin-resistant mutants (Rif^r) were isolated by UV induction as described by Miller (19).

Soils. The soils used in this study were clay loam soils from a corn field (Brandon, Manitoba). T56 soil was from the field exposed to four successive annual applications of Eradicane (commercial formulation of the herbicide EPTC). T56U soil was from an adjacent field which was not exposed to EPTC.

Media. BMN was basal minimal-salts-nitrogen medium (1); BMNE was BMN supplemented with 50 μg of EPTC per ml. The herbicide solution in water was filter sterilized and kept at 4°C as a stock solution of 300 μg/ml. The semisolid media contained 1.2% agar (Difco Laboratories) and the plates were kept at 4°C. The bacterial strains were stored on nutrient agar slants and subcultured in nutrient broth when indicated. Liquid cultures were shaken in a gyrotory shaker (New Brunswick Scientific Co.) at 29°C.

Enrichment. Two grams of T56 soil was shaken at 29°C in 20 ml of BMNE. Four successive transfers at 3-day intervals were made (0.2 to 10 ml) into fresh BMNE medium. Aliquots (0.1 ml) were plated on BMNE agar, and rapidly growing bacterial colonies were selected and purified by streaking on BMNE agar. They were further checked for their ability to grow on EPTC by inoculation into BMNE liquid medium or plating on BMNE-agarose (1%) plates. T56U soil was similarly treated.

Identification and characterization of bacteria. Shape and morphology of the bacterial cells were determined by phase-contrast microscopy. Growth on various carbon sources was determined by inoculation of a loopful of culture into BMN medium supplemented with 1 mg of the carbon source per ml. Further characterization, including determination of the constituents of the cell wall, were carried out as suggested by Keddie and co-workers (13, 14).

Cell growth with EPTC. Utilization of EPTC as the sole carbon source for growth by the isolate was determined by inoculating 5 ml of a log-phase culture of TE1 in BMNE into 30 ml of fresh BMNE in a 300-ml flask. Cell growth was monitored spectrophotometrically (Beckman model DU6) at 600 nm.

Determination of EPTC by gas chromatography. EPTC was determined by withdrawing an aliquot (1 ml) from cultures, centrifuging out the cells in a Microfuge (Beckman Instruments, Inc.) for 3 min, and extracting the supernatant with 2 ml of hexane for 1.5 min. A 10-μl portion of the hexane extract was injected into a gas chromatograph (Varian Series 3700) for quantitation (16).

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TABLE 1. Bacterial strains

Strain	Plasmids (MDa)				Relevant phenotype	Origin or reference
	65.5	60	50.5	2.5		
TE1	+	+	+	+	E^+	Wild-type soil isolate; this study
TE2	+	+	+	+	E^+ Gen^r	Gen^r mutant of TE1
TE3	+	+	−	+	E^-	32°C treatment of TE1
TE4	+	+	−	+	E^- Rif^r Str^e	Antibiotic-resistant mutant of TE3
TE5	+	+	−	+	E^-	Acridine orange treatment of TE1
TE6	+	+	−	+	E^- Rif^r Str^r	Antibiotic-resistant mutant of TE5
TE7	+	−	−	+	E^-	Acridine orange treatment of TE1
TE8	+	−	−	+	E^- Rif^r Str^r	Antibiotic-resistant mutant of TE7
TE9	+	+	−	+	E^-	Spontaneous E^- mutant of TE1
TE10	+	+	+	+	E^+ Gen^s Rif^e Str^r	Transconjugant of TE4 from mating with TE2
TE11	+	+	+	+	E^+ Gen^s Rif^r Str^r	Transconjugant of TE6 from mating with TE2
TE12	+	−	+	+	E^+ Gen^s Rif^r Str^r	Transconjugant of TE8 from mating with TE2
A. globiformis 8010 Collection	Nil				E^-	American Type Culture Collection
A. simplex 6946 Collection	Nil				E^-	American Type Culture Collection
A. oxidans 104					E^- Nic^+	R. Brandsh (2)
Bacillus thuringiensis (see Fig. 3A) subsp. *thuringiensis* HD-2						10

Degradation of EPTC. To determine the degradation of EPTC by the bacteria growing in various media, log-phase cultures were adjusted to an optical density (OD) at 600 nm of 0.07 with the same media. EPTC (final concentration, 30 μg/ml) was added and the cultures were shaken at 28°C. The rate of loss of EPTC was determined during the course of incubation.

Isolation of EPTC mutants. Mutants not able to utilize EPTC as sole carbon source were isolated by transferring a 3- to 4-day-old colony of the EPTC-degrading isolate from nutrient agar to nutrient broth and then growing it under various conditions (see next section) before plating on nutrient agar. Single colonies on the plate were replica plated onto two successive BMNE agar plates (to avoid extraneous carryover of nutrients). Colonies showing poor growth on the second BMNE agar plates were retested by inoculation into liquid BMNE medium. Colonies not able to grow were scored as E^- (EPTC degradation deficient).

Plasmid curing. Acridine orange (filter sterilized) was added to a final concentration of 15 μg/ml to a nutrient broth culture of the isolate at an OD (600 nm) of about 0.02. The culture was shaken in the dark at 29°C for 16 h before plating out for the selection of $EPTC^-$ mutants. Plasmid curing was also carried out by shaking the culture at 32°C for 16 h before plating for E^- mutants.

Agarose gel electrophoresis of cell lysates. The modified Eckhardt (6) lysate electrophoresis method as described by González et al. (10) was used for examining the plasmids of the isolates. This method was further modified as follows. Cells were grown overnight in nutrient broth, and 1 ml of the culture (OD, about 0.9) was harvested. The cells were then frozen at −20°C for 2 h before resuspension in 50 μl of protoplasting medium with 20 mg of lysozyme per ml. After incubation for 2 h at 37°C, a 10-μl aliquot was pipetted under 25 μl of sodium dodecyl sulfate mixture in a well of 0.7% vertical agarose gel (17 by 17 by 0.3 cm). Electrophoresis was carried out at 3 mA for 1 h followed by 7 mA for 0.5 h and then at 28 mA for 3 h. Gels were stained with ethidium bromide and photographed on a UV light box. *Bacillus thuringiensis* subsp. *thuringiensis* strain HD-2 (10) was used in the experiment as a reference source of plasmids of known molecular weights. Determination of relative molecular weights of the plasmids in the isolates was performed with an NA2 nucleic acid analyzer (Bethesda Research Laboratories, Inc.).

Plasmid DNA isolation and electrophoresis. The method described by Heath et al. (11) was used for plasmid DNA isolation from nutrient broth cultures of TE1. Plasmid DNA in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) was mixed with an equal volume of 20% sucrose in TE buffer and loaded onto a 0.7% vertical agarose gel (17 by 17 by 0.3 cm) with wells 6 mm in width. Electrophoresis buffer was Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid, pH 8.3 to 8.5). Electrophoresis was at a constant voltage of 90 V for 5 h.

Bacterial conjugation. Matings between TE2 as donor and E^- Str^r Rif^r mutants as recipients were done at equal cell densities of mid-log or early stationary-phase cells grown in nutrient broth. Cells were filtered on sterile membranes (Millipore Corp.) and placed on soft nutrient agar plates (agar, 0.7%). The plates were incubated for 12 to 16 h for mid-log-phase and 4 to 6 h for stationary-phase cells. The cells on the filters were suspended in and washed once with BMN medium. Aliquots (0.15 ml) or dilutions were plated on BMNE agar plates containing 30 μg of rifampin per ml. After 4 to 5 days of incubation at 29°C, the colonies were picked and purified on the same selective medium. They were subsequently examined for other markers.

Chemicals. EPTC was a gift from Stauffer Chemicals (U.S.A.). The biochemicals used in taxonomic tests were purchased from Sigma Chemical Co. and Aldrich Chemical Co. The microbiological media and stains were obtained from Difco and BDH, Canada.

RESULTS

Bacterial isolation. Plating of the final enrichment culture of T56 soil on BMNE agar (see Materials and Methods) gave rise to a few relatively rapidly growing colonies of identical colony morphology on each plate. These colonies were pale

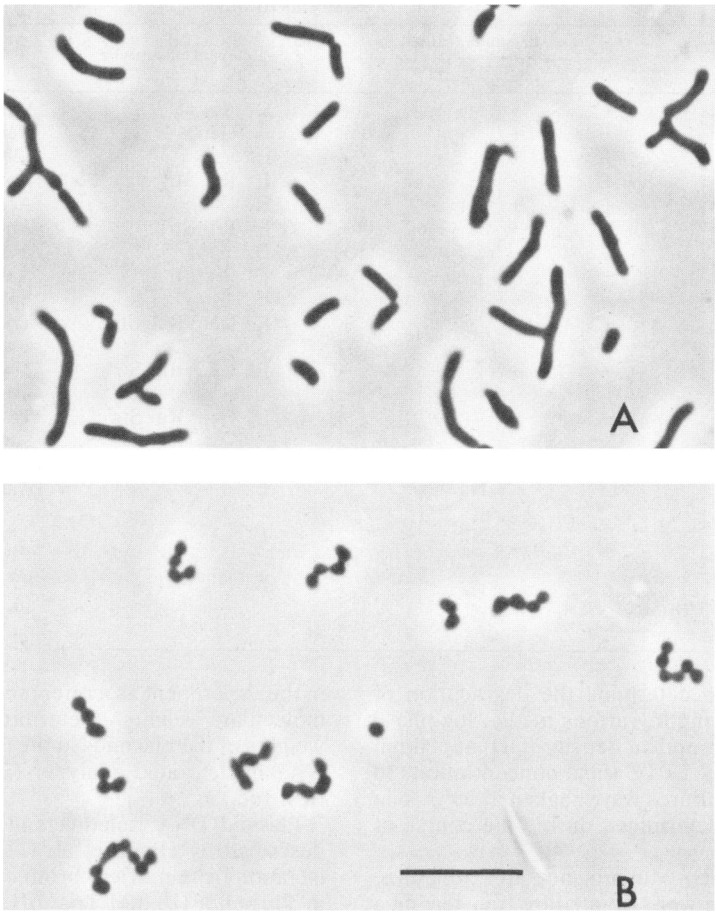

FIG. 1. Phase-contrast micrographs of TE1 growing in nutrient broth: (A) log-phase cells; (B) stationary-phase cells. Magnification bar, 10 μm.

to white and reached a diameter of 2 mm in 6 days. Purified colonies were found to be able to grow in liquid BMNE medium but not in BMN. On BMN agar, the isolate showed some growth but never exceeded a diameter of 1 mm. One isolate, designated TE1, was selected for further studies. Similar rapidly growing colonies on BMNE agar plates were not obtained when T56U soil was subjected to the same enrichment procedure.

Characterization. Culture of strain TE1 in various media such as nutrient broth and BMN with glycerol showed a marked change in morphology with time (Fig. 1). At log phase, the nonmotile cells were pleomorphic long rods, some with rather prominent branchings. V-formation was common during cell division. At stationary phase, the cells were coccoid and reverted to rods when subcultured in fresh nutrient broth, the development cycle typical of *Arthrobacter* spp.

The isolate grew well in a simple defined medium without growth supplements. It could utilize for growth the following compounds as sole carbon source: citrate, malonate, ketoglutarate, D-ribose, D-xylose, *m*-inositol, ornithine, proline, uracil, 4-hydroxybenzoate, 4-aminobutyric acid, 5-aminovaleric acid, and ethanol in addition to acetate, glucose, glycerol, aspartate, and succinate. TE1 could not grow on maltose, rhamnose, *m*-hydroxybenzoate, or ketogluconate as sole carbon source. Cell wall preparations of TE1 contained diaminopimelic acid but not lysine.

Utilization of EPTC for growth. The utilization of EPTC as sole carbon source for growth by TE1 is shown in Fig. 2. A BMNE medium showed a gradual drop in EPTC concentration after inoculation with a log-phase culture of TE1 in BMNE. Evaporation could account for the disappearance of small amounts of EPTC. Cell growth ceased shortly after EPTC was exhausted. The doubling time of TE1 growing in BMNE was estimated to be approximately 8 h.

Degradation of EPTC in the presence of other carbon sources. Addition of EPTC (tested up to 10 μg/ml) to cultures of TE1 growing on various carbon sources did not affect the growth rates of the cultures. The increase in OD as well as the degradation of EPTC in various cultures in 4 h are shown in Table 2. The amount of EPTC degraded was adjusted for the loss of the herbicide through evaporation. Nonspecific binding of EPTC to cells was found to be minimal.

Isolation of mutants of TE1 not able to utilize EPTC. Mutants not able to grow on EPTC as sole carbon source (designated E^-) arose at high frequency from the wild-type strain TE1 (E^+). Three- to four-day-old colonies of wild-type TE1 on nutrient agar, upon subculturing on the same medium, gave rise to colonies which were 100% E^+ (about 200 tested). However, when overnight nutrient broth culture of the same inoculum was plated on nutrient agar, approximately 4% of the colonies tested out to be E^-. The frequency of E^- mutants was increased to about 14% when the nutrient broth culture was treated with the plasmid-curing agent

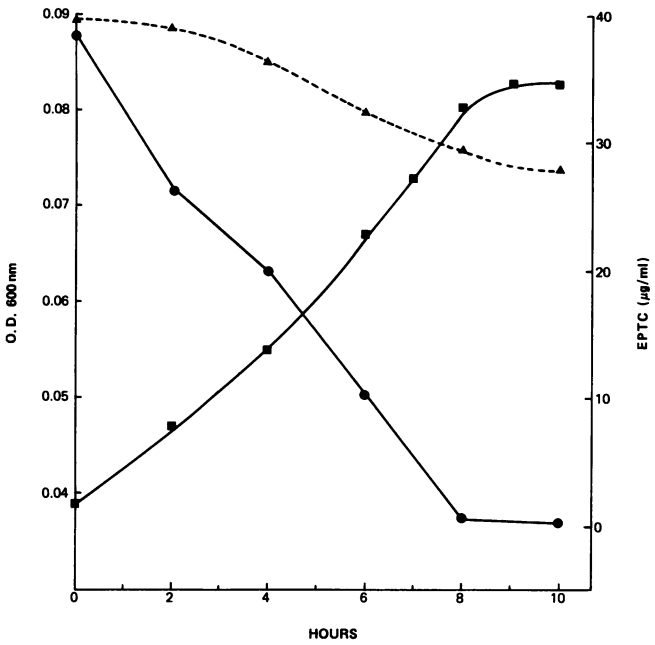

FIG. 2. Utilization of EPTC as sole carbon source for growth by strain TE1. The culture was incubated with shaking at 28°C in a minimal salts medium supplemented with EPTC. Symbols: ■, OD of the culture; ●, EPTC concentration in the medium. Dotted line shows EPTC concentration in uninoculated control.

acridine orange (15 μg/ml) or to about 20% when the culture was incubated at 32°C. Single colonies of TE1, after prolonged storage on nutrient agar plates, also yielded high numbers of E^- cells upon subculturing on nutrient agar. No revertants to the E^+ phenotype were obtained from E^- strains ($<10^{-10}$).

Characterization of E^- mutants of TE1. Morphologically, the E^- mutants were identical to strain TE1. However, on BMNE agar, E^- mutants formed small colonies rarely exceeding 1 mm in diameter. These colonies were similar in size and shape to the colonies of strain TE1 grown on BMN agar.

E^- mutants did not grow in BMN liquid medium. A culture of an E^- mutant in BMN supplemented with glycerol (0.1%) showed, after the addition of EPTC, little decrease in EPTC concentration other than that due to evaporation (see Fig. 5).

Plasmid composition of TE1 and its E^- mutants. The modified agarose gel electrophoresis of cell lysates, which facilitated rapid screening of large numbers of cultures, revealed that strain TE1 carried four plasmids of relative molecular masses of 65.5, 60, 50.5, and 2.5 megadaltons (Fig. 3A). The same four plasmids were clearly seen when plasmid DNA from TE1 purified by the method described by Heath et al. (11) was analyzed by gel electrophoresis (Fig. 4, lane 1). All E^- mutants (Table 1), irrespective of the process of their isolation, were found to lack the 50.5-MDa plasmid (Fig. 3). All but one of the E^- mutants retained the other three plasmids (Fig. 4, lane 4). This particular mutant, TE8, was among the E^- mutants arising out of the treatment with acridine orange. It had, in addition, lost the 60-MDa plasmid.

TABLE 2. Degradation of EPTC by TE1 growing with various carbon sources[a]

Growth medium	EPTC degraded (μg/ml)	Increase in OD
BMN + glycerol (1 mg/ml)	9.2	0.069
BMN + glucose (1 mg/ml)	8.3	0.047
Nutrient broth	0	0.121
BMN + EPTC (60 μg/ml)	30	0.038

[a] All cultures were adjusted to an OD (600 nm) of 0.07. EPTC was added, and the amount degraded after 4-h incubation at 28°C was determined.

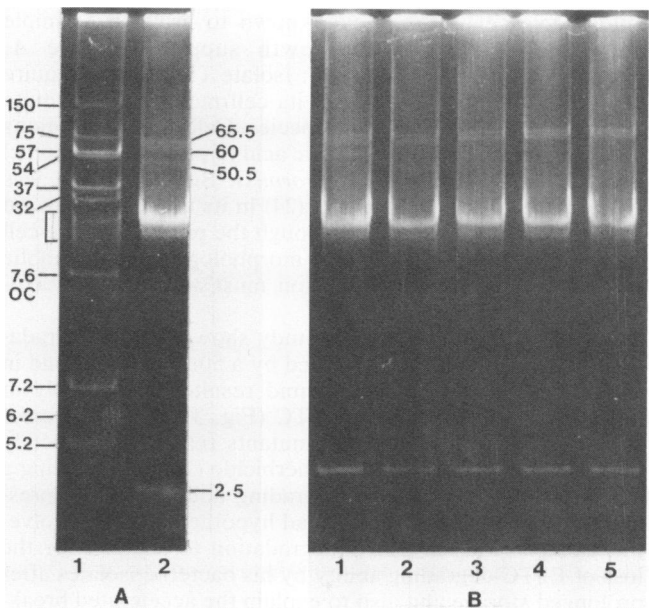

FIG. 3. Plasmid composition of strain TE1 and E^- derivatives of TE1. Modified Eckhardt lysate procedure (see Materials and Methods) was used. (A) Lanes: 1, plasmids of *B. thuringiensis* subsp. *thuringiensis* strain HD-2; 2, TE1. (B) Lanes: 1, TE1; 2, TE9, spontaneous E^- mutant; 3, TE3, E^- mutant obtained from 32°C incubation; 4, TE5, E^- mutant from acridine orange-treated culture; 5, another E^- mutant from a separate experiment of acridine orange treatment. The numbers on the left in (A) indicate the molecular masses (10^3) of the plasmid standards and the numbers on the right indicate the relative molecular masses (10^3) of plasmids of TE1. The bracket shows the position of linear chromosomal DNA.

Plasmid transfer by conjugation. Colonies that arose from the selection plates of the conjugation experiments were found to be E^+, gentamicin sensitive, and rifampin and streptomycin resistant. They had acquired the 50.5-MDa plasmid in the mating process (Fig. 4). When grown in BMN medium supplemented with glycerol (or without the supplement), the transconjugants degraded EPTC as efficiently as the wild-type strain TE1 (Fig. 5). The E^+ transconjugants derived from a mating experiment of TE2 (Gen^r) with TE8 (Rif^r Str^r mutant of TE7) carried the 65.6-, 50.5- and 2.5-MDa plasmids. The 60-MDa plasmid was not transferred into TE8. However, this transconjugant also degraded EPTC as effectively as TE1 (data not shown). The frequency of plasmid transfer from TE1 to its E^- mutants ranged from 0.7×10^{-7} to 1.3×10^{-6} per initial donor cell. No E^+ transconjugants were obtained when *Arthrobacter globiformis*, *A. oxidans*, or *A. simplex* cells were used as recipients.

DISCUSSION

Isolate TE1 falls into the general description of the genus *Arthrobacter* (14). However, the strain could not be categorically assigned to any particular species of this genus. The

only species of *Arthrobacter* known to grow in a simple defined medium without growth supplements are *A. globiformis* and *A. simplex* (18). Isolate TE1 did not require any growth factors. However, its cell morphology is quite distinct from either of the two species. Cell wall preparations of TE1 contained diaminopimelic acid (typical of *A. simplex*) and not lysine (as in *A. globiformis*). But the isolate was more versatile than *A. simplex* (24) in its ability to grow on various carbon substrates. Although the plasmids in the cell could have a bearing on its morphology and metabolic activities, the exact identification must await details taxonomic studies.

The results reported in this study show that the degradation of EPTC by TE1 is mediated by a 50.5-MDa plasmid in the cell. The loss of this plasmid resulted irreversibly in mutants unable to degrade EPTC (Fig. 3). The transfer of this plasmid from TE1 to E^- mutants restored completely their capability to degrade the herbicide (Fig. 5), showing a correlation between EPTC-degrading ability and the presence of this plasmid. Lee (15) had hypothesized the involvement of plasmids in EPTC degradation to account for the loss of EPTC-degrading ability by his bacterial isolates after prolonged storage and also to explain the accelerated breakdown of the herbicide in soils with history of previous exposure to EPTC observed by many workers (16, 20, 30). A similar plasmid-associated mechanism for the transfer and spread of herbicide-degrading ability was proposed to effect the rapid breakdown of 2,4-D in soil (5). Although no

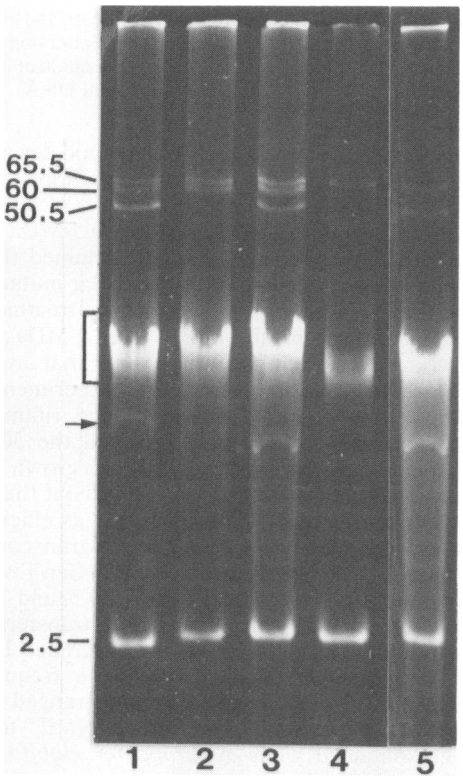

FIG. 4. Plasmid DNA profiles of strain TE2, its E^- derivatives, and the E^+ transconjugants from mating of TE2 with E^- mutants. DNA was isolated as described by Heath et al. (11). Lanes: 1, TE2; 2, E^- mutant (TE4); 3, transconjugant (TE10); 4, E^- mutant (TE8); 5, transconjugant (TE12). The bracket denotes the position of linear chromosomal DNA. The arrow indicates the position of the open circular form of the 2.5-MDa plasmid.

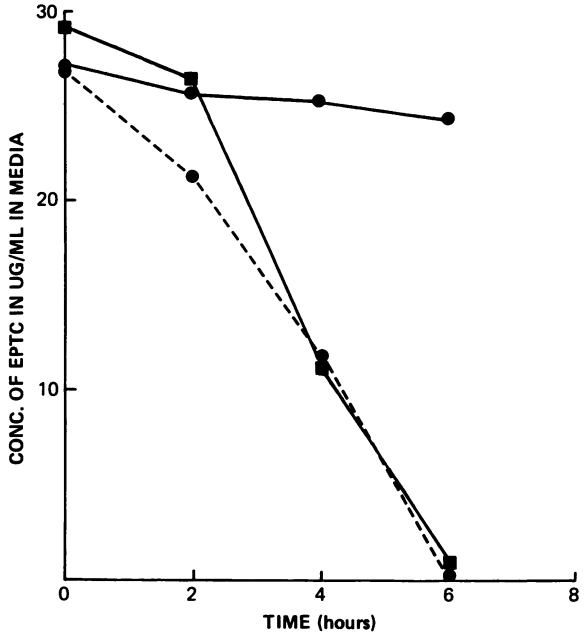

FIG. 5. EPTC degradation by an E^+ transconjugant obtained from matings of TE2 with an E^- mutant (TE4). EPTC (final concentration, 30 μg/ml) was added to the cultures growing in BMN plus glycerol (OD of 0.1 at 600 nm), and disappearance of EPTC from the cultures with time was determined. Symbols: ■, TE2; ●—●, TE4, E^- mutant; ●---●, TE10, E^+ transconjugants. Similar results were obtained with other E^+ transconjugants.

Arthrobacter species were reported among the soil isolates of Lee (15), the results in this paper substantiate his hypothesis that genes for certain herbicide degradation may be localized on the plasmids. This suggestion, which was first made by Waid (28), is becoming increasingly evident (4, 5, 25).

Members of the genus *Arthrobacter* are abundant and ubiquitous in soil. They have been reported to be involved in the utilization of a wide variety of organic chemicals (3, 17, 26, 27, 31), chlorinated biphenyls (9), and certain herbicides (4, 7). Plasmid-associated degradation of chlorinated biphenyls (9), nicotine (2, 3), chlorobenzoic acid (17), and hydroxypyridine (29) in *Arthrobacter* species has been shown. Single plasmid species present in these strains were responsible for the catabolic functions.

The *Arthrobacter* isolate TE1 in this study, however, harbors four plasmids, although only one appeared to be involved in EPTC degradation. This catabollic plasmid exhibited some unique characteristics not shared by the other three plasmids in the cell. It was easily lost as evidenced by a high rate of spontaneous E^- mutant formation; it was readily cured by treatment with acridine orange or incubation at high temperature. This plasmid was conjugally transmissible to the E^- mutants. In this respect, it is similar to the plasmid associated with nicotine degradation (3) but unlike the plasmid involved in parathion hydrolysis (25). The latter plasmid is not transmissible to the cured mutants.

Among the many E^- derivatives obtained by acridine orange treatment, only one, TE7, was also devoid of the 60-MDa plasmid. When TE7 was used as a recipient in mating with TE1, only the 50.5-MDa plasmid was transferred (Fig. 4). However, full restoration of EPTC degrada-

tion ability in the transconjugant indicated that the 60-MDa plasmid was not involved in the catabolism of EPTC.

There was an obvious drawback in our protocol to distinguish the presumptive transconjugants. Our selection was based on the ability of transconjugants to grow on BMNE. We could not, therefore, exclude plasmid transfer if the EPTC degradative trait was not expressed in the recipients. Based on this phenotypic expression, we were unable to transfer the EPTC-degrading plasmid into *A. globiformis* (ATCC 8010), *A. oxidans* ATCC 14358), *A. simplex* (ATCC 6946), nicotine-degrading *A. oxidans*, a cured derivative of this *A. oxidans* (results not presented), and three different *Arthrobacter* strains of similar morphology isolated from untreated T56U soil. This limited our means to isolate the 50.5-MDa plasmid individually in an *Arthrobacter* strain. Alternate methods for purification and genetic characterization of the plasmid are now in progress. Similar methods will be used to isolate the small (2.5 MDa) cryptic plasmid present in TE1. This is the smallest plasmid reported to exist in *Arthrobacter* spp. Plasmids of this size are potentially very useful for the construction of suitable vectors for cloning and DNA recombinant work as demonstrated recently with similar small cryptic plasmids present in *Corynebacterium* spp. (23, 32).

Isolate TE1 growing on various carbon sources degraded EPTC at different rates. This would suggest that the enzyme(s) responsible for the degradation is regulated. Catabolite repression by glucose did not seem to occur since cells growing on glycerol degraded EPTC as fast as those growing on glucose. Although the first step in the metabolism of EPTC has been suggested to be the hydrolytic cleavage of the molecule (8, 12), no specific hydrolytic enzyme has so far been isolated and characterized. The isolation of TE1 should allow detailed examination of EPTC metabolism in bacteria. This would enable us to determine the metabolic defect in E^- mutants and identify the gene(s) on the 50.5-MDa plasmid specifying the EPTC-degrading phenotype.

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