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Title:

**Involvement of Linear Plasmids in Aerobic Biodegradation
of Vinyl Chloride**

Running Title:

Biodegradation of Vinyl Chloride via Linear Plasmids

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ABSTRACT

1
2 *Pseudomonas putida* strain AJ and *Ochrobactrum* strain TD were isolated from
3 hazardous waste sites based on their ability to use vinyl chloride (VC) as a sole source of carbon
4 and energy under aerobic conditions. Strains AJ and TD also use ethene and ethylene oxide as
5 growth substrates. Strain AJ contained a linear megaplasmid (approximately 260 kb) when
6 grown on VC or ethene, but no circular plasmids. While growing on ethylene oxide, the size of
7 the linear plasmid in strain AJ decreased to approximately 100 kb, although its ability to use VC
8 as a substrate was retained. The linear plasmids in strain AJ were cured and its ability to
9 consume VC, ethene, and ethylene oxide was lost following growth on a rich substrate (Luria-
10 Bertani broth) through at least three transfers. Strain TD contained three linear plasmids, ranging
11 in size from approximately 100 kb to 320 kb, when growing on VC or ethene. As with strain AJ,
12 the linear plasmids in strain TD were cured following growth on Luria-Bertani broth and its
13 ability to consume VC and ethene was lost. Further analysis of these linear plasmids may help
14 reveal the pathway for VC biodegradation in strains AJ and TD and explain why this process
15 occurs at many but not all sites where groundwater is contaminated with chloroethenes.
16 Metabolism of VC and ethene by strains AJ and TD is initiated by an alkene monooxygenase.
17 Their yields during growth on VC (0.15-0.20 mg total suspended solids per mg VC) are similar
18 to the yields reported for other isolates (i.e., *Mycobacterium* sp., *Nocardioides* sp., and
19 *Pseudomonas* sp.).

20 (Some preliminary results of this study were presented at the 103rd annual meeting of the
21 American Society for Microbiology, Washington, DC.)

INTRODUCTION

1
2 Millions of tons of vinyl chloride (VC) are produced each year, primarily for the
3 manufacture of polyvinyl chloride (23). However, the occurrence of VC in groundwater is
4 typically not a consequence of direct releases to the environment. VC contamination of
5 groundwater results mainly from transformation of other chlorinated aliphatic compounds,
6 including reductive dechlorination of polychlorinated ethenes and dehydrohalogenation of 1,2-
7 dichloroethane (39). It has recently been demonstrated that VC is also formed naturally in soils,
8 presumptively during oxidative reactions involving humic substances, chloride ions and an
9 oxidant (21). This process may have started as long 400 million years ago (21), so it seems
10 reasonable to expect that biodegradation processes also developed long ago.

11 Reduction of VC to ethene is typically the rate limiting step in the overall reduction of
12 chlorinated ethenes, which can lead to accumulation of VC in groundwater (12, 29). The
13 comparatively slow rate of VC reduction may be related to this reaction being cometabolic in
14 some strains of *Dehalococcoides*, although other strains have recently been shown capable of
15 respiring with VC (8, 18). Oxidative acetogenesis of VC has also been documented in anaerobic
16 sediments (2), although the extent of this process at most locations is not yet known.

17 In locations where anaerobic groundwater transitions to aerobic conditions, VC that
18 migrates from the anaerobic zone may be subject to aerobic biodegradation. Several strains of
19 *Pseudomonas* sp. and *Mycobacterium* sp. along with one *Nocardioides* sp. have been isolated
20 from soil, river water, groundwater, and activated sludge based on their ability to use VC as a
21 sole source of carbon and energy under aerobic conditions (4, 17, 37, 38). While aerobic
22 biodegradation of VC is frequently reported in field studies (7, 11), it is by no means a universal
23 process. Coleman et al. (4) reported a lack of aerobic VC biodegradation activity in 11 of the 31

1 samples tested from chlorinated-ethene contaminated sites. Madl (26) observed no aerobic VC
2 biodegradation activity in three of the six samples tested from an area downgradient of a landfill
3 contaminated with chloroethenes.

4 A better understanding of aerobic VC metabolism is needed to help predict when this
5 process will or will not occur in the environment. Coleman and Spain (6) recently demonstrated
6 that a four-component monooxygenase initiates the oxidation of ethene and VC in
7 *Mycobacterium* strain JS60. The ethylene oxide and VC-epoxide that are formed then react with
8 an epoxyalkane:coenzyme M transferase. The gene for this transferase (JS60 EaCoMT)
9 hybridized to linear megaplasmids in strain JS60 and five other *Mycobacterium* strains grown on
10 VC (5). We hypothesized that the genes associated with a VC⁺ and ethene⁺ phenotype in isolates
11 other than *Mycobacterium* sp. are also carried on a plasmid. While we could have tested the VC⁺
12 and ethene⁺ *Pseudomonas* strains we previously isolated (37, 38), they were derived from
13 activated sludge rather than actual hazardous waste sites, and in the case of strain MF1, it grows
14 very slowly. We therefore obtained two new isolates that grow on VC and ethene from locations
15 that have groundwater contaminated with chlorinated ethenes. Both cultures also grow on
16 ethylene oxide. The VC⁺ and ethene⁺ phenotypes in these isolates are associated with the
17 presence of linear megaplasmids.

18

19

MATERIALS AND METHODS

20 **Chemicals and media.** VC gas (99.5%) was purchased from Fluka, ethene (99.9%)
21 from Matheson, and ethylene oxide (99.5%) from Sigma-Aldrich. All other chemicals used were
22 of reagent grade. Strains AJ and TD were grown in the minimal salts medium (MSM) described
23 by Hartmans et al. (17), but the amount of (NH₄)₂SO₄ was reduced to 0.67 g/liter. No vitamins

1 or other complex growth factors were added to the MSM. *Mycobacterium* strain JS60 was
2 grown in the MSM described by Coleman et al. (3).

3 **Analytical methods.** The total amount of VC, ethene, and ethylene oxide present in
4 serum bottles was determined by gas chromatographic analysis of headspace samples, as
5 previously described (37). The amounts for VC and ethene were converted to aqueous phase
6 concentrations using Henry's law constants of 0.925 for VC and 7.24 for ethene ((mol·m⁻³ gas
7 concentration)/(mol·m⁻³ aqueous concentration)) at 23°C (14). The presence of VC-epoxide was
8 tested based on matching retention times on a gas chromatograph with chemically synthesized
9 authentic material, in addition to a colorimetric procedure involving reaction with 4-(4-
10 nitrobenzyl)pyridine in ethylene glycol, as previously described (37). Chloride ion was
11 measured using an ion-selective electrode (Orion) connected to a pH millivolt meter (Corning)
12 (37). Chemical oxygen demand was determined with a Hach Company (Loveland, CO) kit
13 (range, 5 to 150 mg/liter).

14 **Microcosms and enrichment cultures.** Experiments involving VC or ethene were
15 performed in 70 or 160-ml serum bottles capped with grey butyl rubber septa (Wheaton
16 Scientific Products; Millville, New Jersey). Previous studies demonstrated that minimal losses
17 of VC and ethene occur with these septa (37). Teflon faced red rubber septa or grey butyl rubber
18 septa were used during experiments with ethylene oxide.

19 The two isolates obtained during this study were developed with inocula from different
20 locations. The first source was a former lagoon site in Sacramento, California (7). Reductive
21 dechlorination of chlorinated ethenes and ethanes was documented in the anaerobic source area,
22 along with apparent oxidation of the daughter products (including VC) in the downgradient
23 aerobic region. A sediment and groundwater sample from monitoring well 3037 in the aerobic

1 portion of the plume was used to set-up microcosms (13). An enrichment culture was developed
2 by repeatedly supplying VC as the only source of carbon and energy and then transferring an
3 aliquot to MSM. The sediment-free culture was further enriched by repeated additions of VC as
4 the sole substrate for eight months and periodically diluting the enrichment with fresh MSM.

5 The second source of inoculum was the sanitary landfill at the Department of Energy's
6 Savannah River Site near Aiken, South Carolina. First flush groundwater from monitoring well
7 67D was used to set-up the microcosms; sediment was not available. This well was chosen
8 because it is downgradient of the groundwater that flows beneath the landfill, it has a history of
9 VC contamination, and it is likely aerated by horizontal sparging wells that were installed to
10 control the movement of chlorinated contaminants. The pH of the groundwater was adjusted
11 from 5.04 to 7.02 using dibasic potassium phosphate. One set of microcosms received ethene
12 (50 μ M, aqueous phase) as the sole substrate and a second set received only VC (50 μ M,
13 aqueous phase). The set with VC showed no significant activity, even after 450 days of
14 incubation. A slow rate of ethene utilization occurred relative to killed controls. The contents of
15 these bottles were concentrated by centrifugation and resuspended in MSM. The rate of ethene
16 utilization increased substantially, but VC utilization did not. The ethene-grown enrichment
17 culture became the source from which an isolate was obtained with the ability to grow on VC
18 (see below).

19 Enrichment cultures were maintained by adjusting the pH periodically to 7.2 ± 0.1 using
20 8 M NaOH and supplying oxygen by purging the headspace with air or oxygen after VC or
21 ethene was consumed. The enrichments were incubated at room temperature (23°C) in an
22 inverted position on a gyratory shaker (100-150 rpm).

1 **Pure cultures.** Three pure cultures capable of growing on VC as a sole source of carbon
2 and energy were used in this study. An isolate from the Sacramento site was obtained by
3 streaking an aliquot of the enrichment culture on trypticase soy agar, incubating for 26-38 hours
4 at 22°C, and transferring individual white colonies to serum bottles containing MSM with VC as
5 the sole substrate. VC consumption began after 50-70 days. The isolate was assigned the strain
6 designation “AJ.”

7 An isolate from the SRS ethene-enrichment culture was also obtained by streaking an
8 aliquot on trypticase soy agar and incubating for 18-22 hours at 22°C. Individual white colonies
9 were transferred to MSM and ethene was supplied as the sole substrate. Ethene consumption
10 began after approximately 25 days and was maintained for several months. Samples of the pure
11 culture were then provided with VC as the sole source of carbon and energy. The culture began
12 using VC immediately and has been maintained on VC as the sole substrate ever since. The
13 isolate was assigned the strain designation “TD.” Strains AJ and TD were identified based on
14 the sequence of their 16S rRNA gene, as previously described (37) with minor modifications
15 (10).

16 The third pure culture used in this study, *Mycobacterium* strain JS60, was obtained from
17 the Air Force Research Laboratory at Tyndall Air Force Base. Strain JS60 was isolated from an
18 industrial site contaminated with chlorinated ethenes (4).

19 **Plasmid Analysis.** The presence of large linear plasmids in strains AJ and TD following
20 growth on VC and ethene, as well as in strain AJ following growth on ethylene oxide, was
21 evaluated using a modified procedure for preparation of high molecular weight bacterial DNA
22 embedded in agarose plugs, as previously described (32) with the following modifications. Cells
23 were centrifuged (10 min at 10,000 rpm) and resuspended in MSM (1 ml) to an OD₆₀₀ of 15-300,

1 depending on the isolate and substrate used. The resuspended cell solution (1 ml) was warmed
2 (45°C, 3-4 min) and embedded into 1 ml of 1.2% low-melting temperature agarose containing
3 25% sucrose (45°C). After solidifying, the plugs were removed from the molds, agitated in
4 NaCl/Tris/EDTA solution (200 mM NaCl, 10 mM Tris-Cl at pH 7.2, 100 mM EDTA at pH 8.0),
5 removed from the NaCl/Tris/EDTA solution, agitated with a bacterial cell lysis solution followed
6 by a proteinase K solution, and then cut and placed into a 1% agarose gel.

7 DNA was separated using a clamped homogeneous electric field system (CHEF DR-III;
8 Bio-Rad) at 1-50 sec linear ramp, 6 V/cm, 14°C in 0.5X TBE buffer for 18 h. Concatamers of •
9 DNA (• ladder PFG marker, New England Biolabs) were used as molecular markers. To
10 determine if the plasmids were linear or circular, the pulse times were changed: one gel was run
11 at initial and final switch times of 30 sec; a second gel was run at initial and final switch times of
12 90 sec (31).

13 The presence of linear plasmids was also evaluated following growth of strains AJ and
14 TD on Luria-Bertani broth (LB). Cells were transferred to LB and grown to a maximum optical
15 density. An aliquot was transferred to fresh LB and the process was repeated through at least
16 two more growth cycles before checking for plasmids. Samples were also centrifuged (10,000
17 rpm, 10 min), washed three times in MSM, resuspended in MSM and evaluated for their ability
18 to use VC and ethene. LB-grown strain AJ was also tested for its ability to resume growth on
19 ethylene oxide. In addition to LB, strain AJ was grown through at least three cycles on acetate,
20 ethylene glycol, glyoxylate, glycolate, ethanolamine and glycolaldehyde. These cells were
21 evaluated for linear plasmids, as well as the ability to resume growth on VC.

22 The presence of circular plasmids in strain AJ following growth on VC was evaluated.
23 Circular plasmids were isolated as previously described (30) and separated on a 1% CHEF gel at

1 1-50 sec linear ramp, 6V/cm, 14°C in 0.5X TBE buffer for 18 h. *Pseudomonas aeruginosa*
2 pME290 (ATCC #37412), containing a 6.8 kb circular plasmid, and an *E. coli* clone, containing
3 a 138 kb circular plasmid (a large bee genomic insert carried by a bacterial artificial chromosome
4 (BAC) vector) (36), served as positive controls.

5 **Growth experiments.** The ability of the isolates to grow on various substrates was
6 evaluated based on an increase in optical density (600 nm, Milton Roy Spec 20D
7 spectrophotometer). For volatile compounds (VC, ethene, ethylene oxide and glycolaldehyde),
8 experiments were conducted in sealed serum bottles. Repeated additions of substrate were made
9 (starting at 0.040-0.10 mmol/bottle and proceeding with higher doses until a total of 0.94-1.3
10 mmol/bottle was consumed), along with a sufficient amount of oxygen. Growth on non-volatile
11 substrates (glyoxylate, glycolate, ethylene glycol, ethanolamine, acetate and chloroacetate) was
12 evaluated in shake flasks. For glyoxylate, glycolate, ethylene glycol, ethanolamine and acetate,
13 10-20 mM was added (1.0-2.0 mmol/bottle). To avoid substrate toxicity, initial concentrations
14 of 1 mM (0.1 mmol/bottle) were used for glycolaldehyde and chloroacetate. Substrate
15 consumption was monitored based on changes in chemical oxygen demand (since consumption
16 of 1 mM was not enough to noticeably increase optical density); more substrate was added when
17 less than 10% of the initial amount remained.

18 The observed yields for strains AJ and TD were calculated based on the increase in total
19 suspended solids (TSS) following consumption of repeated additions of VC. TSS was
20 determined according to Standard Methods (16).

21 **Kinetics of VC utilization.** Monod kinetic parameters for utilization of VC by strain AJ
22 were determined as previously described (15, 37). Culture for the kinetic experiments was
23 obtained from a 2.3-L reactor operated in a semi-continuous batch mode at a 36 d hydraulic

1 retention time. After several retention times, the concentration of biomass stabilized at 100 mg
2 TSS per liter. Batch depletion experiments were set up with samples from the reactor. VC
3 depletion curves were evaluated to determine the maximum specific VC utilization rate (k) and
4 the half saturation coefficient (K_S), taking biomass growth into account (15). The initial VC
5 concentration was varied from 6-25 μM , in order to encompass the maximum utilization rate and
6 the region in which the half saturation value becomes important. k and K_S were determined from
7 the batch depletion data by a weighted, nonlinear least-squares method (37). The effect of mass
8 transfer was evaluated by incorporating a mass transfer coefficient for VC ($K_La = 34.5 \text{ hr}^{-1}$) into
9 the Monod equation and comparing the solutions for k and K_S to those without mass transfer
10 (37).

11 **Nucleotide sequence accession number.** The sequences for strains AJ and TD were
12 deposited into GenBank with accession numbers AY391278 and AY623625, respectively.

13

14

RESULTS

15 **Identification of strains TD and AJ.** Strain TD was isolated from an enrichment
16 culture developed with groundwater in an area near the sanitary landfill at the Department of
17 Energy's Savannah River Site in South Carolina, where the groundwater is contaminated with
18 chlorinated ethenes from the landfill leachate. The enrichment was grown on ethene as the sole
19 source of carbon and energy; little or no activity occurred initially with VC alone. However,
20 once the isolate was obtained and grown on ethene, it rapidly transitioned to VC as a sole
21 substrate. Strain TD is a gram negative motile rod. Based the sequence of its 16S rRNA gene
22 (1449 bases), strain TD shares greater than 99.8% identity (using GenBank) with 19

1 *Ochrobactrum* stains, including 4FB9 (accession no. AF229875), which is capable of growing
2 on 4-fluorobenzoate as a sole source of carbon and energy (34).

3 Strain AJ was isolated from an enrichment culture developed with sediment and
4 groundwater from a hazardous waste site in California that is contaminated with chlorinated
5 ethenes (13). The isolate was initially grown in MSM with VC as its sole source of carbon and
6 energy and oxygen as the terminal electron acceptor, through numerous transfers. Strain AJ also
7 uses ethene and ethylene oxide as sole sources of carbon and energy. It is gram negative, rod
8 shaped and motile. Based on the sequence of its 16S rRNA gene (1496 bases), the closest match
9 to strain AJ (using GenBank) is to *Pseudomonas putida*. Strain AJ shares 99.8% identity with *P.*
10 *putida* ATCC 17527 (accession no. AJ249451).

11 **Presence of Linear Plasmids.** Strain AJ contains a linear megaplasmid (approximately
12 260 kb) when grown on VC or ethene as the sole source of carbon and energy (Figure 1, lanes 2
13 and 4). Changing the CHEF gel pulse times did not alter movement of the plasmid, confirming
14 that it is linear (data not shown). When these cells were transferred to LB broth and grown
15 through at least three cycles on this rich substrate (in the absence of VC or ethene), the linear
16 plasmid was no longer present (Figure 1, lanes 3 and 5). A shorter period of growth on LB did
17 not reliably cure the plasmids. Two microbes containing circular plasmids (*P. aeruginosa*
18 pME290 and an *E. coli* bee BAC clone) were used to demonstrate that circular plasmids are not
19 separated from CHEF gel plugs (not shown in Figure 1) when using an extraction procedure for
20 high molecular weight DNA (32). *Mycobacterium* strain JS60 was used as a positive control
21 since it contains a linear megaplasmid when grown on VC (5) or ethene ([Personal](#)
22 [Communication?](#)) (Figure 1, lane 9).

1 To confirm that the presence of the linear plasmid is required for the VC⁺ phenotype, the
2 LB-grown cells (lacking the plasmid, according to Figure 1) were concentrated, washed, and
3 placed back into MSM with VC as the sole substrate. Even after 190 days of incubation, VC
4 utilization did not resume (Figure 2). When strain AJ was growing on VC, trace amounts of VC-
5 epoxide were occasionally detected during headspace analysis. Following growth of strain AJ on
6 LB, there was no transient accumulation of VC-epoxide or ethylene oxide, suggesting that the
7 alkene monooxygenase presumptively needed to initiate aerobic catabolism of VC and ethene
8 (see below) was also absent, or not induced. LB-grown cells cured of the linear megaplasmids
9 also lost the ability to use ethylene oxide as a sole substrate.

10 The linear megaplasmid present in VC-grown cells was also cured when strain AJ was
11 grown through three transfers on acetate, ethanolamine, ethylene glycol, glyoxylate, glycolate,
12 and glycolaldehyde (each a potential downstream intermediate in aerobic catabolism of VC
13 and/or ethene). As with LB, when strain AJ was grown on these simple substrates and then
14 returned to MSM and VC as the sole substrate, use of VC did not resume, even after several
15 months of incubation.

16 Antibiotic resistance in strain AJ with and without the linear megaplasmid was evaluated
17 by plating cells on LB-agar containing ampicillin (100 and 200 mg/l), kanamycin (50 and 100
18 mg/l), and chloramphenicol (25 and 50 mg/l). The VC-grown cells containing the plasmid
19 exhibited no inhibition. When the linear megaplasmid was cured following growth on LB, there
20 was still no inhibition, indicating the genes for resistance to these antibiotics are not carried
21 exclusively on the plasmid.

22 VC-grown strain AJ switched to ethylene oxide as a sole source of carbon and energy
23 without a lag. While growing on ethylene oxide, strain AJ retained a linear plasmid but its size

1 decreased to approximately 100 kb (Figure 1, lane 6). Following growth of strain AJ on ethylene
2 oxide for several months, samples were returned to VC as the sole substrate and use of VC began
3 after a lag of approximately 10 days. Analysis of these cells (i.e., grown on VC, then ethylene
4 oxide, then back to VC) indicated that the single 100 kb linear plasmid present during growth on
5 ethylene oxide was retained. With cultures that were switched from ethylene oxide to ethene, the
6 cells did not retain the single 100 kb plasmid. These cells contained two plasmids,
7 approximately 30 and 45 kb in size. At this point, use of ethene as a sole source of carbon and
8 energy became erratic; in several serum bottles, use of ethene ceased entirely, indicating that
9 extended incubation of strain AJ on ethylene oxide resulted in the loss of one or more genes
10 needed for sustained use of ethene as a sole substrate.

11 The 260 kb linear plasmid initially present in strain AJ was retained following growth on
12 VC for more than 6 months. Two small linear plasmids (approximately 100 kb and 80 kb) and
13 one larger one (approximately 390 kb) appeared in strain AJ after cultivation on VC for more
14 than one year. Long-term incubation of strain AJ on ethene and ethylene oxide as sole substrates
15 also led to changes in the linear plasmid arrangement. Linear plasmids of approximately 200 kb
16 and 300 kb size appeared in cells grown on ethene. In addition to the 100 kb plasmid, three other
17 plasmids (approximately 210 kb, 230 kb, and 320 kb) appeared in cells grown on ethylene oxide.
18 In spite of these changes, the ability of strain AJ to use VC and ethene as growth substrates was
19 retained during nearly three years of incubation. The only instability occurred when ethylene
20 oxide-grown cells were switched to ethene and ethene utilization faltered after several weeks.

21 Like strain AJ, the ethene⁺ and VC⁺ phenotypes in strain TD are associated with linear
22 plasmids. VC-grown strain TD contained two linear plasmids, approximately 190 and 260 kb in
23 size (Figure 1, lane 7). Ethene-grown strain TD contained three linear plasmids, approximately

1 100, 175 and 260 kb in size (Figure 1, lane 8). When samples of strain TD were grown on LB
2 through at least three transfers, the plasmids were cured and the resulting cells no longer had the
3 ability to use VC or ethene as sole sources of carbon and energy. As with strain AJ, there was no
4 accumulation of VC-epoxide or ethylene oxide when LB-grown strain TD was provided with VC
5 or ethene.

6 **Characteristics of Strains AJ and TD.** The observed yields for strains AJ and TD when
7 growing on VC were 0.196 ± 0.037 and 0.147 ± 0.010 mg TSS per mg VC, respectively. These
8 are similar to the values reported for other VC-grown isolates (4, 37) (TSS assumed to contain
9 50% protein). Simultaneous nonlinear fitting of data from four batch depletion experiments
10 resulted in the following values for the Monod kinetic parameters for strain AJ: $K_s = 2.36 \pm$
11 $0.054 \mu\text{M}$ and $k = 1.41 \pm 0.18 \mu\text{mol VC per mg TSS per day}$. Incorporation of a mass transfer
12 coefficient for VC into the Monod equation did not change the resulting values for K_s and k . K_s
13 is within the range of previously reported values (4, 37, 38), while k is an order of magnitude
14 lower compared to several *Mycobacterium* strains and *Nocardioides* strain JS614, but similar to
15 another *Pseudomonas* isolate (38). Differences among the k values may be partly attributable to
16 differences in the conditions used to grow the cultures prior to determining the kinetic
17 parameters (37). The maximum growth rate for strain AJ (calculated from k and the yield, as
18 described previously (37)) is 0.017 d^{-1} . The extent of VC dechlorination by strain AJ was
19 assessed based on triplicate measurements of chloride release: $1.009 \pm 0.054 \text{ mol Cl}^-$ per mol of
20 VC consumed, which is very close the stoichiometric amount expected.

21 The involvement of a monooxygenase in VC catabolism was evaluated with strains AJ
22 and TD. For each isolate, two sets of duplicate serum bottles received VC (60 μM and 150 μM
23 for strains AJ and TD, respectively); one set also received acetylene (5% headspace

1 concentration). All of the VC was consumed in less than two days in the set without acetylene,
2 while less than 10% was consumed in the set with acetylene present. When acetylene and VC
3 were purged from the headspace and VC was added again, VC consumption resumed, indicating
4 the effect was reversible. Strains AJ and TD are not able to use VC or ethene as a substrate in
5 the absence of oxygen. Strain AJ is capable of growth on ethylene oxide using nitrate as a
6 terminal electron acceptor, indicating that oxygen is not used as a reactant in the catabolic
7 pathway beyond ethylene oxide.

8 The ability of strain AJ to resume VC utilization following a period of starvation was
9 evaluated. This was a concern because several of the aerobic isolates capable of using VC as a
10 growth substrate lose the ability to consume VC after starvation for only one day or less (4, 17).
11 However, with strain AJ, VC metabolism resumed even after 60 days without exposure to VC
12 (or any other substrates).

13 In addition to VC, ethene, and ethylene oxide, strains AJ and TD grow on glycolate,
14 glyoxylate, acetate, ethanolamine, and ethylene glycol. Strain AJ also grew on glycolaldehyde,
15 but was unable to use chloroacetate as a substrate, even after 53 days of incubation.

16

17

DISCUSSION

18 This study demonstrated that use of VC as a sole source of carbon and energy under
19 aerobic conditions by two isolates obtained from hazardous waste sites depends on the presence
20 of linear megaplasmids. In addition, linear plasmids are required in strains AJ and TD for use of
21 ethene as a growth substrate, and in strain AJ for use of ethylene oxide. Linear plasmids have
22 been found in both gram positive and gram negative bacteria (19), although no previous reports
23 were found for linear plasmids in *Pseudomonas putida* or *Ochrobactrum* sp. In several strains

1 of *Rhodococcus*, linear plasmids carry the genes for isopropyl benzene and TCE catabolism (9),
2 isopropylcatechol 2,3-dioxygenase (22), an alkene monooxygenase (33), and polychlorinated
3 biphenyl degradation (27). Linear plasmids have also been found in strains of *Xanthobacter* for
4 biodegradation of 1,2-dichloroethane (35) and propylene (24), in strains of *Streptomyces* for
5 mercury resistance (31), and in *Nocardia opaca* for hydrogen autotrophy (20). *Mycobacterium*
6 strain E-1-57, isolated on ethene, contains two linear plasmids (260 and 340 kb); however, a
7 direct link between their presence and ethene metabolism was not established (33).

8 Strain TD appears to be the first *Ochrobactrum* sp. reported that uses VC and ethene as
9 growth substrates. Most of the microbes isolated thus far with this ability are *Mycobacterium* sp.
10 (4, 17), although several strains of *Pseudomonas* (37, 38) and one *Nocardioides* sp. (4) have also
11 been reported. Strain AJ appears to be the first *P. putida* isolate that grows on VC. Several of
12 the *Ochrobactrum* strains that share more than 99.8% identity with strain TD include four *O.*
13 *anthropi*, which are perhaps best known as opportunistic human pathogens. However, several
14 *Ochrobactrum* isolates are known for their ability to biodegrade halogenated organic
15 compounds, including 4-fluorobenzoate (34) and atrazine (25).

16 VC catabolism in strains AJ and TD appears to be initiated by an alkene monooxygenase,
17 based on inhibition of VC utilization by acetylene, a known inhibitor of monooxygenases (1),
18 occasional detection of trace amounts of VC-epoxide during growth on VC, and a lack of VC
19 consumption in the absence of oxygen. Other isolates capable of growth on VC also use an
20 alkene monooxygenase (4, 6, 17, 37, 38). A large plasmid (approximately 310 kb) in VC-grown
21 *Nocardioides* strain JS614 carries the genes for an alkene monooxygenase (28). The
22 monooxygenase and coenzyme M genes used by *Xanthobacter* strain Py2 for propylene
23 metabolism are located on a linear megaplasmid (24). The genes for a monooxygenase and an

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FIGURE LEGENDS

FIG. 1. Evaluation of linear plasmids under difference growth conditions. Lane 1, λ ladder; lane 2, strain AJ grown on VC; lane 3, strain AJ grown on LB following growth on VC; lane 4, strain AJ grown on ethene; lane 5, strain AJ grown on LB following growth on ethene; lane 6, strain AJ grown on ethylene oxide; lane 7, strain TD grown on VC; lane 8, strain TD grown on ethene; and lane 9, *Mycobacterium* strain JS60 grown on ethene.

FIG. 2. VC consumption by strain AJ containing a 260 kb linear megaplasmid (see Figure 1, lane 2) and the lack of VC consumption by strain AJ after the plasmid was cured by culturing on LB (see Figure 1, lane 3).

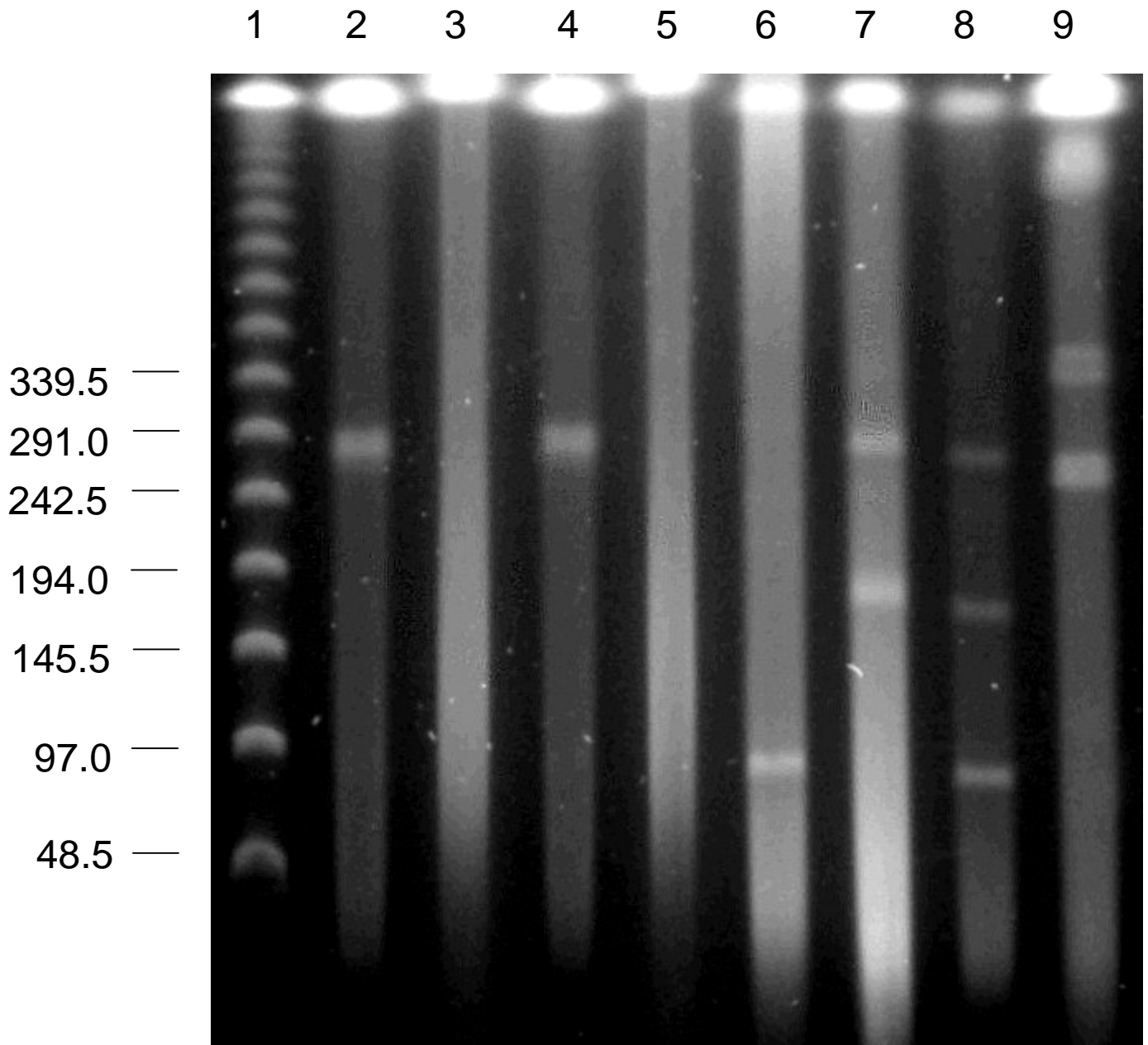


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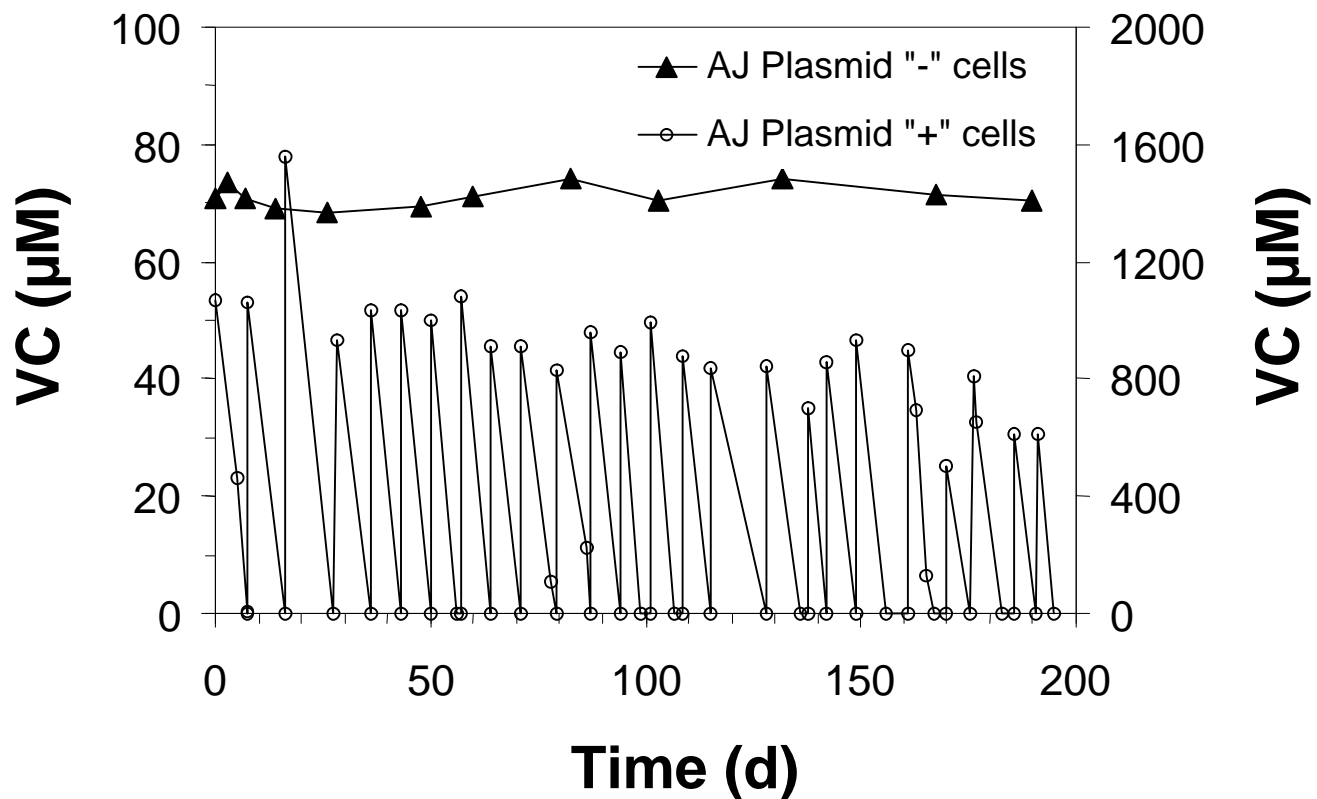


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