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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

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Chemicals and media. VC gas (99.5%) was purchased from Fluka, ethene (99.9%) from Matheson, and ethylene oxide (99.5%) from Sigma-Aldrich. All other chemicals used were of reagent grade. Strains AJ and TD were grown in the minimal salts medium (MSM) described by Hartmans et al. (17), but the amount of $(NH_4)_2SO_4$ was reduced to 0.67 g/liter. No vitamins

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

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

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

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

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

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

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

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

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

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

Plasmid Analysis. The presence of large linear plasmids in strains AJ and TD following growth on VC and ethene, as well as in strain AJ following growth on ethylene oxide, was evaluated using a modified procedure for preparation of high molecular weight bacterial DNA embedded in agarose plugs, as previously described (32) with the following modifications. Cells were centrifuged (10 min at 10,000 rpm) and resuspended in MSM (1 ml) to an OD_{600} of 15-300,

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

epoxyalkane:coenzyme M transferase involved in catabolism of VC by *Mycobacterium* strain JS60 are located on linear megaplasmids (6).

In strains AJ and TD, the pathway for VC and ethene metabolism beyond their respective epoxides is not yet known. Strain AJ grows on several substrates that are potential downstream intermediates, including ethylene glycol, glycolaldehyde, glycolate, glyoxlate, ethanolamine, and acetate. However, growth of strain AJ on these compounds results in loss of the plasmid and the ability to use VC as a substrate. This suggests that the pathway proceeds through other intermediates, e.g., via reaction with an epoxyalkane:coenzyme M transferase (5, 6). Regardless of the pathway, one would expect that only a few reactions are needed to transform VC-epoxide into a compound that can be degraded with enzymes that are not carried on the plasmid. If only a few genes are needed, it raises the issue of why the linear plasmids are so large. Further analysis of these linear plasmids may reveal interesting functions peripherally related to VC, ethene and ethylene-oxide metabolism. For example, given the high degree of reactivity of epoxides with nucleic acids, genes for DNA repair may play a key role in maintenance of the plasmids.

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REFERENCES

1. **Bedard, C., and R. Knowles.** 1989. Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. **53:**68-84.
2. **Bradley, P. M., and F. H. Chapelle.** 2000. Acetogenic microbial degradation of vinyl chloride. Environ. Sci. Technol. **34:**2761-2763.
3. **Coleman, N. V., T. E. Mattes, J. M. Gossett, and J. C. Spain.** 2002. Biodegradation of *cis*-dichloroethene as the sole carbon source by a β-Proteobacterium. Appl. Environ. Microbiol. **68:**2726-2730.
4. **Coleman, N. V., T. E. Mattes, J. M. Gossett, and J. C. Spain.** 2002. Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. Appl. Environ. Microbiol. **68:**6162-6171.
5. **Coleman, N. V., and J. C. Spain.** 2003. Distribution of the coenzyme M pathway of epoxide metabolism among ethene- and vinyl chloride-degrading *Mycobacterium* strains. Appl. Environ. Microbiol. **69:**6041-6046.
6. **Coleman, N. V., and J. C. Spain.** 2003. Epoxyalkane:coenzyme M transferase in the ethene and vinyl chloride biodegradation pathways of *Mycobacterium* Strain JS60. J. Bacteriol. **185:**5536-5545.
7. **Cox, E., E. Edwards, L. Lehmicke, and D. Major.** 1995. Intrinsic biodegradation of trichloroethene and trichloroethane in a sequential anaerobic-aerobic aquifer, p. 223-232. *In* R. E. Hinchee, J. T. Wilson, and D. C. Downey (eds.), Intrinsic Bioremediation. Battelle Press, Columbus, OH.
8. **Cupples, A. M., A. M. Spormann, and P. L. McCarty.** 2003. Growth of a

Dehalococcoides-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. Appl. Environ. Microbiol. **69:**953-959.

9. **Dabrock, B., M. Keßeler, B. Averhoff, and G. Gottschalk.** 1994. Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. Appl. Environ. Microbiol. **60:**853-860.

10. **Danko, A. S.** 2004. Use of Vinyl Chloride as a Growth Substrate by Aerobic Bacteria: Pathway Analysis and Involvement of Linear Plasmids. Ph.D. thesis, Clemson University, Clemson, SC.

11. **Edwards, E. A., and E. E. Cox.** 1997. Field and laboratory studies of sequential anaerobic-aerobic chlorinated solvent biodegradation, p. 261-265. *In* B. C. Alleman and A. Leeson (eds.), In Situ and On-Site Bioremediation, vol. 3. Battelle Press, Columbus, OH.

12. **Fennell, D. E., A. B. Carroll, J. M. Gossett, and S. H. Zinder.** 2001. Assessment of indigenous reductive dechlorination potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. **35:**1830-1839.

13. **Freedman, D. L., A. S. Danko, and M. F. Verce.** 2001. Substrate interactions during aerobic biodegradation of methane, ethene, vinyl chloride and 1,2-dichloroethenes. Wat. Sci. Technol. **43:**333-340.

14. **Freedman, D. L., and S. D. Herz.** 1996. Use of ethylene and ethane as primary substrates for aerobic cometabolism of vinyl chloride. Wat. Environ. Res. **68:**320-328.

15. **Freedman, D. L., M. H. Swamy, N. C. Bell, and M. F. Verce.** 2004. Biodegradation of

chloromethane under nitrate-reducing and aerobic conditions by *Pseudomonas aeruginosa* strain NB1. Appl. Environ. Microbiol. **70**, in press.

16. **Greenberg, A. E., L. S. Clesceri, and A. D. e. Eaton.** 1992. Standard Methods for the Examination of Water and Wastewater, 18th ed. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.

17. **Hartmans, S., and J. A. M. de Bont.** 1992. Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. Appl. Environ. Microbiol. **58:**1220-1226.

18. **He, J., K. M. Ritalahti, M. R. Aiello, and F. E. Löffler.** 2003. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. Appl. Environ. Microbiol. **69:**996-1003.

19. **Hinnebusch, J., and K. Tilly.** 1993. Linear plasmids and chromosomes in bacteria. Molecular Microbiol. **10:**917-922.

20. **Kalkus, J., M. Reh, and H. G. Schlegel.** 1990. Hydrogen autotrophy of *Nocardia opaca* strains is encoded by linear megaplasmids. J. Gen. Microbiol. **136:**1145-1151.

21. **Keppler, F., R. Borchers, J. Parcht, S. Rheinberger, and H. F. Scholer.** 2002. Natural formation of vinyl chloride in the terrestrial environment. Environ. Sci. Technol. **36:**2479-2483.

22. **Kesseler, M., E. R. Dabbs, B. Averhoff, and G. Gottschalk.** 1996. Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2. Microbiol. **142:**3241-3251.

23. **Kielhorn, J., C. Melber, U. Wahnschaffe, A. Aitio, and I. Mangelsdorf.** 2000. Vinyl chloride: still a cause for concern. Environ. Health Perspect. **108:**579-588.

24. **Krum, J. G., and S. A. Ensign.** 2001. Evidence that a linear megaplasmid encodes enzymes of aliphatic alkene and epoxide metabolism and coenzyme M (2-Mercaptoethanesulfonate) biosynthesis in *Xanthobacter* strain Py2. J. Bacteriol. **183:**2172-2177.

25. **Laura, D., G. de Socio, R. Frassanito, and D. Rotilio.** 1996. Effects of atrazine on Ochrobactrum anthropi membrane fatty acids. Appl. Environ. Microbiol. **62:**2644-2646.

26. **Madl, M. D.** 2002. Aerobic Biodegradation of Ethene, *cis*-1,2-Dichloroethene and Vinyl Chloride in Sediments at the Savannah River Site Sanitary Landfill. MS thesis, Clemson University, Clemson, SC.

27. **Masai, E., K. Sugiyama, N. Iwashita, S. Shimizu, J. E. Haushild, T. Hatta, K. Kimbara, K. Yano, and M. Fukuda.** 1997. The *bphDEF meta*-cleavage pathway genes involved in biphenyl/polychlorinated biphenyl degradation are located on a linear plasmid and separated from the initial *bphACB* genes in *Rhodococcus* sp. strain RHA1. Gene **187:**141-149.

28. **Mattes, T. E., N. V. Coleman, J. C. Spain, and J. M. Gossett.** 2003. Evidence that vinyl chloride monooxygenase genes are encoded by a megaplasmid in *Nocardioides* strain JS614, Proceedings of the 103rd General Meeting. American Society of Microbiology, Washington, D. C.

29. **Maymó-Gatell, X., I. Nijenhuis, and S. H. Zinder.** 2001. Reductive dechlorination of *cis*-1,2-dichloroethene and vinyl chloride by "*Dehalococcoides ethenogenes*". Environ. Sci. Technol. **35:**516-521.

30. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol. **59:**2730-2733.

31. **Ravel, J., H. Schrempf, and R. T. Hill.** 1998. Mercury resistance is encoded by transferable giant linear plasmids in two Chesapeake Bay *Streptomyces* strains. Appl. Environ. Microbiol. **64:**3383-3388.

32. **Riethman, H., B. Birren, and A. Gnirke.** 1997. Preparing HMW Bacterial DNA Embedded in Agarose Plugs, p. 136-138. *In* B. Birren, E. D. Green, S. Klapholz, R. M. Myers, and J. Roskams (eds.), Genome Analysis: A Laboratory Manual Volume 1: Analyzing DNA. Cold Spring Harbor Laboratory Press, NY.

33. **Saeki, H., M. Akira, B. Keizo, B. Averhoff, and G. Gottschalk.** 1999. Degradation of trichloroethene by a linear-plasmid-encoded alkene monooxygenase in *Rhodococcus corallinus* (*Nocardia corallina*) B-276. Microbiol. **145:**1721-1730.

34. **Song, B., N. J. Palleroni, and M. M. Haggblom.** 2000. Isolation and Characterization of Diverse Halobenzoate-Degrading Denitrifying Bacteria from Soils and Sediments. Appl. Environ. Microbiol. **66:**3446-3453.

35. **Tardif, G., C. W. Greer, D. Labbe, and P. C. K. Lau.** 1991. Involvement of a large plasmid in the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*. Appl. Environ. Microbiol. **57:**1853-1857.

36. **Tomkins, J. P., M. Luo, G. C. Fang, D. Main, J. L. Goicoechea, M. Atkins, D. A. Frisch, R. E. Page, E. Guzmán-Novoa, Y. Yu, G. Hunt, and R. A. Wing.** 2002. New genomic resources for the honey bee (*Apis mellifera* L.): development of a deep-coverage BAC library and a preliminary STC database. Genet. Mol. Res. **1:**306-316.

37. **Verce, M. F., R. L. Ulrich, and D. L. Freedman.** 2000. Characterization of an isolate that uses vinyl chloride as a growth substrate under aerobic conditions. Appl. Environ. Microbiol. **66:**3535-3542.

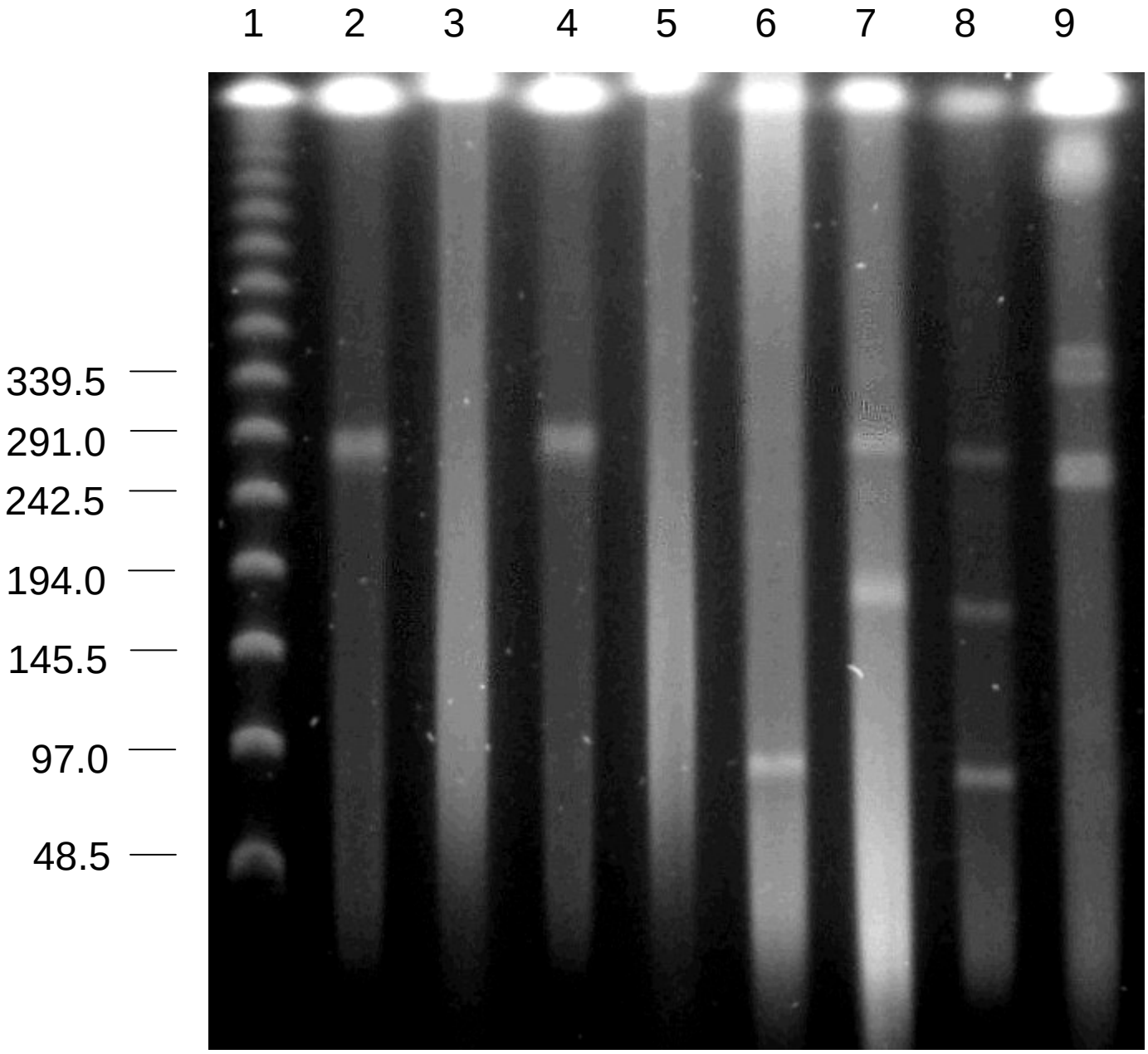
38. **Verce, M. F., R. L. Ulrich, and D. L. Freedman.** 2001. Transition from cometabolic to growth-linked biodegradation of vinyl chloride by a *Pseudomonas* sp. isolated on ethene. Environ. Sci. Technol. **35:**4242-4251.

39. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21:**722-736.

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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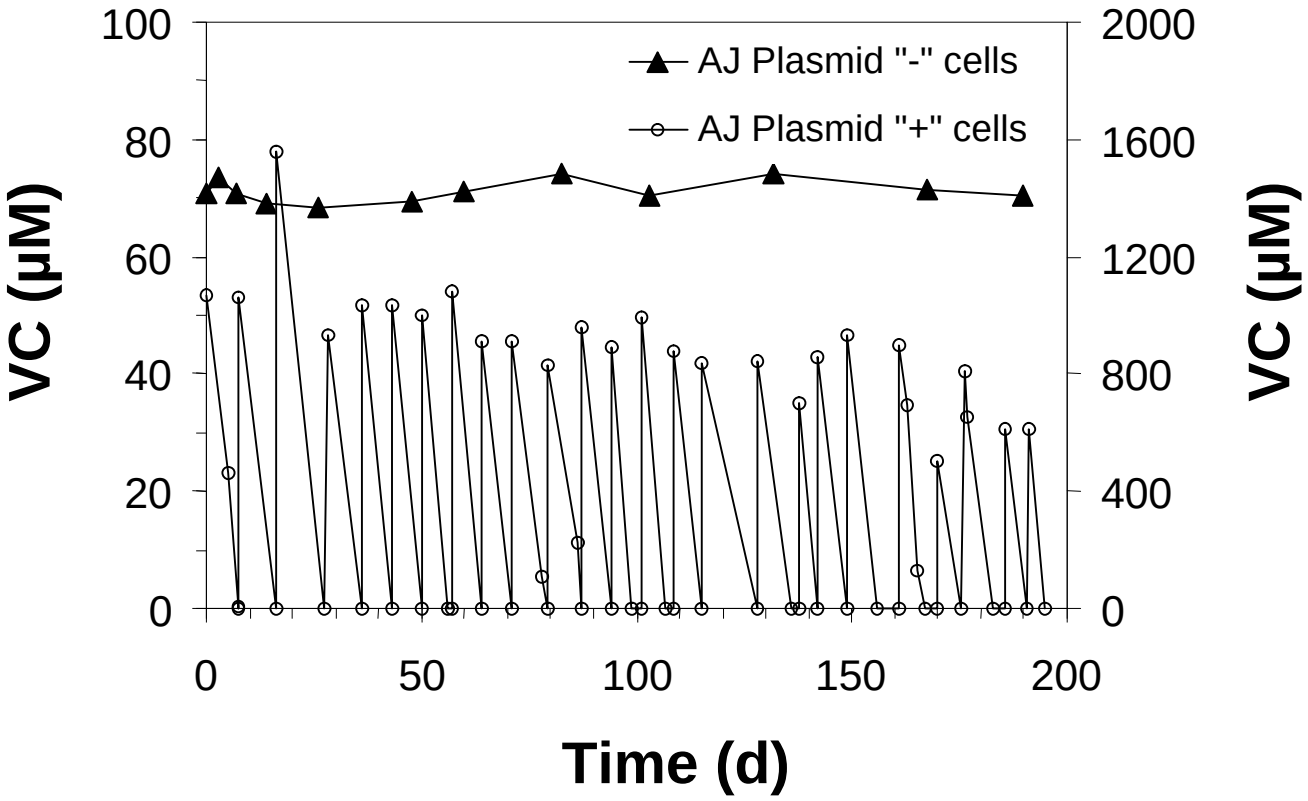

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