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Contrasting regulatory effects of organic acids on aerobic vinyl chloride biodegradation in etheneotrophs

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Abstract

Vinyl chloride (VC) is a common groundwater pollutant generated during anaerobic biodegradation of chlorinated solvents (e.g., trichloroethene (TCE) or tetrachloroethene (PCE)). Aerobic VC biodegradation by etheneotrophs can support anaerobic PCE and TCE bioremediation to achieve complete removal in situ. However, anaerobic bioremediation strategies necessitate biostimulation with electron donors that are fermented in situ, generating organic acids that could influence aerobic VC biodegradation processes. We examined the effect of organic acids (lactate, acetate, propionate, and butyrate) on aerobic VC biodegradation by VC-assimilating etheneotrophs *Mycobacterium* strain JS60 and *Nocardioides* strain JS614. Strain JS60 grew on all organic acids tested, while strain JS614 did not respond to lactate. VC-grown strain JS60 fed VC and one or more organic acids showed carbon catabolite repression (CCR) behavior where VC biodegradation occurred only after organic acids were depleted. In contrast, CCR was not evident in VC-grown strain JS614, which degraded VC and organic acids simultaneously. Acetate-grown JS60 showed similar CCR behavior when fed VC and a single organic acid, except that extended lag periods (5–12 days) occurred before VC oxidation ensued. Acetate-grown JS614 fed VC and either acetate or butyrate displayed 5–8 day lag periods before simultaneous VC and organic acid biodegradation. In contrast, acetate-grown JS614 degraded VC and propionate without a significant lag, suggesting a regulatory link between propionate and VC oxidation in JS614. Different global regulatory mechanisms controlling VC biodegradation in the presence of organic acids in etheneotrophs have implications for developing combined anaerobic–aerobic bioremediation strategies at chlorinated ethene-contaminated sites.

Key points

- *With organic acids present, VC utilization was repressed in JS60, but not in JS614*
- *Strain JS60 grew readily on lactate, while strain JS614 did not*
- *Propionate alleviated lag periods for VC utilization in acetate-grown JS614*

Keywords Etheneotroph · Bioremediation · Global regulatory mechanisms · Diauxic growth

Introduction

Vinyl chloride (VC) is a common contaminant in groundwater and soil (ATSDR 2006; Czinnerova et al. 2020) with highly toxic and carcinogenic properties (Brinker et al. 2015) that necessitate its removal from the environment. Substantial amounts of VC can be generated at hazardous waste sites contaminated with chlorinated ethenes (i.e., tetrachloroethene (PCE) and trichloroethene (TCE)) that are undergoing anaerobic dehalogenation (Kielhorn et al. 2000; Richards et al. 2019; Vainberg et al. 2009). Complete anaerobic dehalogenation of PCE and TCE stepwise through intermediates *cis*-dichloroethene and VC to ethene, the desired end-product, is readily achieved under controlled conditions by organohalide-respiring bacteria (OHRB) (Magnuson et al. 1998; Němeček et al. 2020; Saiyari et al. 2018). However, dechlorination of VC to ethene by OHRB is often slower than dechlorination of PCE and TCE to VC in the field (Aulenta et al. 2002), which results in VC accumulation (Blázquez-Pallí et al. 2019; Fennell et al. 2001; Hunkeler et al. 1999; Lorah and Voytek 2004; Maymó-Gatell et al. 2001; Tiehm and Schmidt 2011) and potential escape from anaerobic treatment.

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Anaerobic bioremediation of chlorinated ethenes in groundwater by OHRB requires addition of electron donors such as emulsified vegetable oils or organic acids (e.g., lactate) (Aulenta et al. 2007; Chomsurin et al. 2008; He et al. 2002; Lee et al. 1998) to biostimulate the microbial community and promote OHRB growth (Czinnerova et al. 2020; Dugat-Bony et al. 2012; Peng et al. 2012). These electron donors are partially converted to volatile fatty acids (VFAs, e.g., acetate, propionate, and butyrate) (Aulenta et al. 2007; Isipato et al. 2020; Wu et al. 2012), which are subsequently fermented to produce hydrogen, the electron donor for many OHRB such as *Dehalococcoides mccartyi* (Aulenta et al. 2007; Lee et al. 1998). A current strategy for addressing VC accumulation issues at chlorinated ethene contaminated sites undergoing anaerobic bioremediation is to add electron donors far in excess of stoichiometric requirements to create more reducing conditions, satisfy the demand of competing organisms, and drive complete dechlorination of chlorinated ethenes to ethene (Haluska and Finneran 2021; Lee et al. 1998).

The difficulties associated with achieving complete anaerobic dechlorination of PCE and TCE to ethene in the field necessitate alternative bioremediation strategies such as combined anaerobic/aerobic treatment (Tiehm and Schmidt 2011). Combining anaerobic and aerobic bioremediation at a site is a plausible approach because VC is readily biodegradable under aerobic conditions by a group of bacteria called "etheneotrophs" which can grow on the ethene and/or VC generated during anaerobic treatment as carbon and energy sources (Chuang et al. 2010; Coleman et al. 2002b; Findlay et al. 2016; Freedman and Herz 1996; Jin and Mattes 2008, 2010; Verce et al. 2001). Etheneotrophs could thus be useful in supporting anaerobic bioremediation strategies and achieving complete removal of PCE and TCE from contaminated groundwater (Mattes et al. 2010).

Unfermented electron donor and generated VFAs can migrate downgradient from the injection point with groundwater flow (Ottosen et al. 2021). Etheneotrophs, known to inhabit VC-contaminated groundwater and sediment (Liang et al. 2017; Richards et al. 2019), can grow on organic acids such as VFAs (e.g., acetate) (Coleman and Spain 2003b; Mattes et al. 2007). Thus, the practice of adding excess electron donor to aquifers during anaerobic bioremediation (Haluska and Finneran 2021; Lee et al. 1998) could lead to migration of organic acids into aerobic aquifer regions and influence aerobic VC biodegradation by etheneotrophs that inhabit those regions. How etheneotrophs respond to mixtures of organic acids and VC is not clear, nor are the implications of organic acid presence on aerobic VC biodegradation processes in the field. There are reports of substrate interactions between VC and ethene (Louarn et al. 2006) and between VC, ethene, methane, and 1,2-dichloroethenes (Freedman et al. 2001), but the effect of organic acids on aerobic VC biodegradation has not yet been considered.

Axenic bacterial cultures fed growth substrate mixtures will typically consume a preferred substrate that facilitates the fastest growth before consuming other substrates in a phenomenon called diauxic growth. Diauxic growth behavior in bacteria is controlled by local and global regulatory mechanisms, e.g., induction and carbon catabolite repression (CCR), respectively (Deutscher 2008; Stülke and Hillen 1999). CCR has been investigated in aerobic alkane and aromatic degraders (Doughty et al. 2006; Moratti et al. 2022; Rojo 2009; Yoshikawa et al. 2017). However, our understanding of regulatory mechanisms such as CCR in alkene-oxidizing bacteria is limited (Mattes et al. 2007; Moratti et al. 2022; Taylor et al. 2010).

The purpose of this study was to examine and compare the effects that organic acids have on aerobic VC biodegradation patterns in VC-assimilating etheneotrophs. Two well-characterized strains, *Mycobacterium* sp. strain JS60 and *Nocardioides* sp. strain JS614, were evaluated under conditions that can occur in VC-contaminated groundwater environments undergoing biostimulation of OHRB with electron donors. The initial steps in the aerobic VC biodegradation pathway in strains JS60 and JS614 involve the enzymes alkene monooxygenase (AkMO), which catalyzes the oxidation of VC into the epoxide chlorooxirane, and epoxyalkane:coenzyme M transferase (EaCoMT), which transforms the epoxide into a CoM conjugate for further metabolism (Coleman and Spain 2003a, 2003b; Mattes et al. 2005). The growth patterns that etheneotrophs like JS60 and JS614 display in response to mixtures of VC and organic acids will inform strategies involving combined anaerobic–aerobic bioremediation of chlorinated ethenes in groundwater.

Materials and Methods

Media and chemicals

A minimal salts medium (MSM), prepared as previously described (Coleman et al. 2002a), was used for cell growth. VC (99%, Synquest Labs, Alachua, FL) was used as a carbon and energy source in most experiments. In some experiments, organic acids (i.e., acetate, propionate, lactate, and/or butyrate (≥ 99%, Sigma-Aldrich, St. Louis, MO)) were added as aqueous solutions of their sodium salts. Ethene (99.5%, Specialty Gases of America, Toledo, OH) was also used.

Bacterial strains, experimental setup, and cell density analyses

VC- and ethene-assimilating *Mycobacterium* strain JS60 (ATCC® BAA-494™) and *Nocardioides* sp. strain JS614 (ATCC® BAA-499™) were grown on VC (150 µmol) and/or acetate (150 µmol) in sterile 160 mL Wheaton® serum

bottles containing 50 mL of sterile MSM. Once the optical density at 600 nm (OD_{600}) of the cultures, as measured with a spectrophotometer, reached ≈ 0.1, cells were then diluted without harvesting and washing into 160 mL bottles with 50 mL sterile MSM to an initial target OD_{600} ≈ 0.01 to start the experiments. All bottles were set up with air containing 21% oxygen in the headspace. Serum bottles were sealed with butyl rubber stoppers and aluminum crimp caps and incubated at 20 °C with orbital shaking at 200 rpm. Bottles were then fed VC (75 µmol–150 µmol), single organic acids (75 µmol–150 µmol), or mixtures of VC, ethene, and organic acids (75 µmol–150 µmol). OD_{600} measurements were routinely recorded to track culture growth over time.

Oxygen demand of the substrates

Because serum bottles were constructed with air in the 110 ml headspace, the amount of oxygen present initially in each serum bottle (headspace + liquid) was approximately 975 µmol. The theoretical chemical oxygen demand (COD) of each substrate used (in terms of µmol COD/µmol substrate) is: 2.5 (VC); 3.0 (ethene); 2.0 (acetate); 3.25 (lactate); 3.75 (propionate); and 5.5 (butyrate). The highest COD of any treatment in this study was 825 µmol (150 µmol butyrate added) which was lower than the total amount of oxygen available initially in each bottle.

Protein analyses

Biomass was measured as total protein to estimate cell yields and maximum substrate utilization rates. To determine protein concentrations, 0.45 ml culture samples were mixed with 0.15 ml of 10 M NaOH and heated for 10 min at 90℃. After cooling the mixture, 0.4 ml of neutralizing solution (3:5 ratio of HCl (10 M) and MSM) was added, followed by centrifuging at 16,000 × g for 5 min. The absorbance of the supernatant was measured at 230 nm and 260 nm and the Eq. $(183 \times A_{230})–(75.8 \times A_{260})$ was used to calculate the protein content in µg/mL as described previously (Coleman et al. 2002a). Calibration curves showing the relationship between OD_{600} and protein concentrations in pure cultures were developed for estimating protein concentrations from OD_{600} readings (Figure S1).

VC and ethene analyses

VC and ethene in headspace samples (100 µl), collected with a gastight syringe, were analyzed by gas chromatography (Agilent 6890) on a Supelco 1% SP-1000 Carbopack B column (6 ft × 1/8-inch diameter, 60/80 mesh), using a flame ionization detector (GC-FID) with separation at 90 °C isothermal. The gas standard curve used for quantifying VC and ethene concentration was developed in serum bottles (30 mL) with known masses of VC, ethene, and methane added (Table S1). Aqueous VC and ethene concentrations were estimated with Henry's law: $Hc = \frac{C_g}{C_w}$ where H_c is the pseudo-dimensionless Henry's coefficient, C_w is the aqueous concentration of the analyte, and C_g is the headspace concentration of the analyte determined by GC-FID. H_c for VC is 0.91 at 20 °C (Gossett 1987) and H_c for ethene is 7.24 at 20 °C (Coleman et al. 2002b). The gas and aqueous concentrations and H_c values were used to determine the total mass of VC and ethene in the bottles using a mass balance.

The specific utilization rates of VC and ethene were estimated by developing a linear regression of VC or ethene concentrations over three consecutive time points (x-1, x, and x + 1). The raw VC and ethene utilization rates (as determined by the slope of the linear regression line) were normalized by the protein concentration at point x as described previously (Choi et al. 2021). The 95% confidence intervals of linear regressions used to estimate the substrate utilization rate and the growth yield were performed using GraphPad Prism 9 software. VC and ethene growth yields were estimated from substrate depletion data and protein values as described previously (Coleman et al. 2002a).

Organic acid analyses

Liquid samples (1 mL) were passed through 0.2 µm filters and organic acids (lactate, acetate, propionate, and butyrate) were analyzed by ion chromatography (Dionex ICS-2100), consisting of an AS-11 column (250 × 4 mm) with an AG11 guard column. The column temperature was 30℃, the cell temperature was 35℃, and the suppressor was set at 41 mA. The eluent flow rate was 1.1 ml/min. The eluent concentration was initially 1 mM KOH with a ramp to 20 mM at 16 min and then 60 mM KOH at 18 min in a total run time of 20 min. Growth yields and maximum utilization rates for organic acids were estimated as described above for VC and ethene.

Results

Biodegradation of VC by *Mycobacterium* sp. strain JS60 and *Nocardioides* sp. strain JS614

Strains JS60 and JS614 both grow aerobically on VC as a carbon and energy source (Chuang and Mattes 2007; Coleman et al. 2002b; Coleman and Spain 2003b). Comparing aerobic VC biodegradation as a sole substrate by VC-grown strains JS60 and JS614 in separate bottles (initial OD_{600} = 0.01) revealed that strain JS614 consumed VC (150 µmol) more quickly and also generated more biomass than strain JS60 (Figure S2). The estimated strain JS614 specific VC utilization rate (18.6 nmol/min-mg protein) was lower than that of strain JS60 (30.8 nmol/min-mg

protein) in this experiment, while the VC growth yield of strain JS614 (12.78 g protein/mol VC) was approximately twice that of strain JS60 (5.55 g protein/mol VC) (Table 1). A higher VC growth yield explains why JS614 degraded VC more quickly than JS60 in this experiment (Fig. S2) despite the lower specific VC utilization rate. This VC only experiment provides a reference point for comparing specific utilization rates and yields for JS614 and JS60 using mixtures of VC and organic acids in subsequent experiments.

Organic acid metabolism by VC-grown strains JS60 and JS614

VC-grown strain JS60 grew aerobically on lactate, acetate, propionate, or butyrate when fed these organic acids individually (Figure S3). VC-grown strain JS614 also grew aerobically on acetate, propionate, and butyrate but did not readily grow on lactate within the experimental time frame (Figure S3A). Aerobic growth of strains JS60 and JS614 on VFAs (acetate, propionate, and butyrate) (150 µmol) (Figure S3B–D) is consistent with observations made in previous studies (Coleman et al. 2002b; Coleman and Spain 2003b; Mattes et al. 2007; Taylor et al. 2010). Strain JS60 displayed much higher estimated maximum utilization rates for organic acids (147–491 nmol/min-mg protein) than strain JS164 (30.2–49.3 nmol/min-mg protein) (Table 1). However, the growth yields of strain JS60 on organic acids (6.39 g–17.4 g protein/mol) were slightly lower than strain JS614 (8.33–28.7 g protein/mol), except for lactate (Table 1).

Table 1 Estimated maximum substrate utilization rates and growth yields with a sole substrate by VC-grown strains JS60 and JS614. A "-" indicates that the substrate utilization rate and growth yield was not estimated because JS614 did not respond to lactate during the experimental period

Carbon source	Maximum substrate utilization rate (nmol/min/mg of protein)[a, b]		Growth yield (g of protein/mol of substrate) [a, b]	
	JS60	JS614	JS60	JS614
VC	30.8 ± 1.2[c]	18.6 ± 5.6	5.55 ± 0.4[d]	12.8 ± 1.0
Lactate	491 ± 202	-	13.6 ± 1.7	-
Acetate	201 ± 33.5	30.5 ± 9.1	6.39 ± 1.2	8.33 ± 1.8
Propionate	181 ± 15.4	49.3 ± 8.0	12.3 ± 2.6	14.9 ± 1.1
Butyrate	147 ± 62.7	30.2 ± 13.3	17.4 ± 3.2	28.7 ± 1.5

[a]The theoretical chemical oxygen demand (COD) of each substrate used (in terms of µmol COD/µmol substrate) is: 2.5 (VC); 3.0 (ethene); 2.0 (acetate); 3.25 (lactate); 3.75 (propionate); and 5.5 (butyrate)

[b]Assuming cells are 55% protein and 1.42 mg COD/mg cells, the conversion factor is 2.58 mg COD/mg protein

[c]Values are the average of two replicates ± 95% confidence intervals of the substrate utilization rate linear regression

[d]Values are the average of two replicates ± 95% confidence intervals of the growth yield linear regression

VC biodegradation by VC-grown strains JS614 and JS60 in the presence of organic acids

We hypothesized that a CCR mechanism in both JS60 and JS614 cultures fed VC-organic acid mixtures would repress VC biodegradation in the presence of organic acids (i.e., they will display a diauxic growth pattern). This hypothesis was tested using VC-grown strain JS60 and JS614 cultures to ensure VC biodegradation enzymes (i.e., AkMO and EaCoMT) were induced at the start of the experiment.

VC-grown strains JS60 and JS614 were subsequently fed VC (75 µmol/bottle) and an individual organic acid (75 µmol/bottle). CCR behavior in VC-grown strain JS60 was evident with a mixture of lactate and VC as lactate was consumed initially in two days followed by VC three days later (Fig. 1A). As expected, VC-grown strain JS614 did not utilize lactate in the VC-lactate mixture, and degraded VC without any apparent inhibition by the presence of lactate (Fig. 1B).

When fed a mixture of VC and acetate, VC-grown strain JS60 consumed 75 µmol acetate in one day and then initiated VC consumption after a 1-day lag period (Fig. 2A). Similarly, VC-grown strain JS60 consumed propionate and butyrate within 2 days and then switched to VC utilization almost immediately (Figs. 2C, 2E). In contrast, VC-grown strain JS614 did not display CCR behavior and instead depleted VC and acetate simultaneously, consuming both substrates after 5 days (Fig. 2B). A similar substrate utilization pattern was observed when VC-grown strain JS614 was fed mixtures of VC and propionate or butyrate (Figs. 2D and 2F).

When consuming VC and VFAs simultaneously, VC-grown strain JS614 utilized VC at consistently slower rates (13.4–14.2 nmol/min-mg protein) (Table 2) than when VC was the sole substrate (18.6 nmol/min-mg protein) (Table 1). However, after initially utilizing organic acids, strain JS60 also subsequently utilized VC at similar or slightly lower rates (18.3–31.9 nmol/min-mg protein) (Table 2) than when VC was the sole substrate (30.6 nmol/min-mg protein) (Table 1). In the case of VC-grown JS60, apparent acceleration of VC biodegradation rates (before normalization to biomass) after growth on organic acids, as compared to growth on VC as sole substrate (Fig. S1A), could be explained by the initial growth on organic acids generating more active biomass for subsequent VC utilization.

After utilizing organic acids, the estimated VC growth yield of strain JS60 was variable (4.3–8.83 g protein/mol) (Table 2), but comparable to the estimated VC growth yield of strain JS60 when VC was the sole substrate (5.55 g protein/mol) (Table 1). In the presence of VC, the estimated JS60 growth yields on organic acids (6.65–9.94 g protein/mol) (Table S2) were lower than

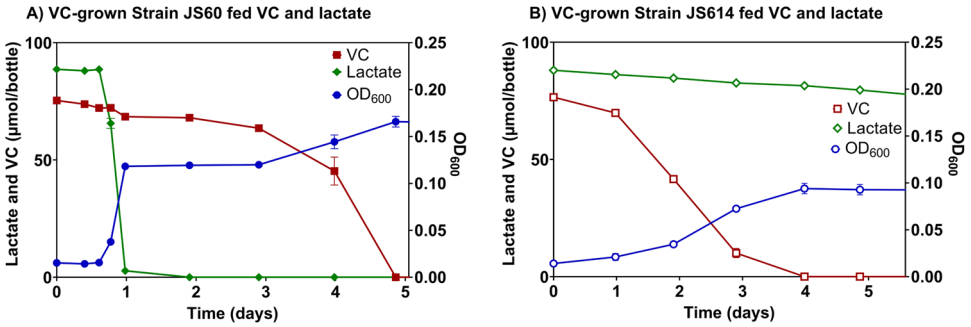

Fig. 1 Effect of lactate on VC biodegradation by (**A**) VC-grown *Mycobacterium* strain JS60 and (**B**) *Nocardioides* strain JS614 cultures. Both cultures were fed a mixture of VC (75 µmol) and lactate (75 µmol). Data points are the average of two replicates and the error bars show the standard deviation. To convert VC mass units (µmoles) to aqueous VC concentrations multiply by 6.66 (for µM) or 0.416 (for mg/L)

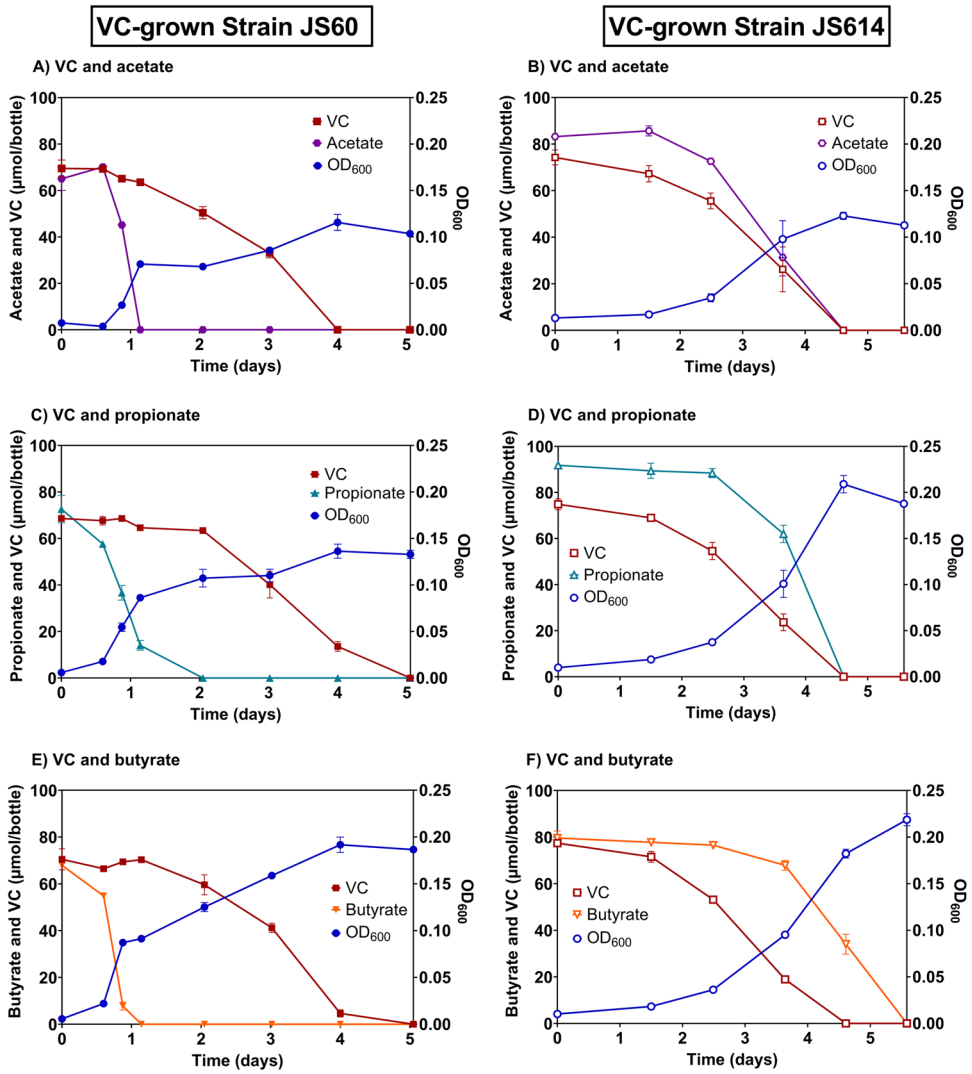

Fig. 2 Effect of VFAs on VC biodegradation by VC-grown *Mycobacterium* strain JS60 and *Nocardioides* strain JS614. VC-grown cultures were fed with an initial mixture of a single VFA (75 µmol acetate, propionate, or butyrate) and VC (75 µmol). (**A**) VC-grown strain JS60 fed acetate and VC; (**B**) VC-grown strain JS614 fed acetate and VC; (**C**) VC-grown strain JS60 fed propionate and VC; and (**D**) VC-grown strain JS614 fed propionate and VC; (**E**) VC-grown strain JS60 fed butyrate and VC; and (**F**) VC-grown strain JS614 fed butyrate and VC. Data points are the average of two replicates and the error bars show the standard deviation. To convert VC mass units (µmoles) to aqueous VC concentrations multiply by 6.66 (for µM) or 0.416 (for mg/L)

those when JS60 was fed those same organic acids alone (6.39–17.37 g protein/mol) (Table 1). Estimated growth yields of VC-grown strain JS614 simultaneously utilizing VC plus an organic acid ranged from 7.15–13.53 g protein/mol (Table 2). These yields were lower than observed with any substrate alone (Table 1).

Table 2 Estimated maximum VC utilization rates (nmol/min-mg protein) and estimated growth yield during the VC consuming period (g protein/mol) with mixtures containing both VC and an organic acid (lactate, acetate, propionate, butyrate) by VC-grown and acetate-grown strains JS60 and JS614

Treatment	Maximum substrate utilization rate (nmol/min/mg of protein)[a,b]				Growth yield (g of protein/mol of substrate)[a,b]			
	VC-grown		Acetate-grown		VC-grown		Acetate-grown	
	JS60	JS614	JS60	JS614	JS60	JS614	JS60	JS614
VC + lactate	22.8 ± 10.0[c]	13.4 ± 6.3	10.5 ± 3.8	24.6 ± 8.9	4.7 ± 0.4[d]	8.99 ± 1.7	5.81 ± 0.7	8.95 ± 1.5
VC + acetate	31.9 ± 4.7	14.2 ± 4.7	14.3 ± 3.5	19.1 ± 8.1	6.48 ± 1.7	7.15 ± 1.1	5.91 ± 0.7	7.47 ± 1.3
VC + propionate	24.2 ± 3.1	13.7 ± 2.5	15.4 ± 3.3	10.2 ± 4.8	4.3 ± 3.9	11.72 ± 0.8	5.7 ± 1.5	12.87 ± 1.1
VC + butyrate	18.3 ± 5.6	14.0 ± 2.9	20.2 ± 2.8	11.8 ± 3.8	8.83 ± 2.2	13.53 ± 0.7	5.77 ± 0.9	14.85 ± 2.4

[a] The theoretical chemical oxygen demand (COD) of each substrate used (in terms of µmol COD/µmol substrate) is: 2.5 (VC); 3.0 (ethene); 2.0 (acetate); 3.25 (lactate); 3.75 (propionate); and 5.5 (butyrate)

[b] Assuming cells are 55% protein and 1.42 mg COD/mg cells, the conversion factor is 2.58 mg COD/mg protein

[c] Values are the average of two replicates ± 95% confidence intervals of the substrate utilization rate linear regression

[d] Values are the average of two replicates ± 95% confidence intervals of the growth yield linear regression

VC biodegradation in the presence of organic acids by acetate-grown strains JS614 and JS60

Experiments described thus far used VC-grown cells to ensure that VC biodegradation enzymes (i.e., AkMO) were induced when cultures were exposed to VC-organic acid mixtures. However, VC-assimilating bacteria living in contaminated subsurface environments could also encounter conditions that repress VC biodegradation gene and enzyme expression, such as transiently high VFA concentrations in the absence of VC or ethene. Previous RT-PCR and RT-qPCR experiments indicate that growth on acetate does not induce VC biodegradation genes (i.e., genes encoding AkMO and EaCoMT) in both strains JS60 (Coleman and Spain 2003a) and JS614 (Mattes et al. 2007, 2005, 2015). To explore these potentially repressing conditions in a laboratory setting, we investigated the response of acetate-grown strain JS60 and strain JS614 cultures to mixtures of VC (75 µmol/bottle) and a single organic acid (75 µmol/bottle).

As observed with VC-grown strain JS60 cultures fed VC and organic acids, acetate-grown strain JS60 displayed CCR behavior (i.e., diauxic growth patterns) when fed a VC-lactate mixture (Figure S4A) and when fed VC-acetate, VC-propionate, and VC-butyrate mixtures (Fig. 3A, C, and E). Acetate-grown strain JS60 cultures fed VC with an organic acid immediately consumed the organic acid but in contrast to VC-grown JS60 cultures, displayed longer lag periods prior to initiating growth on VC. The length of the lag period varied with the organic acid present (i.e., lactate—9 days, acetate—12 days, propionate—5 days, butyrate—9 days) (Fig. S4A, 3A, 3C, and 3E).

Once VC biodegradation initiated, specific VC utilization rates in acetate-grown JS60 cells were lower (9.7–20.2 nmol/min-mg protein) than in VC-grown JS60 cells fed VC and an organic acid (18.3–31.9 nmol/min-mg protein) (Table 2) and when VC was the sole substrate (30.6 nmol/min-mg protein) (Table 1). The lower utilization rates could perhaps be caused by only a fraction of the acetate-grown biomass switching to growth on VC. The range of estimated VC growth yield (5.7–5.91 g protein/mol) in acetate-grown strain JS60 fed VC and an organic acid (Table 2) was very similar to the estimated VC growth yield of strain JS60 when VC was the sole substrate (5.55 g protein/mol) (Table 1). These yield estimates indicate that initial growth on organic acids has no effect on subsequent VC growth yield in strain JS60.

As previously observed with VC-grown strain JS614 cultures, acetate-grown strain JS614 did not utilize lactate when fed a VC-lactate mixture (Fig. S4B). Like acetate-grown JS60 cultures, acetate-grown JS614 cultures fed VC with C2 and C4 organic acids (i.e., acetate and butyrate) displayed variable extended lag periods (acetate—8 days; butyrate—5 days) prior to utilizing VC,

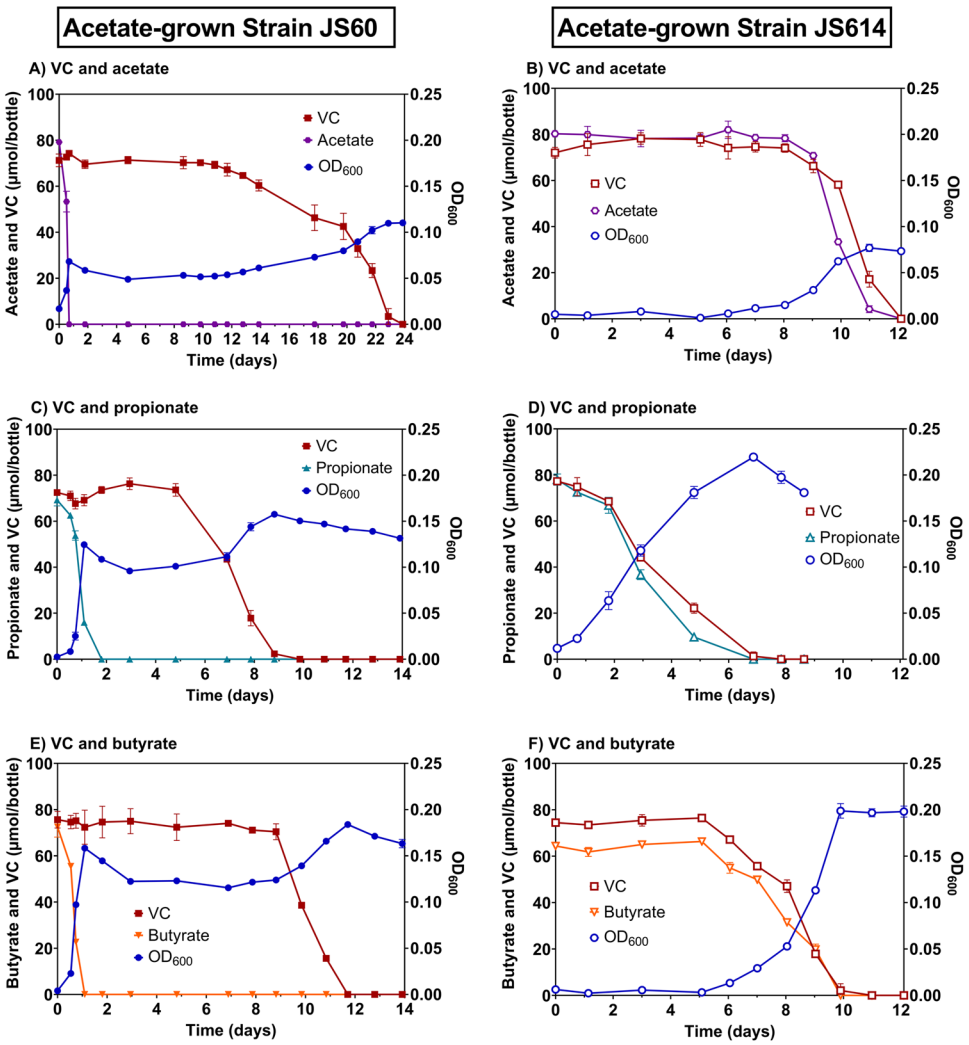

Fig. 3 Effect of VFAs on VC biodegradation by acetate-grown *Mycobacterium* strain JS60 and *Nocardioides* strain JS614. Acetate-grown cultures were fed with an initial mixture of a single VFA (75 µmol acetate, propionate, or butyrate) and VC (75 µmol). (**A**) Acetate-grown strain JS60 fed acetate and VC; (**B**) acetate-grown strain JS614 fed acetate and VC; (**C**) acetate-grown strain JS60 fed propionate and VC; and (**D**) acetate-grown strain JS614 fed propionate and VC; (**E**) VC acetate-grown strain JS60 fed butyrate and VC; and (**F**) acetate-grown strain JS614 fed butyrate and VC. Data points are the average of two replicates and the error bars show the standard deviation. To convert VC mass units (µmoles) to aqueous VC concentrations multiply by 6.66 (for µM) or 0.416 (for mg/L)

which was degraded simultaneously with the added VFA (Figs. 3B and 3F). However, acetate and butyrate were readily consumed by acetate-grown JS614 cells in the absence of VC (Fig. S5). Conversely, acetate-grown strain JS614 fed VC and propionate did not display an extended lag period and consumed the VC and the propionate simultaneously (Fig. 3D).

Acetate-grown strain JS614 cells simultaneously utilizing VC plus a VFA displayed a wider range of estimated utilization rates (10.2–24.6 nmol/min-mg protein) than VC-grown JS614 (13.4–14.2 nmol/min-mg protein; Table 2). Growth yields in acetate-grown JS614 utilizing both VC and VFAs (7.47–14.85 g protein/mol) were like those observed in VC-grown strain JS614 (7.15–13.53 g protein/mol; Table 2), but lower than observed with any VFA alone (8.33–28.7 g protein/mol; Table 1). Thus, JS614 growth yields were affected by simultaneous degradation of a VFA and VC after growth on acetate, most likely because VC was being used as a carbon source serving to decrease apparent growth yields in the presence of VFAs.

VC biodegradation by VC-grown strains JS614 and JS60 in the presence of ethene and multiple organic acids

Aerobic VC-assimilating bacteria inhabiting a chlorinated-ethene contaminated site biostimulated with electron donor will encounter VC, ethene, and multiple organic acids simultaneously. We investigated the effect of multiple carbon sources on VC biodegradation by VC-grown strains JS614 and JS60 (initial $OD_{600} = 0.01$). We hypothesized that in mixtures of 6 carbon sources (25 µmol each of VC, ethene, lactate, acetate, propionate, and butyrate), VC-grown strains JS60 and JS614 would show similar growth patterns as observed in the presence of a single organic acid (i.e., strain JS60 will display a diauxic growth pattern, and strain JS614 will consume all substrates simultaneously).

As expected, VC-grown strain JS60 displayed CCR in the presence of multiple carbon sources. Strain JS60 first consumed all organic acids in about 1 day and then

degraded ethene in 2 days prior to initiating VC utilization (Fig. 4A). In contrast, VC-grown strain JS614 degraded VC, ethene, and VFAs simultaneously and without a lag period, and still did not utilize lactate. VC and ethene were consumed first (in about 3 days) while the VFAs were consumed later, after 3–5 days (Fig. 4B). Strain JS614 completely degraded 25 µmol VC more quickly (3 days) than strain JS60 (5 days) in the presence of multiple carbon sources (Fig. 4). This finding differs from VC biodegradation patterns in VC-grown strains JS614 and JS60 in the presence of a single organic acid. This suggests that while the presence of a single organic acid can appear to accelerate VC degradation in strain JS60 by increasing active biomass concentration, the presence of multiple carbon sources delays the onset of VC utilization. In contrast, the presence of multiple carbon sources seems to improve the rate of VC biodegradation by strain JS614.

Discussion

Migration of excess electron donor (e.g., organic acids such as lactate and generated VFAs) from anaerobic chlorinated ethene treatment zones (Ottosen et al. 2021) into aerobic aquifer regions inhabited by etheneotrophs could influence supporting aerobic VC biodegradation processes. Thus, a better understanding of the impact of organic acids on aerobic VC biodegradation processes could inform bioremediation strategies such as combined aerobic/anaerobic treatment (Tiehm and Schmidt 2011). Although regulatory and CCR mechanisms in microorganisms are relevant to the success of field-scale bioremediation strategies, they are seldom investigated in that context (Mattes et al. 2007; Moratti et al. 2022; Taylor et al. 2010). Our findings could inform future combined anaerobic/aerobic bioremediation strategies that support complete degradation of PCE and TCE and alleviate VC accumulation at chlorinated ethene contaminated sites (Mattes et al. 2010).

The mass of VC, ethene, and organic acids added to bottles correspond to maximum initial aqueous concentrations of 31–62 mg/L (VC), 0.8 mg/L (ethene), 8.9 mg/L (acetate), 11.1 mg/L (propionate), 13.2 mg/L (butyrate), and 13.5 mg/L (lactate). The initial VC (and ethene) concentrations used in these experiments are generally higher than typically observed in groundwater, although VC concentrations as high as 50 mg/L and ethene concentrations up to 4 mg/L have been measured in situ (Liang et al. 2017). The relatively high mass of VC provided in this study was done so to provide sufficient VC to investigate cell growth and substrate utilization under laboratory conditions and so that kinetic and yield parameter estimation can be performed. While in situ VFA and lactate concentrations are not typically measured and reported, in one field study in situ VFA concentrations of up to 100 mg/L were observed downgradient at least 271 days after injection (Ottosen et al. 2021), thus the organic acid concentrations used here could be considered environmentally relevant.

The experiments in this study were also performed under fully aerobic conditions. However, dissolved oxygen (DO) concentrations may be very low at the periphery of chlorinated solvent plumes undergoing biostimulation. Several strains of etheneotrophs, including JS60 and JS614, display very low oxygen half-saturation constants (Ks values; 0.03–0.3 mg/L) when grown on VC (Coleman et al. 2002b). Oxygen Ks values have not been determined for etheneotrophs growing on VFAs. Because VC metabolism requires oxygen as a reactant for the initial epoxidation as well as a terminal electron acceptor, while VFA metabolism only requires oxygen as a terminal electron acceptor, the oxygen Ks values for growth on VFAs are likely the same or lower as those seen with growth on

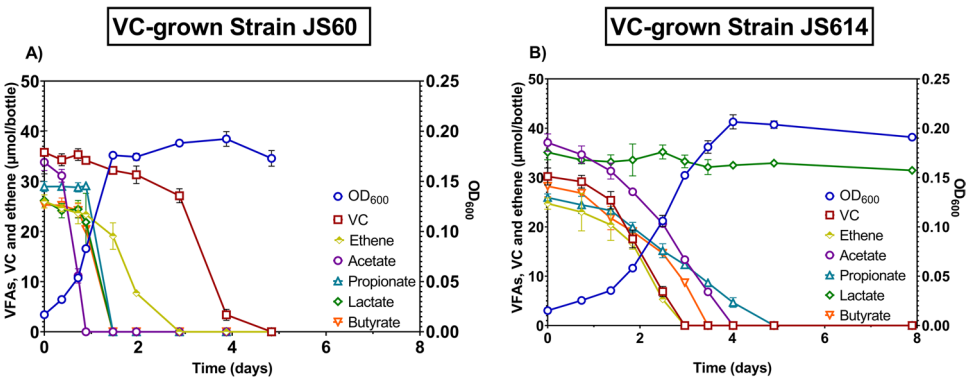

Fig. 4 VC biodegradation in the presence of organic acids and ethene by A) VC-grown strain JS60 and B) VC-grown strain JS614. VC-grown cultures were fed with an approximately equimolar mixture (25–35 µmol each) of multiple organic acids (lactate, acetate, propionate, and butyrate), ethene and VC. Data points are the average of two replicates and the error bars show the standard deviation. To convert VC mass units (µmoles) to aqueous VC concentrations multiply by 6.66 (for µM) or 0.416 (for mg/L). To convert ethene mass (µmoles) to aqueous ethene concentrations multiply by 1.18 (for µM) or 0.033 (for mg/L)

VC. This suggests that oxygen concentrations will have little impact on VC and VFA biodegradation kinetics under even microaerophilic conditions (DO ~ 1 mg/L).

The experiments conducted here were performed in batch reactors while the data were interpreted in the context of a groundwater systems, where water is flowing continuously. This is reasonable because reaction kinetics in plug flow reactors, which are used to model groundwater flow in porous media, are essentially the same as in batch reactors. Because both VC and the VFAs are soluble in water with little affinity for organic matter, these chemicals will likely migrate with the groundwater at similar rates. Thus, it is realistic for etheneotrophs to encounter mixtures of VC and VFAs in the environment, particularly near the aerobic fringe of a chlorinated solvent plume. However, when oxygen is less limiting in a contaminated groundwater environment, other indigenous heterotrophic bacteria would likely rapidly consume VFAs before the VC, possibly allowing VC to travel farther down gradient where competition between these substrates is no longer a concern.

We found that pure VC-assimilating JS60 and JS614 cultures displayed different VC biodegradation patterns in the presence of organic acids. We speculate that these different patterns can be explained by different global regulatory mechanisms controlling VC biodegradation pathways in strains JS60 and JS614. Strain JS60 displayed classic CCR behavior in the presence of VC and organic acids, while strain JS614 ignored lactate, and consumed VFAs and VC simultaneously. The finding that VC-assimilating etheneotrophs respond differently to lactate is relevant for field applications where lactate is used as an electron donor to stimulate anaerobic bioremediation of chlorinated ethenes (Lee et al. 1998) and where aerobic polishing of VC is desired.

Even though strain JS60 consumed the same amount of VC faster than strain JS614 in the presence of a single organic acid after a diauxic growth phase, strain JS614 consumed VC more quickly than JS60 in the presence of ethene and multiple organic acids. These results suggest that when VFAs are present, aerobic VC degradation is inhibited in strain JS60 but not in strain JS614. However, since JS60 can grow rapidly on VFAs, the increase in active biomass could facilitate a greater VC removal capacity by "JS60-like" etheneotrophs in situ once VFAs are exhausted.

Further, the observation that VC-grown strain JS614 consumes VC at slightly lower rates with lower VC growth yields in the presence of organic acids (as compared to VC alone) suggests that organic acids are not only used as carbon sources for growth in the presence of VC, but could also be used as energy sources to maintain AkMO activity (Mattes et al. 2007, Mattes et al. 2005) albeit at lower rates. This behavior could be useful in field applications if JS614-like etheneotrophs are present, in that VFAs generated during anaerobic treatment or possibly added exogenously could be useful for maintaining AkMO activity for VC oxidation.

Both JS60 and JS614 displayed extended lag periods before initiating VC biodegradation after growth on a VFA (i.e., acetate), with exception of strain JS614 fed propionate. Extended lag periods (10–12 days) prior to VC biodegradation in acetate-grown JS614 cultures have been observed in a previous study of VC starvation behavior in JS614 (Mattes et al. 2007). In that study, adding 1 mM acetate alleviated the VC lag period in starved VC-grown cells, but not in acetate-grown cells (Mattes et al. 2007). Surprisingly in the present study, and in contrast to acetate-grown JS60 cultures, acetate-grown JS614 cells fed VC plus acetate or butyrate did not consume either VFA for the duration of the lag period. This indicates that the presence of VC inhibits acetate and butyrate utilization in acetate-grown JS614 cells.

The alleviation of the extended lag period for VC utilization in acetate-grown JS614 cells fed VC and propionate suggests that an active propionate biodegradation pathway benefits AkMO induction in JS614. A similar regulatory phenomenon was observed in alkane-degrading *Thauera butanivorans*, which utilizes C2 to C9 alkane substrates with a soluble butane monooxygenase (sBMO)(Dubbels et al. 2009). Experiments with a *T. butanivorans* sBMO reporter strain, constructed so that the sBMO promoter controls beta-galactosidase expression, showed that growth on acetate and butyrate induced beta-galactosidase (i.e., induced sBMO) primarily in response to even-chain-length alcohols (C2 to C8). However, sBMO induction in response to both even- (C2 to C8) and odd-chain-length (C3 to C7) alcohols was observed in the propionate-grown *T. butanivorans* sBMO reporter strain (Doughty et al. 2006). A primary conclusion of that work was that an induced propionate pathway overcomes propionate-dependent sBMO repression in *T. butanivorans*. Our results suggest that AkMO induction in strain JS614 is similarly linked as propionate degradation by strain JS614 is readily induced in the presence of VC, while acetate or butyrate is not. The apparent linkage between propionate and VC metabolism in strain JS614 suggests that propionate could be useful in biostimulating "JS614-like" etheneotrophs in situ.

The pathway encoding AkMO and EaCoMT is carried on a plasmid in both JS60 and JS614 (Coleman and Spain 2003a; Mattes et al. 2005). Prolonged growth on non-alkene substrates has been shown to cure plasmids carrying these genes from other etheneotrophs, resulting in the loss of the ability to degrade VC (Danko et al. 2004; Mattes et al. 2005). Although the plasmid in strain JS614 was cured after extended growth (> 60 days) on non-selective, rich media plates (Mattes et al. 2005), no attempt was made in this study to cure the plasmids from these strains, and it is unclear how they would react to extended growth on VFAs in liquid culture in the absence of VC or ethene.

Application of CCR mechanisms in combined anaerobic/aerobic VC bioremediation strategies would require

demonstrating that etheneotrophs are in contact with both VC and VFAs in groundwater, determining the AkMO and EaCoMT induction status of etheneotrophs, and identifying the predominant CCR mechanism possessed by etheneotrophs in situ. VFAs have been measured in groundwater to evaluate the status of electron donors for reductive dechlorination processes (Ottosen et al. 2021), but generation and transport of VFAs in groundwater have not yet been considered in the context of aerobic VC oxidation. Determining the induction status of etheneotrophs is possible by applying reverse-transcription (RT)-qPCR to groundwater samples. For example, *etnC* and *etnE* expression was observed in over 50% of 95 VC-contaminated groundwater samples (Liang et al. 2017) which suggests that aerobic VC biodegradation genes in etheneotrophs are induced in situ. Determining the predominant CCR mechanism controlling etheneotrophs in situ is more difficult, because current knowledge of the regulation of AkMO and EaCoMT expression in strains JS60 and JS614 and other etheneotrophs is limited (Mattes et al. 2007; Moratti et al. 2022). Whether the CCR behavior in strain JS60 extends to other mycobacterial VC-assimilators and the lack of CCR in strain JS614 is found in other VC-assimilating *Nocardioides* strains is yet to be determined. More work is needed to delineate global regulatory mechanisms in VC-assimilating bacteria and perhaps identify biomarkers of different CCR mechanisms.

However, if we speculate that "JS60-like" mycobacterial VC-assimilators display CCR behavior with VC and organic acid mixtures and "JS614-like" VC-assimilators display simultaneous degradation of VC and organic acids, an analysis of *etnE* diversity in the microbial community might provide some clues as to the predominant etheneotroph CCR mechanism present at a VC-contaminated site. For instance, *etnE* diversity studies have revealed at least two major and distinct *etnE* clades—the "*Mycobacterium* clade" and the "*Nocardioides* clade" (Liu and Mattes 2016). Environmental *etnE* sequences from the *Mycobacterium etnE* clade appear to be more common at VC-contaminated sites (Liu and Mattes 2016), but certain sites (e.g., a contaminated site in Fairbanks, AK) seem to harbor predominantly *Nocardioides*-like VC-assimilators (Liu et al. 2018).

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Author contribution PMR and TEM conceived and designed research. WZ conducted experiments, analyzed data, developed figures and tables, and wrote the initial manuscript. TEM and PMR edited the manuscript. All authors read and approved the manuscript.

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Data availability All processed data are available without restriction upon inquiry.

Declarations

Ethics Approval This article does not report any studies with human participants or animals performed by the authors.

Conflict of interest The authors declare no competing interests.

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