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Kinetics of Bacterial Growth on Chlorinated Aliphatic Compounds

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With the pure bacterial cultures *Ancylobacter aquaticus* AD20 and AD25, *Xanthobacter autotrophicus* GJ10, and *Pseudomonas* sp. strain AD1, Monod kinetics was observed during growth in chemostat cultures on 1,2-dichloroethane (AD20, AD25, and GJ10), 2-chloroethanol (AD20 and GJ10), and 1,3-dichloro-2-propanol (AD1). Both the Michaelis-Menten constants (K_m) of the first catabolic (dehalogenating) enzyme and the Monod half-saturation constants (K_s) followed the order 2-chloroethanol, 1,3-dichloro-2-propanol, epichlorohydrin, and 1,2-dichloroethane. The K_s values of strains GJ10, AD20, and AD25 for 1,2-dichloroethane were 260, 222, and 24 μM , respectively. The low K_s value of strain AD25 was correlated with a higher haloalkane dehalogenase content of this bacterium. The growth rates of strains AD20 and GJ10 in continuous cultures on 1,2-dichloroethane were higher than the rates predicted from the kinetics of the haloalkane dehalogenase and the concentration of the enzyme in the cells. The results indicate that the efficiency of chlorinated compound removal is indeed influenced by the kinetic properties and cellular content of the first catabolic enzyme. The cell envelope did not seem to act as a barrier for permeation of 1,2-dichloroethane.

Halogenated aliphatic compounds are frequent constituents of industrial waste gases. Several halogenated aliphatics are produced in millions of tons annually, mainly for the use of solvents and cleaning agents. Because of the toxic effects that these compounds may have on humans as well as on the natural environment, there is growing interest in technologies for their removal. Biological waste gas purification is an option, provided that efficient elimination of these potentially recalcitrant substrates can be achieved. For this, specialized bacterial strains that are able to use halogenated aliphatics as sole carbon and energy sources can be used (9–11, 16). The elimination efficiency depends not only on the process technology used but also on the degradation properties of the bacterial strains involved. Important aspects of bacterial growth are the Monod half-saturation constant (K_s) and the maximum growth rate (μ_{max}). If in a treatment system the concentration of the growth substrate and K_s value of the dominant strain are in the same order of magnitude, the degree of the elimination is influenced by the K_s value. The kinetic parameters also affect the rate of the removal in biofilms and thus influence reactor design (8).

Data about the kinetic constants of pure cultures growing on halogenated aliphatics are scarce (7). Moreover, the factors that determine the K_s values are unknown. In general, the K_s value of a strain could be influenced by the hydrophobicity of the volatile compound used as growth substrate, by the kinetic properties (K_m and V_{max}) of uptake proteins or catabolic enzymes, and by the concentrations of these enzymes in the cells. To test whether these factors determine K_s values of bacterial cultures degrading volatile chlorinated substrates, several well-studied specialized strains (21, 30, 32) were grown in continuous cultures on 1,2-dichloroethane (DCE), 2-chloroethanol, epichlorohydrin, or 1,3-dichloro-2-propanol.

The pathways of the degradation of these halogenated aliphatic compounds have been elucidated (21, 30). Pure cultures of *Xanthobacter autotrophicus* GJ10 and of *Ancy-*

lobacter aquaticus AD20 and AD25 degrade DCE to 2-chloroethanol by a constitutively produced haloalkane dehalogenase (21, 23, 32) (Fig. 1A). The DNA sequences encoding this enzyme are identical in the three strains (20, 32). 2-Chloroethanol is converted via chloroacetaldehyde to chloroacetic acid by a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase (19, 21, 32). The second dehalogenase, specific for halocarboxylic acids, dechlorinates 2-chloroacetic acid to glycolic acid (21, 32).

Pseudomonas sp. strain AD1, isolated on epichlorohydrin, converts 1,3-dichloro-2-propanol to this epoxide by a haloalcohol dehalogenase (30) (Fig. 1B). Epichlorohydrin is converted to 3-chloro-1,2-propanediol by an epoxide hydrolase (17, 30). Dehalogenation of 3-chloro-1,2-propanediol, catalyzed by the haloalcohol dehalogenase (30, 31), proceeds by the formation of the epoxide glycidol, which may be converted to glycerol by an epoxide hydrolase.

The determination of K_s values in chemostat cultures is considered one of the best methods available (28). In this study, the kinetic properties of the organisms were measured during growth in continuous cultures and were compared with the kinetic properties of the first catabolic enzyme involved in the degradation of the growth substrate.

The results obtained indicate that growth of the strains examined followed Monod kinetics. A low K_s value of a pure culture was correlated with a low K_m value of the first catabolic enzyme. Strain AD25, which overproduced the haloalkane dehalogenase, had a much lower K_s value for growth on DCE. Furthermore, strains AD20 and GJ10 showed growth rates on DCE somewhat higher than predicted from the amount of haloalkane dehalogenase present in the cells.

MATERIALS AND METHODS

Organisms. *X. autotrophicus* GJ10 (21), *A. aquaticus* AD20 (32), and *A. aquaticus* AD25 (32) were maintained on nutrient broth (NB) agar plates or on brain heart infusion (BHI) agar plates. *Pseudomonas* sp. strain AD1 (30) was

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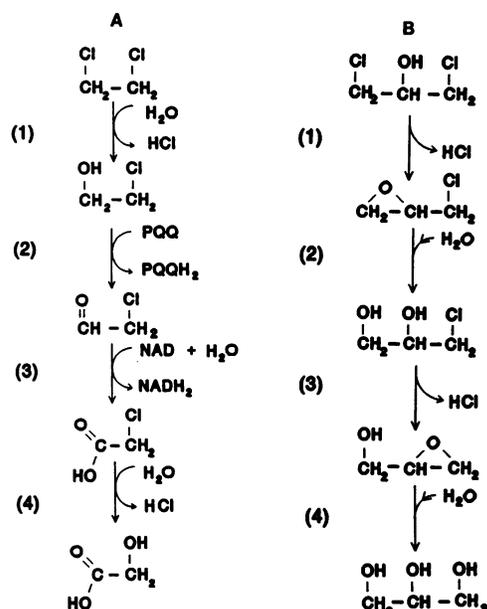


FIG. 1. (A) Degradation pathway of DCE by *X. autotrophicus* GJ10 and *A. aquaticus* AD20 and AD25. Enzymes: 1, haloalkane dehalogenase; 2, alcohol dehydrogenase; 3, aldehyde dehydrogenase; 4, chloroacetic acid dehalogenase. (B) Degradation pathway of 1,3-dichloro-2-propanol by *Pseudomonas* sp. strain AD1. Enzymes: 1 and 3, haloalcohol dehalogenase; 2 and 4, epoxide hydrolase.

maintained on MMY (21) agar plates supplemented with epichlorohydrin.

Media and growth conditions. For routine use, all strains were cultured in MMY medium (21) supplemented with 30 mg of yeast extract (Difco Laboratories) per liter and incubated under conditions described previously (21).

The medium used for continuous cultures was a modified MMY medium (21) that contained, per liter, 0.16 g of KH_2PO_4 , 0.46 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g of $\text{MgSO}_4 \cdot$

$7\text{H}_2\text{O}$, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 5 ml of trace metals solution, and 30 mg of yeast extract. The phosphate buffer was autoclaved after it was brought to pH 5.0 with 6 N H_2SO_4 . The other components were autoclaved separately.

Growth rates were determined by measuring the A_{450} . In case of strain AD25, which grew in flocks, the A_{450} was determined after destruction of the flocks by pulling 8 ml of culture fluid 20 times through a syringe (volume, 10 ml) with a needle that had a diameter of 1 mm (32).

Chloride levels of culture media were measured with an ion-selective electrode (Orion type 94-17) or by the colorimetric assay of Bergmann and Sanik (5).

Continuous cultures. Continuous growth of bacteria was accomplished in a 1-liter fermentor filled with 750 ml of medium. Culture medium was supplied via silicon rubber tubing. When the storage medium was supplemented with 2-chloroethanol (5 mM) or with 1,3-dichloro-2-propanol (6.5 mM), the medium was supplied via viton rubber tubing (Iso-versinic; Rubber B. V., Hilversum, The Netherlands). Culture medium was added to the fermentor with a Cole-Parmer peristaltic pump (model 7554-30). The reactor was stirred by an electronic motor coupled to a double-blade rotor. The stirring rate was 800 rpm unless stated otherwise. The level of the culture fluid in the fermentor was adjusted by a medium outlet (Fig. 2). The chemostat cultures were maintained at pH 7 by automatic addition of sterile 2 N KOH. The reactor was kept at 30°C by a temperature sensor and controller, which was connected to an infrared lamp.

The volatile halogenated compounds DCE and epichlorohydrin were supplied via the gas phase. Air was bubbled through a water column that had a length of approximately 10 cm, to minimize evaporation of medium from the culture vessel. A second airflow was bubbled through a flask containing halogenated substrate (Fig. 2). Both gas flows were adjusted by two independent flow controllers. The total gas flow rate was 900 to 3,500 ml/h, depending on the desired biomass concentration in the fermentor and the dilution rate. The air-substrate flow rate was 60 to 500 ml/h. The ratio of the oxygen/DCE or oxygen/epichlorohydrin mixtures that entered the fermentor was always at least 20:1.

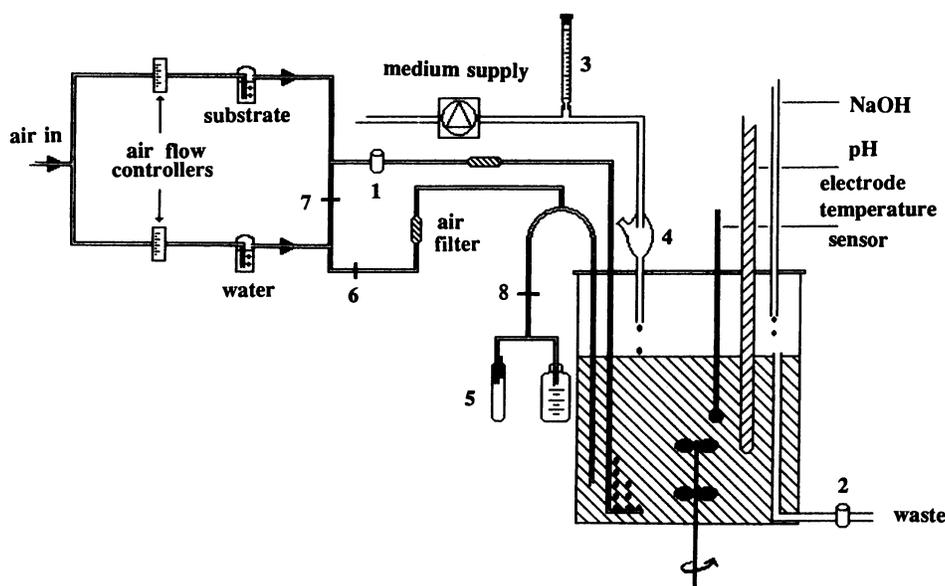


FIG. 2. Continuous culture setup. For explanation, see Materials and Methods.

As shown in Fig. 2, gas samples were taken at point 1 (gas inlet) and point 2 (gas outlet). The dilution rate was measured with a sterile pipet, which was connected at point 3 with the system. At point 4, the medium entered the culture vessel. Before an aqueous sample was taken at point 5, the pipes were cleaned for several seconds with air that contained no volatile substrate. For this, clamp 6 and 8 were opened several seconds, while clamp 7 was closed. Samples of approximately 8 to 12 ml were collected in sterile glass tubes containing 0.5 ml of concentrated phosphoric acid. For this, clamp 6 was closed, and clamp 7 and 8 were opened. The sampling rate was approximately 2 ml/s. In this way, a rapid inactivation of the cells was accomplished.

Acidification of culture fluid containing epichlorohydrin caused hydrolysis of the epoxide and subsequent formation of 3-chloro-1,2-propanediol. To avoid this, aliquots of 4.5 ml of cells grown in chemostat cultures on epichlorohydrin were collected in 1.5 ml of ice-cold diethyl ether that contained 0.05 mM 1-bromohexane as the internal standard.

Cell numbers in chemostat cultures were estimated by dilution of the culture in MMY medium (without yeast extract), after which 0.1-ml aliquots were spread on BHI agar. The colonies formed were counted after 1 week of growth at 30°C.

Growth kinetics. Precultures used for inoculation of continuous cultures were grown from single colonies taken from NB or BHI agar plates. Maximum growth rates were determined in fermentors under batch-fed conditions. The concentration of substrate in the water phase of the culture was checked by gas chromatography and was high enough (8 to 10 times the K_s values) to ensure that the cells were able to grow with μ_{max} .

The Monod half-saturation constant was determined by analysis of substrate concentrations in the water phase of cultures growing at three to five different dilution rates. From these determinations, K_s values were estimated by nonlinear regression analysis (25). Data were collected after at least five volume changes had occurred after adjustment of the dilution rate.

In the case of *Pseudomonas* sp. strain AD1 growing on epichlorohydrin, the K_s value was determined by measuring the substrate concentration in the culture fluid at one dilution rate ($D = 1/2 \mu_{max}$). Cell dry weight was determined by harvesting 75 to 100 ml of culture from a chemostat or from a batch culture. After centrifugation (10,000 $\times g$, 10 min) and resuspension of the cells in 10 ml of ice-cold demineralized water, fractions of 2.5 ml were filtered through a 0.25- μ m-pore-size filter (Schleicher & Schuell). Filters were washed with 2 ml of ice-cold demineralized water, whereafter they were dried at 60°C for several days until a constant weight was reached.

Preparation of extracts and enzyme assays. Crude extracts were prepared as described earlier (21).

The K_m of the haloalcohol dehalogenase of strain AD1 for 1,3-dichloro-2-propanol was determined by analysis of the initial chloride production rates at different substrate concentrations. This was accomplished by continuous measurement of the chloride concentration, using an ion-selective electrode. Incubations were stirred magnetically, and the temperature was kept constant at 30°C by incubation in a water bath. The incubation mixture (volume of 8 ml) consisted of 50 mM sodium phosphate buffer (pH 7.5), 50 to 200 μ l of crude extract, and halogenated substrate. Crude extracts were prepared from cells grown in batch cultures on 1,3-dichloro-2-propanol. Before addition of the enzyme so-

lution, the initial substrate concentration of the solution was checked by gas chromatographic analysis.

The K_m and V_{max} of purified haloalkane dehalogenase for DCE were obtained by determination of the initial rate of 2-chloroethanol formation in screw-cap closed glass tubes containing various concentrations of DCE (0.1 to 4 mM). The reactions in 4.5 ml of Tris-sulfate buffer (50 mM, pH 8.2) were stopped by the addition of 1.5 ml diethyl ether and then subjected to gas chromatographic analysis.

The K_m values of alcohol dehydrogenase for 2-chloroethanol or methanol were determined by using an oxygen electrode as described earlier (19). The activity of the alcohol dehydrogenase was calculated from the oxygen consumption rate. Crude extracts were prepared from cells grown in batch cultures on 2-chloroethanol.

The kinetic parameters K_m and V_{max} of the catabolic enzymes were estimated by nonlinear regression analysis (25) of the initial rates found at different substrate concentrations.

Protein was measured according to the method of Bradford (6).

Gas chromatography. Halogenated compounds were quantitatively determined by capillary gas chromatography analysis. Equipment and temperature program were as described before (21, 30).

Culture samples (4.5 ml) were extracted with 1.5 ml of diethyl ether containing 0.05 mM 1-bromohexane as the internal standard. The upper layer was analyzed by split injection of 1- μ l samples in the gas chromatograph.

The DCE or epichlorohydrin concentrations in the ingoing and outgoing gases of the continuous culture were analyzed by injection of gas samples of 200 μ l in the gas chromatograph. Each DCE or epichlorohydrin concentration determined in the gas phase was the mean of at least four separate gas samples. The maximum variation in the concentration between these samples was less than 10%.

Calibration of DCE or epichlorohydrin in the gas phase was performed by injection of four gas samples of 200 μ l. The samples were taken with a 1-ml syringe from a 1-liter serum flask which contained a known concentration DCE or epichlorohydrin in the gas phase. To ensure that DCE or epichlorohydrin was homogeneously distributed over the gas phase, the flasks were stirred during sampling. The flasks were closed gastight with screw caps containing viton septa.

Chemicals. The chemicals used in this study were all obtained from commercial sources (Janssen Chimica and Aldrich) and were found pure as stated before (21, 30).

RESULTS

Kinetics of DCE degradation of *X. autotrophicus* GJ10. Strain GJ10 was grown in continuous culture under DCE limitation. Because DCE is a volatile compound, it was supplied via the gas phase. The μ_{max} of strain GJ10 on DCE, determined under batch-fed conditions, was 0.104 h⁻¹. The initial concentration of DCE in the water phase of the culture was 2.5 mM. At this concentration, DCE did not inhibit the growth of strain GJ10, as was confirmed by batch culture experiments in which the growth rate of strain GJ10 did not decrease up to 5 mM DCE.

The concentration of DCE in the water phase of the culture was measured during steady states obtained at five different dilution rates. The growth of strain GJ10 followed Monod kinetics. The K_s estimated by nonlinear regression analysis was 260 \pm 54 μ M (Table 1), which is a lower value than reported by others (15).

TABLE 1. Kinetic constants of degradation of chlorinated compounds

Strain	Substrate	μ_{\max} (h ⁻¹)	K_s (μ M)	No. of different steady states	K_m^a (μ M)
GJ10	DCE	0.104	260 \pm 54	4	571
GJ10MR0	DCE	0.060	51 \pm 33	3	571
GJ10MR1	DCE	0.104	81	1	571
GJ10	2-Chloroethanol	0.158	7 \pm 0.5	4	55 \pm 7
AD20	2-Chloroethanol	0.140	4 \pm 1	4	114 \pm 12
AD25	DCE	0.098	24 \pm 4	4	571
AD20	DCE	0.117	222 \pm 35	4	571
AD1	1,3-Dichloro-2-propanol	0.198	12 \pm 1	3	50
	Epichlorohydrin	0.146	8 \pm 1	1	300

^a K_m , Michaelis-Menten constant of the first catabolic enzyme.

According to the Monod equation, the K_s value is equal to the steady-state DCE concentration at a dilution rate of $1/2 \mu_{\max}$. During growth of strain GJ10 at a dilution rate of 0.052 h^{-1} ($1/2 \mu_{\max}$), the concentration of DCE in the water phase was around 279 μ M (Table 2).

The first step in the conversion of DCE by *X. autotrophicus* GJ10 is catalyzed by a haloalkane dehalogenase yielding 2-chloroethanol (21). The K_m of the purified enzyme of strain GJ10 for DCE was 571 μ M (Table 1), which was a lower value than described earlier (23) but was a more reliable result since evaporation of the volatile substrate from the glass tubes could be avoided by the method used here.

The K_s value of strain GJ10 found during growth on DCE was lower than the K_m of the first catabolic enzyme. This correlation between K_s and K_m has also been found with heterotrophic bacteria growing on carbohydrates (13).

Mutants of strain GJ10. Serial transfer of single colonies of strain GJ10 on NB agar plates repeatedly led to the isolation of mutants that showed a reduced growth rate on DCE. Spontaneous DCE-negative mutants were not observed. One of the slowly growing mutants, designated strain GJ10MR0, was studied for its kinetic properties of growth on DCE in a continuous culture.

The μ_{\max} on DCE of strain GJ10MR0 was 0.060 h^{-1} . From four different steady states, a K_s of 51 μ M was calculated (Table 1). During the fifth steady state ($D = 0.048 \text{ h}^{-1}$), a DCE concentration of 232 μ M in the water phase was expected according to Monod kinetics, but a DCE concentration of 105 μ M was found, indicating that a new mutation had developed. The fermentor had been operational at that moment for 10 weeks. Cells of the culture were streaked on NB agar to isolate single colonies. One of the colonies was inoculated in an autoclaved fermentor and grown batch fed

TABLE 3. Growth rates of strains GJ10MR0 and GJ10MR1 determined in batch cultures

Medium	Extra addition	Carbon source ^a	Generation time (h)	
			GJ10MR0	GJ10MR1
MM	Y ^b	DCE	10.6	6.8
MM	Y	2-Chloroethanol	6.8	3.7
MM	Vitamins ^c	2-Chloroethanol	5.7	3.4
MM	Y	Citrate	3.8	2.7
MM	Y	Methanol	5.5	5.0
NB			8.9	5.3

^a Added to a concentration of 4 mM.

^b Y, 30 mg of yeast extract per liter.

^c Added according to reference 21.

with DCE (2 mM). The μ_{\max} of this strain was 0.104 h^{-1} , confirming that a new mutant was isolated. It was named strain GJ10MR1.

Batch culture experiments revealed that the μ_{\max} values of strain GJ10MR1 with 2-chloroethanol, methanol, citrate, and NB were higher than those observed with strain GJ10MR0 (Table 3). From the fact that the growth rates with nonchlorinated compounds had increased too, it can be concluded that the mutations that were responsible for the higher growth rate of strain GJ10MR1 on DCE were not on the level of the DCE catabolic pathway. Moreover, addition of vitamins or yeast extract, or even growth in rich medium (Table 3), still gave higher growth rates for strain GJ10MR1, showing that loss of an auxotrophy also was not the reason for the increased growth rate. Apparently, strain GJ10 could undergo mutations leading to slowly growing variants that can revert to the wild-type phenotype.

The steady-state concentration of DCE during growth of strain GJ10MR1 at a dilution rate of 0.052 h^{-1} ($1/2 \mu_{\max}$) was 81 μ M (Table 1). Thus, the higher maximum growth rate of the revertant led to a lower steady-state concentrations of DCE.

Kinetics of DCE degradation of strains AD20 and AD25. The μ_{\max} values of *A. aquaticus* AD25 and AD20 growing on DCE under batch-fed conditions were 0.098 and 0.117 h^{-1} , respectively. These values were in the same range as found with strain GJ10 (Table 1).

The K_s value of strain AD20 during growth on DCE was 222 μ M. In contrast, strain AD25 had a K_s of 24 μ M for growth on DCE, which is about 10 times lower than the values found with strains AD20 and GJ10. During growth in continuous culture, the efficiency of DCE utilization of strain AD25 was much higher. At a DCE concentration of 279 μ M in the water phase of the continuous culture, the DCE utilization rate of strain GJ10 was 37 nmol/min/mg (dry

TABLE 2. Degradation of volatile halogenated substrates in continuous cultures

Strain	Growth rate (h ⁻¹)	Dilution rate (h ⁻¹)	$S_{\text{air in}}$ (μ mol/h)	$S_{\text{air out}}$ (μ mol/h)	S_{water} (μ M)	Chloride concn (mM)	Yield (g/g)		Conversion (nmol/min/mg)	Elimination (%)
							Continuous culture	Batch culture		
AD20	DCE	0.048	180	10.4	140	9.8	0.17	0.17	43	92
AD25	DCE	0.070	654	16.5	50	24.2	0.16	0.17	67	97
GJ10	DCE	0.052	139	24.5	279	5.7	0.24	— ^a	37	72
GJ10MR0	DCE	0.030	95	5	53	9.3	0.28	—	15	94
GJ10MR1	DCE	0.052	155	6	81	7.3	—	—	24	94
AD1	Epichlorohydrin	0.073	207	7	8	3.5	0.25	—	54	98

^a —, not determined.

TABLE 4. Effect of mixing rate on steady-state DCE concentrations in a continuous culture of *A. aquaticus* AD20^a

Mixing rate (rpm)	DCE _{air in} (μM)	DCE _{air out} (μM)	DCE _{water} (μM)	DCE _{water} /DCE _{air}	Elimination (%)
400	311 ± 10	33 ± 4	585 ± 25	18	78
500	322 ± 3	39 ± 1	620 ± 20	16	77
700	332 ± 10	32 ± 2	612 ± 30	19	79

^a Strain AD20 was cultivated in a chemostat with a dilution rate of 0.090 h⁻¹. The air flow was 1,150 ml/h. Values are averages of at least eight different determinations.

weight) of cells (Table 2). Even at a concentration of 50 μM DCE in the water phase, the rate observed with strain AD25 was 67 nmol/min/mg (dry weight) of cells (Table 2). The amount of the haloalkane dehalogenase of strain AD25 was 30 to 40% of total soluble protein (32), which is 10 to 15 times higher than observed with strain GJ10 (23). Thus, the improved affinity and extent of DCE removal with strain AD25 seemed to be caused by the high haloalkane dehalogenase content of this bacterium.

During all continuous-culture experiments of strains AD20 and AD25 growing on DCE, 2-chloroethanol could not be detected in the culture medium by gas chromatographic analysis.

Competition between *A. aquaticus* AD25 and *X. autotrophicus* GJ10 for DCE. A competition experiment between strains AD25 and GJ10 for DCE as the limiting growth substrate was carried out in a continuous culture. The favorable kinetics of strain AD25 for DCE led to the expectation that strain GJ10 would be competed out at dilution rates up to the μ_{\max} (0.098 h⁻¹) of strain AD25. The competition experiment was carried out at a dilution rate of 0.052 h⁻¹.

Initially, a pure culture of strain GJ10 was inoculated, and a steady state was reached after about 4 days. The concentration of DCE in the water phase was 266 μM, which is in agreement with earlier observations (Table 1). After 6 days, 50 ml of a culture of strain AD25 (10⁷ cells per ml) grown on 2-chloroethanol was inoculated. During the next 15 days, the initial amount of cells of strain GJ10 dropped from 4.5 × 10⁸ to 2 × 10⁴/ml. The color of the culture turned from yellow to white, and the number of cells of strain AD25 increased to 4 × 10⁸/ml. Seven days after the inoculation of strain AD25, the DCE concentration in the water phase had decreased to about 30 μM.

Transport of DCE from gas to water phase in continuous culture. To investigate whether the transport rate of DCE from the air phase to the water phase was rate limiting for the elimination rate of DCE, we measured the DCE concentration in the water and air phases in a continuous culture during steady-state growth of strain AD20 at different stirring rates. The results (Table 4) indicated that the concentration of DCE in the outlet gas of the fermentor showed no significant variation when the stirring rate set at 400, 500, or 700 rpm. Moreover, the ratio DCE_{water}/DCE_{air out} was 16 to 19, which was in agreement with the partition coefficient of DCE (3, 12, 26). If the transport rate of DCE from the gas phase to the water phase was rate limiting, then the outlet DCE concentration at 400 rpm would have been significantly higher than that at 700 rpm. From these results, it was concluded that the transport rate of DCE from the gas phase to the water phase was not rate limiting.

Kinetics of 2-chloroethanol degradation with strains AD20

and GJ10. In continuous cultures of strains AD20 and GJ10 growing on DCE, 2-chloroethanol did not accumulate above the detection level of 2 μM. Consequently, the affinity of the strains for 2-chloroethanol seemed to exceed the affinity for DCE.

Batch experiments in a fermentor on 5 mM 2-chloroethanol revealed that both strains possessed a higher μ_{\max} on 2-chloroethanol than on DCE (Table 1). Furthermore, the K_s values of strains GJ10 and AD20 for 2-chloroethanol were 7 and 4 μM, respectively (Table 1), which is much lower than the values for DCE. The K_m values of the alcohol dehydrogenase of strain GJ10 for 2-chloroethanol and methanol were 55 and 20 μM, respectively. The K_m value of the alcohol dehydrogenase of strain AD20 for 2-chloroethanol was 114 μM (Table 1). Both values are much lower than the K_m value of the haloalkane dehalogenase for DCE.

Kinetics of *Pseudomonas* sp. strain AD1. The correlation between enzyme kinetics and the Monod half-saturation constant was further investigated by using continuous cultures of *Pseudomonas* sp. strain AD1 growing on 1,3-dichloro-2-propanol or on epichlorohydrin.

1,3-Dichloro-2-propanol, a nonvolatile compound, was added via the medium. Strain AD1 growing under batch conditions in a fermentor on 6.5 mM 1,3-dichloro-2-propanol had a μ_{\max} of 0.198 h⁻¹. During growth on 1,3-dichloro-2-propanol, a K_s of 12 μM, based on the results of three different steady states, was calculated (Table 1). Epichlorohydrin could not be detected (<2 μM) in the culture fluid. The K_m of the dehalogenase in crude extracts of strain AD1 with 1,3-dichloro-2-propanol was 50 μM (Table 1), in agreement with earlier observations that a low K_m of the first catabolic enzyme corresponds with a low K_s value.

Pseudomonas sp. strain AD1 converted epichlorohydrin to 3-chloro-1,2-propanediol, a reaction catalyzed by an epoxide hydrolase (30). The K_m of the enzyme was 300 μM, which was determined with purified epoxide hydrolase of strain AD1 (17). The K_s of strain AD1 with epichlorohydrin, determined at a dilution rate of 0.073 h⁻¹ (1/2 μ_{\max}), was 8 μM. In this case, the K_m of the first catabolic enzyme was also much higher than the corresponding K_s value.

In batch cultures of strain AD1, most of the added epichlorohydrin was already converted during the lag phase of growth to 3-chloro-1,2-propanediol (30). In continuous cultures fed with epichlorohydrin, however, no significant accumulation of 3-chloro-1,2-propanediol (<10 μM) was observed. As a consequence of the relatively high affinity for epichlorohydrin, strain AD1 grown at 1/2 μ_{\max} eliminated about 98% of the total amount of epichlorohydrin that entered the culture (Table 2). This percentage could be increased by lowering the dilution rate.

DISCUSSION

The degree of the removal of halogenated aliphatic hydrocarbons from waste gases in biofilters or trickling filters is influenced by the kinetic properties of the bacterial strains that utilize these compounds as growth substrate (8, 9, 16). For application purposes, specialized bacterial cultures with high affinities for halogenated aliphatics are desirable because the allowable concentrations in waste gases are very low. This observation prompted us to study which characteristics of microorganisms influence the kinetics of degradation of chlorinated substrates. Three environmentally important halogenated compounds, DCE, 2-chloroethanol, and epichlorohydrin, were used as growth substrates.

A low affinity for DCE was found with strains AD20 and

GJ10. This was expected to be caused by the low affinity of the haloalkane dehalogenase, which has a K_m of 571 μM for DCE. A correlation between the K_m value of the first catabolic enzyme and the Monod constant was also observed with the strains that grew on 2-chloroethanol, 1,3-dichloro-2-propanol, and epichlorohydrin.

For growth on volatile and nonvolatile halogenated compounds, however, not only the affinity of the first enzyme is important. It was found that the K_s of strain AD25 for DCE was approximately 9 to 10 times lower than that found with strains AD20 and GJ10, although the haloalkane dehalogenases of the three strains are identical (20, 32). This can be explained by the much higher haloalkane dehalogenase content in strain AD25, which is 30 to 40% of total soluble protein, as was shown in an earlier study (32). Because of the high dehalogenase content, strain AD25 achieved relatively high conversion rates at DCE concentrations far below the K_m of the dehalogenase and thus obtained much lower steady-state DCE levels. This provided strain AD25 with a selective advantage over strain GJ10, as found in a competition experiment. In agreement with these observations, it has been found that during growth on xenobiotic compounds in continuous cultures, mutants that have a higher concentration of the first catabolic enzyme may arise. Mutants of *Pseudomonas putida* that had increased levels of chloroacetic acid dehalogenase (33) were obtained during growth in chemostat cultures on chloroacetic acid. In our chemostat experiments, we have not observed overexpression mutants, but during growth on DCE, reversion of a mutant with a lowered μ_{max} to a higher μ_{max} was observed. These kind of mutants have also been described by other authors (14).

In general, cells may increase their affinity for a growth substrate by active accumulation of these compounds in the cells, but with hydrophobic substrates such as DCE, information about active uptake is lacking. On the other hand, it has been reported that the cell membrane may act as a barrier for the permeation of hydrophobic compounds (29). In one of the scarce studies on this question, it was proposed that the rate-determining step in the biodegradation of alkyl esters of *p*-aminobenzoic acid by a *Pseudomonas fluorescens* isolate was diffusion into the cells since the conversion of the esters increased with increasing chain length (29). However, the kinetics of the catabolic enzymes for the growth substrates were not measured.

To examine whether DCE accumulates in the cells of strains AD20, AD25, and GJ10 during growth in chemostats, a comparison was made between the observed growth rates of the strains on DCE and the growth rates allowed by the amount of haloalkane dehalogenase in the cells. The latter were calculated on the basis of the kinetics of purified haloalkane dehalogenase in vitro, the level of the enzyme in the cells, and the growth yield of the strains on DCE. The amounts of the constitutively produced haloalkane dehalogenase in the cells of strains AD25, AD20, and GJ10 are known (23, 32). The predicted maximum growth rate at a given DCE concentration can be described by

$$\mu_a = Y \times E \times V_{\text{max}} \times 60 \times S / (K_m + S) \quad (1)$$

The observed growth rate of the strains can be given by

$$\mu_o = \mu_{\text{max}} \times S / (K_s + S) \quad (2)$$

In equations 1 and 2, Y is the growth yield (milligrams of cells per micromole of substrate), V_{max} is the V_{max} of the haloalkane dehalogenase (micromoles per minute per milligram of dehalogenase protein), E is the haloalkane dehalogenase concentration in the cells (milligrams of dehalogenase

per milligram of cells), S is the DCE concentration (millimolar) in the medium, and K_m and K_s are expressed in millimolar values.

In the cases of strains GJ10 (Fig. 3A) and AD20 (Fig. 3B), the observed growth rate on DCE appeared to be higher than the growth rate theoretically allowed on the basis of the amount of haloalkane dehalogenase in the cells. From the results, it can be concluded that either DCE accumulates in the cells or the affinity of the haloalkane dehalogenase for DCE is somewhat higher in vivo than in vitro. It also follows that the cell membrane of the strains AD20 and GJ10 does not seem to act as a barrier for the permeation of DCE.

The observed growth rates of strain AD25 on DCE, up to 0.060 h^{-1} , are not considerably higher than the growth rates based on the amount of haloalkane dehalogenase in the cells (Fig. 3C). At growth rates higher than 0.060 h^{-1} , the observed growth rates become much lower than the growth rate allowed by the amount of haloalkane dehalogenase in the cells of strain AD25. This may be due to other steps in the metabolism of DCE becoming rate limiting.

The product of DCE, 2-chloroethanol, is oxidized by strains AD20, AD25, and GJ10 by a quinoprotein alcohol dehydrogenase to chloroacetaldehyde. This type of alcohol dehydrogenase is located in the periplasmic space, as shown with other methylotrophic bacteria (1, 22), whereas haloalkane dehalogenase is a cytoplasmic enzyme. Consequently, 2-chloroethanol formed in the cytoplasm during growth on DCE has to be transported to the periplasmic space where the compound is oxidized. The mechanism of transport of this hydrophilic compound over the inner membrane is unknown. There is no indication for active uptake of methanol in mutants *Pseudomonas* sp. strain AM1 that lack the alcohol dehydrogenase (4).

For the compounds used in this study, a correlation between the K_s values of bacterial strains and the hydrophobicity of the growth substrates probably does not exist. We found that compounds that are less hydrophobic than DCE or epichlorohydrin, such as 2-chloroethanol and 1,3-dichloro-2-propanol, gave rise to lower K_s values. Compounds that are more hydrophobic than epichlorohydrin and DCE, such as toluene and chlorobenzene, are degraded by *Pseudomonas* sp. strains GJ40 and GJ31, respectively, and both strains have a K_s value lower than 0.5 μM for these growth substrates (18), as determined in continuous-culture experiments. Furthermore, in an aerobic fixed film reactor, the K_s measured for growth on toluene was 0.07 mg/liter (2). With the halogenated aliphatic compound dichloromethane as the growth substrate, K_s values found with pure and mixed bacterial cultures were lower than 5 μM (8, 16). These observations suggest that physical-chemical properties of hydrocarbons, such as hydrophobicity, do not determine the K_s of a bacterial strain. Probably, the kinetic properties of the first enzyme for a compound are more important for the affinity of a bacterial strain, as shown in this study. The low K_s value for dichloromethane probably is also due to the low K_m of dichloromethane dehalogenase, which is 20 μM (24).

It has been described that bacteria selected in a chemostat with a simple carbon source at a low concentration have a low K_s and a low μ_{max} , and vice versa (27). We did not observe this correlation for different cultures that degrade chlorinated substrates. For the treatment of waste gases contaminated with low concentrations of halogenated aliphatic compounds, strains that possess a low K_s and a high μ_{max} are favorable. The better affinity of strain AD25 for DCE should make this organism more suitable for waste gas purification than strains AD20 and GJ10. Moreover, strain

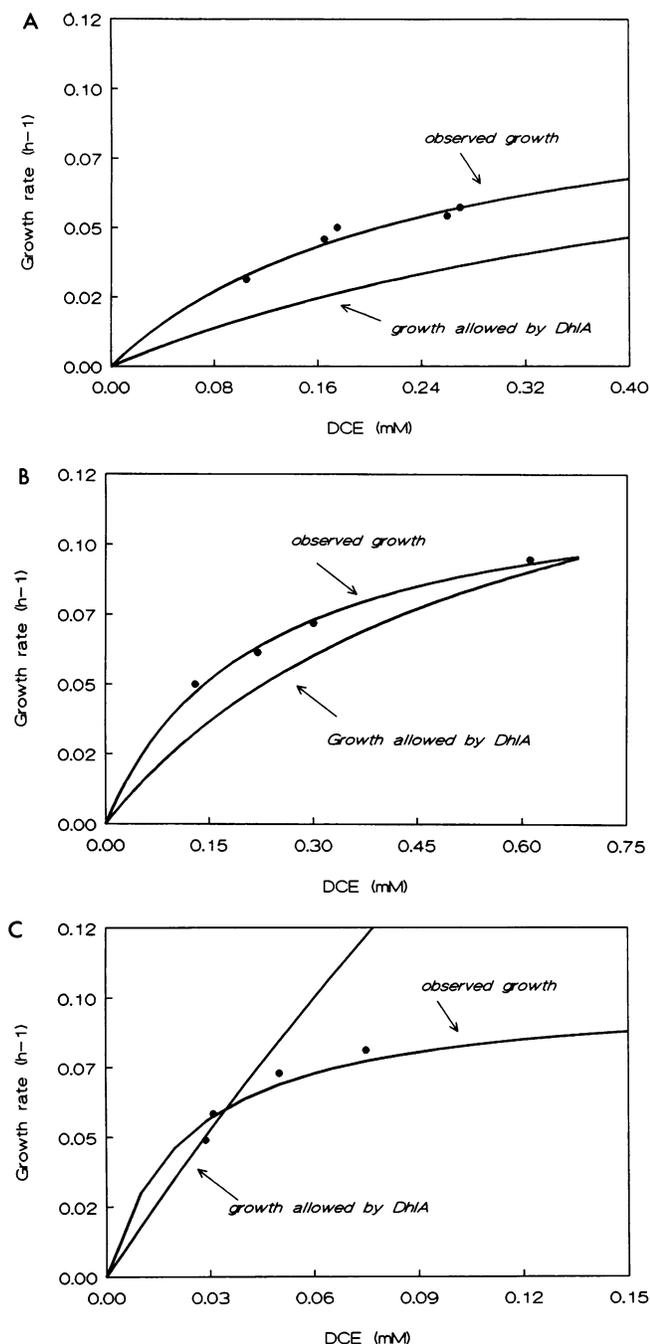


FIG. 3. Kinetics of growth on DCE of the strains GJ10 (A), AD20 (B), and AD25 (C). The observed growth rate is calculated according to equation 2. Symbols represent DCE concentrations in the water phase during steady states at different dilution rates. The predicted growth rate is calculated according to equation 1. For haloalkane dehalogenase, $V_{\max} = 10 \mu\text{mol}/\text{min}/\text{mg}$ of protein and $K_m = 0.57 \text{ mM}$. For strain AD20, $\mu_{\max} = 0.117 \text{ h}^{-1}$, $Y = 0.017 \text{ mg}/\mu\text{mol}$, $K_s = 0.222 \text{ mM}$, and $E = 0.0165 \text{ mg}/\text{mg}$. For strain AD25, $\mu_{\max} = 0.098 \text{ h}^{-1}$, $Y = 0.016 \text{ mg}/\mu\text{mol}$, $K_s = 0.024 \text{ mM}$, and $E = 0.105 \text{ mg}/\text{mg}$. For strain GJ10, $\mu_{\max} = 0.104 \text{ h}^{-1}$, $Y = 0.024 \text{ mg}/\mu\text{mol}$, $K_s = 0.260 \text{ mM}$, and $E = 0.0075 \text{ mg}/\text{mg}$.

AD25 produces extracellular fibers (31) and adheres quickly to glass and steel. Furthermore, strain AD25 produces no slime, which is important since slime production is recognized as a disadvantage when the strain is applied in trickling filters (8). These properties seem to make strain AD25 suitable for use in application systems like trickling filters. As most waste gases often contain a mixture of compounds, further work will be focused on the cometabolic degradation of other short chain halogenated compounds such as 1,2-dichloropropane and dichloromethane, which are substrates of the haloalkane dehalogenase.

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