Three Dehalogenases and Physiological Restraints in the Biodegradation of Haloalkanes by *Arthrobacter* sp. Strain HA1

RUDOLF SCHOLTZ, FERRUCCIO MESSI,† THOMAS LEISINGER, and ALASDAIR M. COOK*

Department of Microbiology, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zurich, Switzerland

Arthrobacter sp. strain HA1 utilizes 18 C_2-to-C_8 1-haloalkanes for growth and synthesizes an inducible 1-bromoalkane debrominase of unknown physiological function (R. Scholtz, T. Leisinger, F. Suter, and A. M. Cook, J. Bacteriol. 169:5016–5021, 1987) in addition to an inducible 1-chlorohexane halidohydrolase which dehalogenates some 50 substrates, including α,ω-dihaloalkanes. α,ω-Dihaloalkanes were utilized by cultures of strain HA1 under certain conditions only. C_9 and C_8 homologs prevented growth. At suitable concentrations, C_7-to-C_5 homologs could serve as sole sources of carbon and energy for growth. C_4 and C_3 homologs could be utilized only in the presence of a second substrate (e.g., butanol), and the C_2 homolog was not degraded. Kinetics of growth and substrate utilization indicated that cells of strain HA1 growing in butanol-salts medium could be used to test whether compounds induced the 1-chlorohexane halidohydrolase. No gratuitous induction of synthesis of the enzyme was observed. Many enzyme substrates (e.g., bromobenzene) did not induce synthesis of the enzyme, though the enzyme sequence to degrade the product (phenol) was present. Some inducers (e.g., bromomethane) were enzyme substrates but not growth substrates. In an attempt to find a physiological role for the 1-bromoalkane debrominase, we observed that several long-chain haloaliphatic compounds ($>C_9$; e.g., 1-bromohexadecane and 1-chlorohexadecane) were utilized for growth and that induced cells could dehalogenate several 1-haloalkanes (at least C_4 to C_{16}). The dehalogenation of the long-chain compounds could not be assayed in the cell extract, so we presume that a third haloalkane dehalogenase was present. All dehalogenations were equally active in the presence or absence of molecular oxygen and were presumed to be hydrolytic.

Haloaliphatic compounds, most of which are haloalkanes, make up the largest group of chemicals in the U.S. Environmental Protection Agency list of priority pollutants (10). Whereas these compounds and homologs were regarded as essentially nonbiodegradable when the list was promulgated, dehalogenative transformations of these compounds by pure cultures of aerobic organisms and by mixed and pure cultures of anaerobic organisms have been observed, as has growth of aerobes with haloalkanes as sole sources of carbon and energy (4, 16; see also reference 11).

Recognition of the bioaccessibility of the haloalkane carbon-halogen bond has revealed the following three groups of cleavage mechanisms: (i) hydrolytic (one class cofactor [glutathione] mediated, one class not requiring a cofactor), (ii) oxygenolytic (one class leading to the corresponding alcohol and the other lending to the aldehyde), and (iii) reductive (one class reducing a C-halogen bond to C–H, the other class reducing halogen—C—C–halogen to C=C) (4, 16). Only group i and a limited selection from group ii are known cell free, the others are still poorly defined, and little is known about the physiology of the utilization of haloalkanes.

Arthrobacter sp. strain HA1 has the widest known range of haloalkane growth substrates (18 1-haloalkanes from C_2 to C_8) (15). The enzyme responsible for converting the xenobiotic to a normal metabolic intermediate, 1-chlorohexane halidohydrolase, hydrolyzes the alkyl halide to the corresponding alcohol, which is utilized for growth and energy production. The purified 1-chlorohexane halidohydrolase has at least 50 halogenated substrates. Strain HA1 also synthesizes a 1-bromoalkane debrominase which is active on

close homologs of 1-bromononane (C_6 to at last C_9), whose physiological function is uncertain (14).

We now report on problems of enzyme induction, poor enzyme affinity for some substrates, the lack of suitable catabolic enzymes, and toxic effects of enzyme substrates on *Arthrobacter* sp. The range of haloalkane growth substrates was found to be larger than previously measured, and evidence for the presence of further dehalogenase activities was obtained.

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MATERIALS AND METHODS

Materials. The sources, identification, and high purity of many of the chemicals used were described elsewhere (14, 15); additional halogenated compounds (99%) were from Fluka, except for the C_7-to-C_9 α,ω-dichloroalkanes, which were kindly supplied by T. Omori. Unless otherwise stated, all experiments with volatile substrates were done in screw-cap vessels closed with Mininert valves (Precision Sampling, Baton Rouge, La.).

Apparatus and analytical methods. The equipment used for gas chromatography-flame ionization detection, spectrophotometry, electrophoresis, and anaerobic incubations has been described elsewhere (14, 15). Analyses of volatile compounds in portions of culture fluid (diols, alcohols, and chloroalcohols) or of the gas phase above experiments involved Porapak P, Chromosorb 101, Tenax, or phenyl-methyl silicone columns (14, 15). Halide ions were quantified as ferrithiocyanate (14) in an adaptation of a standard method (1). Protein in cell extracts was measured by the method of Bradford (2); protein in whole cells was assayed turbidimetrically with reference to a standard curve (15).

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* Corresponding author.

† Present address: Institut für Biotechnologie, ETH-Hönggerberg, CH-8093 Zurich, Switzerland.

TABLE 1. Qualitative[a] and quantitative data on haloalkanes, haloalcohols, and dihydroxyalkanes as growth substrates for *Arthrobacter* sp. strain HA1

Compound	Efficacy as a substrate for halidohydrolase	Growth with the compound (2 mM) as the sole substrate	Growth with 4 mM butanol plus 2 mM haloalkane	Fate of haloalkane[b]		
				Loss	Yield from haloalkane[c] (g of protein/mol of C)	Cl$^-$ (mM)
Butanol	−	+				
1-Chlorobutane	+	+				
1,2-Dichloroethane	−	−	+	−	—	—
1,3-Dichloropropane	+	−	+	+	5	4.3
1,4-Dichlorobutane	+	−	+	+	3	4.5
1,5-Dichloropropane	+	−	−	−	—	—
1,6-Dichlorohexane	+	−	−	−	—	—
2-Chloroethanol	−	−	+			
1,2-Dihydroxyethane	−	−	+			
3-Chloropropane	+	−	+			
1,3-Dihydroxypropane	−	+	+			
4-Chlorobutanol	+	−	+			
1,4-Dihydroxybutane	−	−	+			
1,5-Dihydroxypentane	−	+	+			
6-Chlorohexanol	+	+	+			
1,6-Dihydroxyhexane	−	+	+			

[a] The substrates for the halidohydrolase were evaluated as no substrate disappearance (−) or substrate disappearance concomitant with product formation (+). Growth substrates were either completely degraded (+) or identical to the sterile control (−). There were no ambiguous data.

[b] This set of columns refers to 2 mM haloalkane added to a culture growing in the presence of 4 mM butanol under standard conditions. Cultures were examined after 3 days; further incubation (3 days) had no effect. —, No protein yield or no Cl$^-$.

[c] The yield from growth with butanol (5 g of protein per mol of C) was subtracted from the total yield to give the amount of growth from the haloalkane.

Sodium dodecyl sulfate-gel electrophoresis was done in 12% gels (9). Electrophoresis under native conditions was done in 7.3% gels (5), and enzyme activity was located by precipitating the reaction product, bromide ion, as AgBr (14). Electron micrographs were prepared as described elsewhere (7).

1-Chlorohexane halidohydrolase was assayed as release of chloride ion from 1-chlorobutane in cell extract (14). The test for 1-bromoalkane debrominase in crude extract was similar to that for 1-chlorohexane halidohydrolase and involved 1-bromononane as the substrate (14). The dehalogenase for long-chain 1-haloalkanes ($\geq$C$_9$) was active only in whole cells. Assays with whole cells were analogous to those with cell extracts (14). Experiments under anaerobic conditions were described previously (15).

Organism, growth conditions, storage, and disruption of cells. *Arthrobacter* sp. strain HA1 (15) was used. The salts medium and growth conditions were described by Scholtz et al. (15). Growth, growth kinetics, and induction experiments were done in 150-ml cultures in 1-liter Erlenmeyer flasks. Large amounts of butanol- or 1-chlorobutane-grown cells were prepared as described previously (14); 1-bromohexadecane-grown cells were cultured with 0.5 mM substrate alone. Harvested cells were routinely used immediately for experiments, because the debrominase activity was lost in storage (14). Cells were disrupted by passage through a French pressure cell, and the supernatant fluid was used for experiments with cell extracts (14).

RESULTS AND DISCUSSION

Dichloroalkanes and growth of *Arthrobacter* sp. strain HA1. Growth of strain HA1 with butanol or 1-chlorobutane as the sole carbon source with quantitative substrate utilization and yields of about 5 g of protein per mol of C was confirmed (Table 1). 1,2-Dichloroethane, which was not a substrate for 1-chlorohexane halidohydrolase, was not a growth substrate and neither significantly altered nor was altered by growth of

strain HA1 with butanol (Table 1). 1,3-Dichloropropane and 1,4-dichlorobutane were substrates for the enzyme but not for growth; nevertheless, each compound was totally dechlorinated during growth on butanol, and growth equivalent to 5 and 3 g of protein per mol of C, respectively, due to the presence of the chlorinated compounds was observed. 1,3-Dichloropropane was thus utilized quantitatively, whereas carbon from the butane homolog was incompletely used for growth, and the fate of the residual carbon is unknown. 1,5-Dichloropentane or 1,6-dichlorohexane, each a substrate for the enzyme and subject to dechlorination by suspensions of whole cells under these conditions, was not a growth substrate and inhibited growth with 4 mM butanol; similar effects were observed with the C$_7$, C$_8$, and C$_9$ α,ω-dihaloalkanes.

1,6-Dichlorohexane need not be inhibitory to bacteria (8). When provided for strain HA1 at 0.5 mM in salts medium, 1,5-dichloropentane, 1,6-dichlorohexane, and (after 2 weeks), 1,7-dichloroheptane supported growth, which was indirectly detected as chloride release in stoichiometric amounts; further carbon source additions produced visible growth of strain HA1. 1,8-Dichlorooctane and 1,9-dichlorononane at 0.5 mM neither supported growth nor allowed growth in the presence of butanol.

None of the products tested from the dechlorination of the dichloroalkanes (the chloroalcohol intermediate or the diol product) inhibited growth of strain HA1 with butanol (Table 1). Some chloroalcohols were unstable at 2 weeks of incubation, and no quantitative data about them are presented. C$_3$, C$_5$, and C$_6$ diols, but not the C$_4$ diol, were utilized quantitatively as sole carbon and energy sources with growth yields of about 5 g of protein per mol of C.

At least two general phenomena can be deduced from these data. (i) The C$_5$-to-C$_9$ α,ω-dichloroalkanes themselves, not dechlorination products, are toxic to the organism when the concentration is high enough. The toxicity is not due to a class of substances but to individual compounds in that
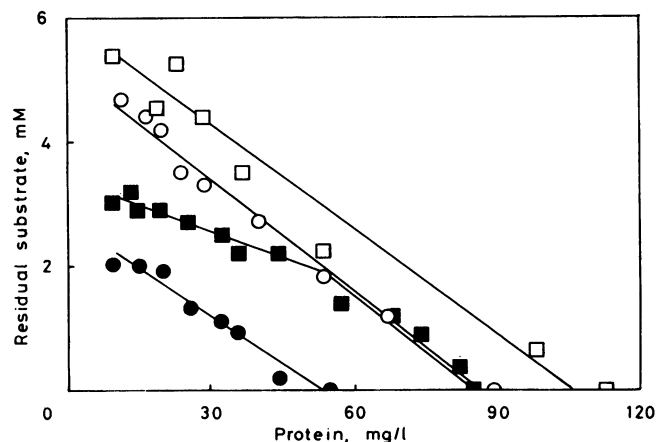
FIG. 1. Substrate utilization as a function of growth of three cultures of *Arthrobacter* sp. strain HA1 in carbon-limited salts medium. A culture of strain HA1 was grown in 4 mM butanol-salts medium at 30°C, and when the butanol was just exhausted, they were used to inoculate (2% [vol/vol]) 150-ml cultures for the growth experiments. Samples of the gaseous and aqueous phases were taken at intervals for analyses of volatile components, bromide release, and protein (as turbidity). For two cultures, single sources of carbon and energy were provided: butanol (O) or 1-bromobutane (□). The third culture contained two carbon sources: butanol (●) plus 1-bromobutane (■).

TABLE 2. Levels of induction of 1-chlorohexane halidohydrolase activity in *Arthrobacter* sp. strain HA1 growing in 4 mM butanol–salts medium in the presence of halogenated compounds[a]

Potential inducer (concn [mM])	Level of induction (%)[b]	Time for growth (days)[c]	Disappearance of potential inducer (%)
Butanol control[d]	<5	3	100
1-Chlorohexane control[d]	160	3	100
1-Chlorohexane (2.0)	100	3	100
6-Chlorohexanol (2.0)	37	7	100
1,3-Dichloropropane (2.0)	29	3	100
1,4-Dichlorobutane (2.0)	30	7	100
Bromomethane (1.0)	76	14	40
1-Chloropropane (2.0)	12	3	0
1-Chloroethane (2.0)	<5	3	0
3-Chloroheptane (1.0)	<5	14	0
1,2-Dibromoethane (2.0)	<5	7	0
1,2-Dibromopropane (2.0)	<5	7	0
Bromobenzene (0.5)	<5	3	N.A.[e]
(Bromomethyl)benzene (0.5)	<5	3	N.A.
(2-Bromoethyl)benzene (0.1)	<5	3	N.A.
(3-Bromopropyl)benzene (0.1)	<5	3	N.A.
1-Fluoropentane (2.0)	7	3	0
1,1-Dichloroethane (2.0)	5	7	0
1,2-Dichloroethane (2.0)	<5	3	0
2-Chloroacetate (2.0)	<5	14	N.A.

[a] Enzyme activity was measured when butanol was exhausted.
[b] The level of induction was compared with that in the presence of 1-chlorobutane (500 µkat/kg of protein), which was considered to be 100%.
[c] Time required for culture to exhaust butanol.
[d] Only one carbon source was present in these control cultures.
[e] N.A., No assay of this compound; no halide was released.

class. (ii) The failure of a nontoxic compound to serve as a sole carbon and energy source does not mean that it is not biodegradable. Degradation depends on one or more of the following factors; (i) a second substrate, (ii) the inducibility of the halidohydrolase, (iii) the interaction of the enzyme with substrate, and (iv) the presence of degradative enzymes for the product of dehalogenation. We presume that there is no transport system for these lipophilic substances.

Kinetics of growth of *Arthrobacter* sp. strain HA1 with mixed substrates. Strain HA1 from butanol-salts medium grew exponentially with butanol ($\mu = 0.19$ h^{-1}) or, after a lag phase (10 h), with 1-bromobutane ($\mu = 0.11$ h^{-1}) as the sole source of carbon and energy for growth (data not shown). Growth was proportional to and concomitant with substrate utilization (Fig. 1). In medium with two substrates, butanol and 1-chlorobutane, cells grew initially with both substrates (Fig. 1) at about the same rate as with butanol alone (data not shown), but butanol was utilized about twice as fast as 1-bromobutane (Fig. 1; compare the different slopes of the lines [● and ■]). After the butanol was exhausted, the cells utilized 1-bromobutane solely (Fig. 1) and the growth rate (data not shown) decreased to about that of the control with 1-bromobutane. A similar set of data with dual substrate utilization (butanol faster than 1-bromobutane) was obtained when the inoculum was from 1-bromobutane–salts medium.

It is clear that synthesis of the broad substrate range 1-chlorohexane halidohydrolase is induced during growth on a second substrate (Table 1). Catabolite repression exists (Table 2, lines 2 and 3), but it is not strong with butanol as a carbon source, and inducers of the enzyme can be evaluated by determining enzyme activity in cultures grown in butanol-salts medium containing the potential inducer.

Level of induction of 1-chlorohexane halidohydrolase by enzyme substrates during growth in butanol-salts medium. Butanol-grown cells of strain HA1 contained no measurable 1-chlorohexane halidohydrolase; cells growing with butanol in the presence of 1-chlorobutane contained the halidohy-

drolase at a level that was considered to be 100% (500 µkat/ kg of protein) (Table 2). This level of the 1-chlorohexane halidohydrolase completely hydrolyzed the compound, causing its induction (Table 2). Four other enzyme substrates which were tested caused significant induction of the enzyme and were subject to dehalogenation. 6-Chlorohexanol, a growth substrate, and two α,ω-dihaloalkanes, each a secondary substrate for growth (Table 1), were completely dehalogenated (consistent with the data in Table 1), whereas bromomethane was a nongrowth substrate causing induction. Bromomethane was presumably toxic, causing markedly slower growth than in the control (Table 2), and the incomplete hydrolysis presumably reflects poor affinity of the enzyme for the substrate (14). Another nongrowth substrate, 1-chloropropane, caused slight induction but was not subject to hydrolysis; the combination of poor induction (Table 2), a high K_m, and a low rate of hydrolysis (14) presumably explains why this compound is not a growth substrate. Several enzyme substrates failed to cause induction of 1-chlorohexane halidohydrolase (the next nine compounds in Table 2). Several nonsubstrates of the enzyme also failed to cause significant induction, so we failed to find gratuitous inducers of the enzyme.

It is clear that the wide substrate range of 1-chlorohexane halidohydrolase (about 50 compounds) is very different from the narrow specificity of the regulatory protein governing enzyme synthesis. Whereas the failure of bromobenzene, (2-bromoethyl)benzene, or (3-bromopropyl)benzene and the effective failure of 1-chloropropane to cause induction, prevents growth on these compounds (the corresponding alcohols being carbon sources), the failure of, e.g., (bromomethyl)benzene to induce the enzyme is less important to the organism, which was unable to utilize benzyl alcohol

TABLE 3. Growth of *Arthrobacter* sp. strain HA1 with 0.5 mM long-chain *n*-alkanes, 1-haloalkanes, and analog[a]

Substrate (chain length)	Time (days) required to reach stationary phase
Dodecane (12)	6
Hexadecane (16)	6
Octadecane (18)	2
Octacosane (28)	6
1-Dodecanol (12)	No growth
1-Hexadecanol (16)	14
1-Octacosanol (28)	14
Dodecanoic acid (12)	No growth
Hexadecanoic acid (16)	No growth
Octadecanoic acid (18)	2
Octacosanic acid (28)	No growth
1-Chlorodecane (10)	6
1-Chlorodocecane (12)	6
1-Chlorohexadecane (16)	6
1-Bromodecane (10)	14
1-Bromododecane (12)	14
1-Bromotetradecane (14)	14
1-Bromohexadecane (16)	6

[a] When appropriate, halide release was measured to confirm substrate utilization.

TABLE 4. Characterization of dehalogenases in cell suspensions of *Arthrobacter* sp. strain HA1 as a function of oxygen requirement

Substrate	Dehalogenation activity $(\mu\text{mol/h})^a$ of cells grown with:			
	1-Chlorobutane		1-Bromohexadecane	
	Aerobic	Anaerobic	Aerobic	Anaerobic
1-Chlorobutane	2.4	2.4	0.46	0.46
1-Chlorononane	2.2	2.2	0.23	0.40
1-Chlorododecane	1.6	1.4	0.26	0.26
1-Bromononane	2.4	2.6	0.50	0.46
1-Bromododecane	1.7	1.7	0.38	0.40
1-Bromohexadecane	0.44	0.14	0.30	0.36

[a] The dehalogenation activity of cells grown aerobically with hexadecane was below the limit of detectability (0.05 μmol/h). The dehalogenation activity of these cells with 1-chlorododecane as a substrate was not assayed.

reproducibly as a carbon source. We thus observed poor affinity for the regulatory protein, poor affinity for the enzyme (see the previous paragraph), and the absence of degradative enzymes as reasons for the failure of strain HA1 to utilize substrates of the 1-chlorohexane halidohydrolase for growth.

Additional haloalkane growth substrates and evidence for further dehalogenase activities. Strain HA1 does not utilize for growth the *n*-alkanes pentane and hexane or nonanol, 1-chlorononane, 1-bromononane, and 1-iodooctane (15), even though it contains a characterized 1-chlorohexane halidohydrolase able to dehalogenate these haloalkanes and a 1-bromoalkane debrominase activity for 1-bromononane (14). We extended the range of long-chain compounds ($\geq C_8$) tested as sole sources of carbon and energy for growth in the search for a physiological role for the debrominase activity. Alkanes, alcohols, a fatty acid, and at least seven long-chain 1-haloalkanes were utilized for growth (Table 3); halide ion was released from the otherwise stable haloalkanes during growth. The substrate range of strain HA1 for unsubstituted haloalkanes as sole carbon and energy sources is thus at least 28 compounds (18 short-chain 1-haloalkanes (C_2 to C_8), 3 α,ω-dihaloalkanes (see above), and 7 long-chain 1-haloalkanes). When strain HA1 utilized these long-chain growth substrates (Table 3), yet another pleomorphic form (cf. reference 15) was observed by phase-contrast and electron microscopy: very long cells (15 to 20 by 1 to 1.4 μm) in chains with a tendency to branching. These cells clumped together to form particles which precipitated immediately on removal of the culture flasks from the shaker. Normal morphology was resumed on transfer to medium containing, e.g., 1-chlorobutane.

The nature of the dehalogenases involved in the degradation of long-chain haloalkanes was examined in cells grown in salts medium containing (i) 1-chlorobutane to induce 1-chlorohexane halidohydrolase and 1-bromoalkane debrominase activity (14, 15), (ii) hexadecane, or (iii) 1-bromohex-

adecane. Strain HA1 was grown to the stationary phase in 30 ml of salts medium containing 10 mM 1-chlorobutane, 1 mM 1-bromohexadecane (added in two portions at 0.5 mM each), or 1 mM hexadecane (added in two portions at 0.5 mM each), harvested, and washed twice in 50 mM Tris buffer (pH 8.2). Each pellet was suspended in the same buffer and brought to 2.5 ml. Portions (200 μl each) of the cell suspension were used to start reaction mixtures (2-ml final volume) which were incubated for 1 h at 30°C and then analyzed for halid release. Hexadecane-grown cells did not dehalogenate any haloalkane, in contrast to 1-bromohexadecane-grown cells (Table 4). Thus, strain HA1 does not contain an alkane monooxygenase (3) that is able to dehalogenate 1-haloalkanes because of its wide substrate specificity, a system claimed by Murphy and Perry (12) and supported by Yokota et al. (17), and that is analogous to the more readily available methane monooxygenase (13).

The presence of an oxygen requirement distinguishes between hydrolytic and oxygenolytic cleavage mechanisms (15, 17). Cells grown in 1-chlorobutane–salts medium catalyzed dehalogenation of six substrates at effectively equal rates in the presence or absence of molecular oxygen (Table 4). These reactions were thus all hydrolytic. Similarly, the reactions catalyzed by cells from 1-bromohexadecane–salts medium were hydrolytic (Table 4).

Butanol-grown cells had no 1-chlorohexane halidohydrolase activity (Table 2), 1-bromoalkane debrominase activity (14), or activity on 1-bromododecane or 1-bromohexadecane. Whereas cells grown in 1-chlorobutane–salts medium were expected to dehalogenate 1-chlorobutane and 1-bromononane, given the analyses of cell-free enzyme activity (14), the dehalogenation of long-chain compounds and the rapid dechlorination of 1-chlorononane were unexpected. This observation may be rationalized by the stability of 1-chlorohexane halidohydrolase and the lability of 1-bromoalkane debrominase (14); a further and more labile dehalogenase may be present that is inactive in cell extracts. Further, the different ratios of activities between the dehalogenation of 1-chlorobutane and 1-bromohexadecane (Table 4) indicate different enzyme complements in cells grown under different inducing conditions.

Extracts of cells from salts medium containing butanol (i.e., not induced for dehalogenases), 1-chlorobutane, or 1-bromohexadecane were prepared. 1-Chlorohexane halidohydrolase (active on C_2 to C_{10}) and 1-bromoalkane debrominase (active on C_6 to at least C_9), but no activities on long-chain 1-halohydrocarbons, were observed in extracts

from 1-chlorobutane-grown cells. No dehalogenating activity (C_4 to C_{16}) was observed in extracts from 1-bromohexadecane-grown cells.

Electrophoresis of the different extracts showed that, under native conditions, only the 1-chlorohexane halidohydrolase was active after separation. Comparison of extracts from butanol-grown and 1-chlorobutane-grown cells showed an increase of four protein bands in the induced cells, and only one of those bands, that for 1-chlorohexane halidohydrolase, could be identified. We presume that at least one of the remaining bands represents 1-bromoalkane debrominase activity. None of those four bands was visible in extracts from 1-bromohexadecane-grown cells, which contained other novel bands.

We thus postulate the presence of the following three inducible haloalkane dehalogenases: (i) the 1-chlorohexane halidohydrolase present only in 1-chlorobutane-grown cells, (ii) the 1-bromoalkane debrominase present only in 1-chlorobutane-grown cells, and (iii) the activity present in 1-bromohexadecane-grown cells. *Arthrobacter* sp. strain HA1 thus contains at least four dehalogenases, since a chloropropionate halidohydrolase has also been observed (15). This organism is thus among those with the largest known complement of halidohydrolases (6). Strain HA1 is also the first organism to degrade long-chain 1-haloalkanes by hydrolysis of the carbon-halogen bond.

ACKNOWLEDGMENTS

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