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Adaptation of *Pseudomonas* sp. GJ1 to 2-bromoethanol caused by overexpression of an NAD-dependent aldehyde dehydrogenase with low affinity for halogenated aldehydes

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Abstract *Pseudomonas* sp. GJ1 is able to grow with 2-chloroethanol as the sole carbon and energy source, but not with 2-bromoethanol, which is toxic at low concentrations (1 mM). A mutant that could grow on 2-bromoethanol with a growth rate of 0.034 h^{-1} at concentrations up to 5 mM was isolated and designated strain GJ1M9. Measurement of enzyme activities showed that mutant and wild-type strains contained a PMS-linked alcohol dehydrogenase that was active with halogenated alcohols and that was threefold overexpressed in the mutant when grown on 2-chloroethanol, but only slightly overproduced when grown on 2-bromoethanol. Both strains also contained an NAD-dependent alcohol dehydrogenase that had no activity with halogenated alcohols. Haloacetate dehalogenase levels were similar in the wild-type and the mutant. Activities of NAD-dependent aldehyde dehydrogenase were only slightly higher in extracts of the mutant grown with 2-bromoethanol than in those of the wild-type grown with 2-chloroethanol. SDS-PAGE, however, showed that this enzyme amounted to more than 50% of the total cellular protein in extracts of the mutant from 2-bromoethanol-grown cells, which was fourfold higher than in extracts of the wild-type strain grown on 2-chloroethanol. The enzyme was purified and shown to be a tetrameric protein consisting of subunits of 55 kDa. The enzyme had low K_m values for acetaldehyde and other non-halogenated aldehydes (0.8–4 μM), but much higher K_m values for chloroacetaldehyde (1.7 mM) and bromoacetaldehyde (10.5 mM), while V_{max} values were similar for halogenated and non-halogenated aldehydes. Cultures that were pregrown on 2-chloroethanol rapidly lost aldehyde dehydrogenase activity after addition of 2-bromoethanol and chloroamphenicol, which indicates that bromoacetaldehyde inactivates the enzyme. To achieve growth with 2-bromoethanol, the high expression of the enzyme thus appears to be necessary in order to compensate for the high K_m for bromoacetaldehyde and for inactivation of the enzyme by bromoacetaldehyde.

Key words 2-Chloroethanol · 2-Bromoethanol · 1,2-Dibromoethane · Dehalogenase · Aldehyde dehydrogenase · Genetic adaptation · Biodegradation

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Introduction

Specific strains of gram-negative bacteria belonging to the genera *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* are able to grow with the synthetic organochlorine compounds 2-chloroethanol and 1,2-dichloroethane as the sole carbon and energy source (Janssen et al. 1984, 1985; Van den Wijngaard et al. 1992). Degradation of 2-chloroethanol has also been found with other bacterial strains, mostly of the genus *Pseudomonas* (Stucki and Leisinger 1983; Janssen et al. 1984; Strotmann et al. 1990). The degradation of 1,2-dichloroethane proceeds via 2-chloroethanol, chloroacetaldehyde, and chloroacetate to glycolate. None of the 1,2-dichloroethane- or 2-chloroethanol utilizing organisms have been reported to also grow with 2-bromoethanol or 1,2-dibromoethane. In principle, these compounds should be degradable by the same pathway as 2-chloroethanol and 1,2-dichloroethane, respectively. The poor degradability of the brominated compounds is unexpected because of the relative ease in breaking carbon-bromine bonds as compared to carbon-chlorine bonds. 1,2-Dibromoethane is a good substrate for the haloalkane dehalogenase that converts 1,2-dichloroethane (Keuning et al. 1985; Van den Wijngaard et al. 1992), and bromoacetic acid is as rapidly dehalogenated by halocarboxylic acid dehalogenase as is chloroacetic acid (Van der Ploeg et al. 1991).

The relative recalcitrance of the bromoaliphatics is also interesting from an environmental point of view. 1,2-

Dibromoethane is an important groundwater pollutant. It has been used as a soil fumigant and as a lead scavenger in gasoline. It is now suspected to be carcinogenic (Rannug 1980) and, therefore, is no longer used for these purposes. Mineralization and growth do not occur because 1,2-dibromoethane is extremely toxic for haloalkane dehalogenase-producing organisms at concentrations of 10 μM or higher (Van den Wijngaard et al. 1992). This could be caused by accumulation of bromoacetaldehyde, which is expected to be the most reactive intermediate. Various attempts to isolate 2-bromoethanol- or 1,2-dibromoethane-degrading organisms from polluted environmental samples have been unsuccessful (D.B. Janssen, unpublished results).

We have previously described the isolation and characterization of the 2-chloroethanol-utilizing *Pseudomonas* sp. strain GJ1 (Janssen et al. 1984). This strain is able to grow on 2-chloroethanol at concentrations of up to 15 mM, but not on 2-bromoethanol. In this study, we report the isolation of a mutant of strain GJ1 able to grow with 2-bromoethanol and describe the overexpression, purification, and characterization of an NAD-dependent aldehyde dehydrogenase involved in bromoethanol metabolism from this mutant.

Materials and methods

Organism and growth conditions

Pseudomonas sp. strain GJ1 (Janssen et al. 1984) and mutants thereof (see below) were cultivated in MMY medium (Van den Wijngaard et al. 1992) in serum flasks at 30° C with rotary shaking at 200 rpm. Mutant GJ1M9 was isolated as described below. Substrates were added at 5 mM unless stated otherwise. For plates, 1.5% agar was added. Crude extracts for enzyme assays were obtained from batch cultures of 100–400 ml grown to the late exponential phase as described by Janssen et al. (1985). Cells were washed with 25 mM Tris-H_2SO_4 buffer, pH 7.5, and lysed by sonication. Extract was obtained by centrifugation (10 min; 10,000 × *g*).

For purification of chloroacetaldehyde dehydrogenase, mutant GJ1M9 was grown for 5 days at 30° C in a 10-l fermentor (Braun Biostat E, Melsungen, Germany) at 60% oxygen saturation in MMY medium supplemented with additional yeast extract (10 mg/l) and $MgSO_4$ (10 mM). The pH was kept at 7.0 by addition of 10% (w/v) NH_4OH. A total amount of 115 mM 2-chloroethanol was added as the carbon and energy source at different times. Two days before harvesting, 3 mM of 2-bromoethanol was added. The final density of the culture was 1.7 mg cell dry weight/ml.

Isolation of a 2-bromoethanol-utilizing mutant

A 2-bromoethanol-resistant mutant of strain GJ1, designated strain GJ1M2, was isolated on a plate containing 5 mM 2-chloroethanol and 3 mM 2-bromoethanol. This mutant was used to isolate new mutants that were able to grow with 2-bromoethanol by repeatedly subculturing and gradually decreasing the concentration of 2-chloroethanol. From time to time, the growth rate was determined with different mixtures of 2-chloroethanol and 2-bromoethanol. When the growth rate had increased, the strain was purified and grown with a lower concentration of 2-chloroethanol and a higher concentration of 2-bromoethanol. Eventually a mutant that was able to grow with 5 mM 2-bromoethanol as the sole carbon and energy source was isolated and designated strain GJ1M9.

Enzyme purification

For the purification of NAD-dependent chloroacetaldehyde dehydrogenase, 8.4 g cells were harvested by continuous centrifugation with a Sharples TIP, resuspended in 200 ml of PEMAG buffer [20 mM potassium phosphate (pH 7.0) containing 1 mM EDTA, 5 mM β-mercaptoethanol, 0.03% NaN_3, and 20% (w/v) glycerol], and sonicated for 12 min in an Ultrasonics W-375 sonicator at 250 W output under permanent cooling. Unbroken cells and debris were removed by centrifugation in a 60 Ti rotor at 143,000 × *g* for 2.5 h. The crude extract was applied to a DEAE-cellulose column (2.5 × 20 cm) that had been equilibrated with PEMAG. The column was washed with 400 ml PEMAG and then eluted with a linear gradient of 0 to 350 mM KCl in PEMAG (total volume 1000 ml). Active fractions were pooled and dialyzed against PEMAG. The dialyzed solution was applied to a hydroxylapatite column (4 × 15 cm) equilibrated with PEMAG and washed with 100 ml of PEMAG. Elution was carried out with a linear gradient of the same buffer with the phosphate concentration increasing from 20 to 150 mM (total volume 1500 ml). Active fractions were pooled and concentrated to a volume of 60 ml with a Diaflow filter PM30 (Amicon). The concentrated enzyme solution was applied in three portions to a Sephacryl S-300 gelfiltration column (4 × 60 cm) equilibrated with PEMAG containing 200 mM KCl. Active fractions were combined and dialyzed against PEMAG.

Determination of enzyme activities

NAD-dependent aldehyde dehydrogenase activities in crude extracts were determined spectrophotometrically at 30° C by following NADH production at 340 nm in 200 mM glycine-NaOH (pH 9.0), 50 mM NH_4Cl, 10% (w/v) glycerol, 1 mM NAD, 80 mM β-mercaptoethanol, 1 mM DTT, and 4.6 mM of substrate, unless stated otherwise.

K_m and V_{max} values for chloroacetaldehyde and bromoacetaldehyde were calculated from initial velocities measured at varying substrate concentrations. Data were directly fitted to the Michaelis-Menten equation by non-linear regression analysis. With the other aldehydes, for which the enzyme has very low K_m values, data were obtained by following NADH production at low initial substrate concentrations. Aldehyde concentrations at 20–50 time points were calculated from NADH concentrations, and the resulting aldehyde concentration progress curves were directly fitted to the integrated Michaelis-Menten equation by non-linear regression.

Since aldehydes are unstable compounds, the concentration of non-halogenated aldehydes was confirmed by calculating the total amount of NADH formed when a limiting amount of substrate was used in an aldehyde dehydrogenase assay. The concentrations of chloroacetaldehyde and bromoacetaldehyde were determined by NMR.

Phenazine-methosulfate (PMS)-dependent alcohol dehydrogenase activities were measured at 30° C by following DCPIP reduction in a spectrophotometer at 600 nm in a reaction mixture (1 ml) containing 100 mM Tris-H_2SO_4 (pH 9.0), 90 mM NH_4Cl, 200 μM NaCN, 100 μM DCPIP, 250 μM PMS, 25 mM of substrate, and a suitable amount of crude extract.

NAD-dependent alcohol dehydrogenase was determined at 30° C by following NADH production spectrophotometrically at 340 nm in a reaction mixture containing 100 mM Tris-H_2SO_4 (pH 9.0), 90 mM NH_4Cl, 1 mM NAD, and 25 mM of substrate. The assay was started by the addition of enzyme.

Haloacetate dehalogenase activity was determined by following halide liberation in a colorimetric assay with 5 mM chloroacetate or bromoacetate as the substrate, as described previously (Janssen et al. 1985). Protein concentrations were measured with Coomassie brilliant blue using bovine serum albumin as a standard.

Gelelectrophoresis

SDS-PAGE was carried out on 7.5 or 10%-polyacrylamide gels. Molecular mass markers used were ovotransferrin (78 kDa),

bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa). To determine the relative amount of aldehyde dehydrogenase in crude extracts, SDS-PAGE gels were scanned with a desktop scanner and analyzed with the Quantiscan program (Biosoft Inc., Cambridge, UK).

Estimation of molecular mass

The molecular mass of the native enzyme was determined by gel filtration on Sephacryl S-300 as described above. Horse spleen ferritin (470 kDa), bovine catalase (247 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and horse liver cytochrome *c* (12.7 kDa) were used as reference proteins.

N-terminal sequence analysis

The N-terminal sequence of the purified protein was determined with a gas-phase protein sequencer (Applied Biosystems, model 477A) with on-line phenylthiohydantoin amino acid identification by ABI model 120A (Eurosequence, Groningen, The Netherlands). Prior to sequence analysis, free amino acids and salts were removed by centrifugation on polyvinylidenedifluoride (PVDF).

Preparation of bromoacetaldehyde

Bromoacetaldehyde was synthesized by hydrolysis of its diethyl acetal. A mixture of 10 ml (67 mmol) bromoacetaldehyde diethyl acetal (Aldrich Chemical, Axel, The Netherlands) and 0.5 M H_2SO_4 was boiled under reflux for 1 h. The turbid mixture was allowed to cool, connected with a claisen head, and carefully distilled during 2 h. The fraction boiling at 75–80° C was collected and adjusted to pH 7 with aqueous NaOH. This solution was extracted using t-butyl methyl ether (3×10 ml), dried (Na_2SO_4), and filtered. The ether was evaporated by a stream of nitrogen. The resulting solution showed reducing power towards Fehling's solution. The 200 MHz ^{1}H-NMR spectrum of this solution showed resonances for the methylene protons of bromoacetaldehyde and for ethanol. The characteristic triplet and quartet of the starting material at respectively 4.67 and 3.39 ppm, respectively, were absent. ^{1}H-NMR data (CD_3OD): δ 4.92 (bs, 2 H, CH_2BrCHO), 3.61 (q, 2 H, $HOCH_2CH_3$), 1.18 (t, 3 H, $HOCH_2CH_3$). The concentration of the bromoacetaldehyde was calibrated using ^{1}H-NMR spectroscopy with acetone as the standard.

Results

Isolation of mutants resistant to 2-bromoethanol

2-Bromoethanol is toxic for *Pseudomonas* sp. GJ1 at concentrations of 1 mM and higher. A mutant that was resistant to 2-bromoethanol (designated strain GJ1M2) was isolated on a MMY plate containing 5 mM of 2-chloroethanol and 3 mM of 2-bromoethanol, but the mutant was not able to grow with the latter compound as sole carbon and energy source. Mutant GJ1M2 was used to isolate mutant M9 by repeatedly subculturing with increasing concentrations of 2-bromoethanol and decreasing levels of 2-chloroethanol, as described in Material and methods. Mutant GJ1M9 was able to grow with 2-bromoethanol as the sole carbon and energy source and was used for further characterization.

Characterization of strain GJ1M9

Mutant M9 grew with 5 mM of 2-bromoethanol at a growth rate of 0.034 h^{-1}. Concentrations higher than 6 mM were toxic. The growth rate of strain GJ1M9 on 5 mM 2-chloroethanol was 0.102 h^{-1}, which is slightly higher than that of the wild-type strain (0.093 h^{-1}). Strain GJ1M9 appeared to grow better on plates containing *n*-butanol or allyl alcohol than did the wild-type strain.

Table 1 Enzyme activities in crude extracts. Extracts of strains GJ1 and GJ1M9 cultivated on different substrates were prepared and activities were measured with ethanol, haloethanol, haloacetaldehyde, or haloacetic acid as the substrate. Activities are expressed in mU/mg of protein

Growth substrate	Enzyme substrate	NAD-dependent alcohol dehydrogenase		PMS-dependent alcohol dehydrogenase		NAD-dependent alcohol dehydrogenase[a]		Haloacid dehalogenase	
		GJ1	GJ1M9	GJ1	GJ1M9	GJ1	GJ1M9	GJ1	GJ1M9
Citrate	Ethan(ol/al)	771	662	93	35	240	180	–	–
	2-Chloroethan(ol/al/oic acid)	< 10[b]	< 10	< 10	< 10	140	120	< 5	< 5
	2-Bromoethan(ol/al/oic acid)	< 10	< 10	< 10	< 10	240	200	< 5	< 5
Ethanol	Ethan(ol/al)	66	99	156	249	1940	4020	–	–
	2-Chloroethan(ol/al/oic acid)	< 10	< 10	195	306	1190	2850	< 5	< 5
	2-Bromoethan(ol/al/oic acid)	< 10	< 10	220	330	800	1480	< 5	< 5
2-Chloroethanol	Ethan(ol/al)	52	180	52	148	2710	4220	–	–
	2-Chloroethan(ol/al/oic acid)	< 10	< 10	64	184	1580	2900	505	481
	2-Bromoethan(ol/al/oic acid)	< 10	< 10	68	198	1000	1300	493	439
2-Bromoethanol	Ethan(ol/al)	–[c]	18	–	67	–	3220	–	–
	2-Chloroethan(ol/al/oic acid)	–	< 10	–	95	–	1840	–	570
	2-Bomoethan(ol/al/oic acid)	–	< 10	–	97	–	1010	–	502

[a] Substrate concentrations: acetaldehyde, 160 μM; chloracetaldehyde, 4.6 mM; bromoacetaldehyde, 20 mM
[b] <, Below detection limit
[c] –, No growth, not applicable

To investigate what caused the ability to grow with 2-bromoethanol, activities of enzymes involved in the degradation of 2-chloroethanol and 2-bromoethanol were determined in crude extracts of wild-type GJ1 and mutant GJ1M9. Mutant and wild-type cells contained an NAD-dependent alcohol dehydrogenase and a PMS-linked alcohol dehydrogenase after growth on citrate or ethanol (Table 1). Only the PMS-linked enzyme was active with 2-chloroethanol and 2-bromoethanol. Although the NAD-dependent enzyme was induced by 2-chloroethanol and 2-bromoethanol, it was not active with these substrates. Moreover, enzyme activity was inhibited by 2-chloroethanol (results not shown). The highest activity of the NAD-dependent enzyme in wild-type and mutant was found in extracts from citrate-grown cells. In the wild-type, activity of the PMS-linked alcohol dehydrogenase was highest in cells grown with ethanol. Cells of strain GJ1M9 produced higher amounts of the PMS-linked enzyme than the wild-type when the growth substrate was ethanol or 2-chloroethanol. Extracts of strain GJ1M9 from 2-bromoethanol-grown cells, however, contained a twofold lower amount of PMS-linked alcohol dehydrogenase than did extracts from 2-chloroethanol-grown cells.

Strains GJ1 and GJ1M9 contained an NAD-dependent aldehyde dehydrogenase activity that was elevated in cells grown on alcohols as compared to citrate-grown cells. A significant difference between wild-type and mutant GJ1M9 was observed when the activities of this enzyme were compared (Table 1). Activities with acetaldehyde and chloroacetaldehyde were about twofold higher in mutant GJ1M9 grown with ethanol or 2-chloroethanol. However, extracts of strain GJ1M9 grown on 2-bromoethanol contained lower activities than did extracts of strain GJ1M9 grown with ethanol or 2-chloroethanol, and only slightly higher activities than did extracts of the wild-type strain cultivated with 2-chloroethanol.

There was no significant difference in the levels of haloacetate dehalogenase between wild-type and mutant strains grown on 2-chloroethanol (Table 1). Haloacetate dehalogenase activity was inducible with 2-chloroethanol in both strains and with 2-bromoethanol in strain GJ1M9.

Crude extracts of wild-type and strain GJ1M9 were subjected to SDS-PAGE (Fig. 1A). A prominent band with a molecular mass of about 55 kDa was visible in extracts of strain GJ1M9 grown on 2-chloroethanol or 2-bromoethanol (Fig. 1A; *lanes* 3 and 4, respectively). This band was present in the highest amount in extracts of cells grown on 2-bromoethanol, and absent or present in smaller amounts in extracts of cells cultivated on citrate or ethanol. By scanning the gel, it was determined that this band represented 57% of the total protein in extracts from 2-bromoethanol-grown cells of strain GJ1M9. This is at least fourfold higher than extracts of the wild-type grown with 2-chloroethanol.

Purification and characterization of chloroacetaldehyde dehydrogenase

We hypothesized that the strongly overproduced protein in 2-bromoethanol-grown cells of strain GJ1M9 was

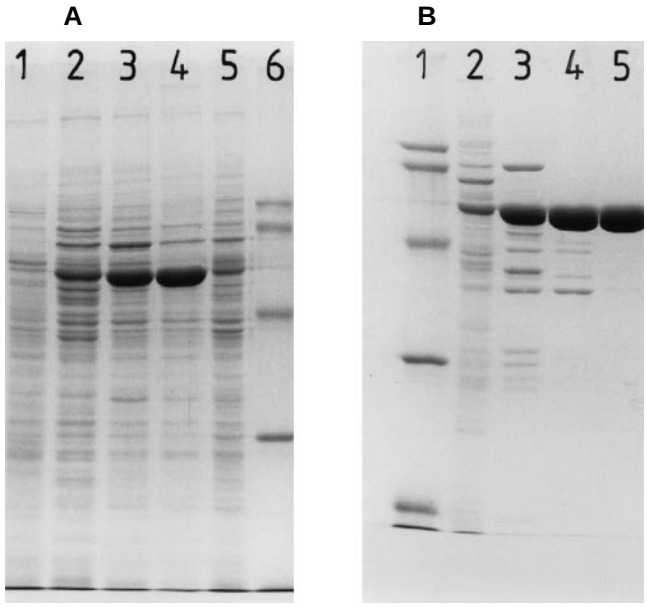

Fig. 1 **A** SDS-PAGE of crude extracts of wild-type *Pseudomonas* sp. GJ1 and mutant GJ1M9 grown on different substrates. *Lanes 1* GJ1M9 grown on citrate, *2* GJ1M9 grown on ethanol, *3* GJ1M9 grown on 2-chloroethanol, *4* GJ1M9 grown on 2-bromoethanol, *5* GJ1 grown on 2-chloroethanol, *6* marker proteins. **B** SDS-PAGE of chloroacetaldehyde dehydrogenase containing fractions during purification from mutant GJ1M9. *Lanes 1* Marker proteins, *2* crude extract, *3* DEAE-cellulose, *4* hydroxylapatite, *5* Sephacryl S-300

NAD-dependent chloroacetaldehyde dehydrogenase. This enzyme was therefore purified from mutant GJ1M9 as described in Materials and methods (Table 2, Fig. 1B). Initial experiments with crude extracts showed that glycerol had a stabilizing effect on enzyme activity. Without glycerol, 50% of the activity was lost in 24 h at 4° C. When 20% (w/v) glycerol was added, the enzyme lost only 25% of its activity in one month.

The enzyme was purified form a 10-l culture grown in a batch fermentor on 2-chloroethanol and induced prior to harvesting with 2-bromoethanol. After five purification steps, a single band on SDS-polyacrylamide gels was obtained (Fig. 1B, lane 5). The protein was purified eightfold, with a yield of 27% of the total cellular protein. Obviously, the expression of the enzyme was not as high in the fermentor culture as in crude extracts from cells grown on 2-bromoethanol in batch culture.

The molecular mass of the purified enzyme was estimated to be 55 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1B), in agreement with the band that was found to be overexpressed in crude extract (Fig. 1A). The native enzyme had a molecular mass of 220 kDa as determined by gel filtration on Sephacryl S-300. This indicates that the enzyme is a tetrameric protein.

The first 30 amino acids of the N-terminal sequence of the purified enzyme were determined and compared with those of other bacterial aldehyde dehydrogenases present in the SWISS-PROT protein database or whose amino acid sequence was derived from the nucleotide sequence. There was homology with the aldehyde dehydrogenases

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Pseudomonas sp. GJ1M9      MITAQPGTPGAVVSFKPRTGNFIGGEFVQP
Alcaligenes eutrophus      MNMAEIAQLGVSNPYKQQYENYIGGAWVPP
Vibrio cholerae            MIYPIPNSETSTVHFKDVYDNYIGGQWMKP
                           *  •          ••     *••**  • *
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Fig. 2 N-terminal sequence of the purified enzyme from strain GJ1M9, and ClustalV alignment with aldehyde dehydrogenases from *Alcaligenes eutrophus* (Priefert et al. 1992) and *Vibrio cholerae* (Parsot and Mekalanos 1991). *Asterisk* Identical residue, *dot* similar residue

from *Alcaligenes eutrophus* (Priefert et al. 1992) and *Vibrio cholerae* (Parsot and Mekalanos 1991; Fig. 2). There was no detectable homology between N-terminal sequences of the enzyme of strain GJ1 and the chloroacetaldehyde dehydrogenase from *X. autotrophicus* strain GJ10.

Kinetic constants

The K_m and V_{max} values of the purified enzyme for several aldehydes were determined in the presence of 1 mM NAD. Low K_m values were found for non-halogenated aldehydes, which made it necessary to use substrate consumption progress curves to obtain accurate kinetic data.

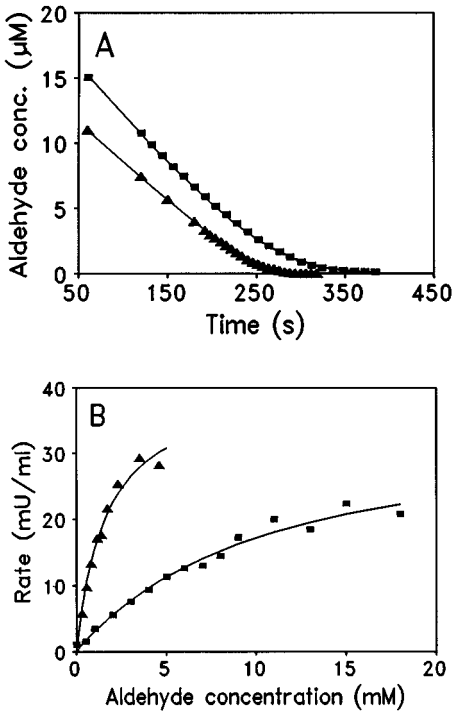

Fig. 3A, B Kinetics of purified aldehyde dehydrogenase. **A** Substrate depletion curves for acetaldehyde (*squares*) and propionaldehyde (triangles); **B** velocity vs. substrate concentration curves for chloroacetaldehyde (*triangles*) and bromoacetaldehyde (*squares*). Solid lines indicate fitted curves calculated by non-linear regression

Table 2 Purification of NAD-dependent chloroacetaldehyde dehydrogenase from strain GJ1M9

Step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	4165	6000	1.44	100	1.0
DEAE-cellulose	968	4900	5.7	82	3.5
Hydroxylapatite	312	3090	9.87	52	6.9
Sephacryl S-300	144.3	1590	11.1	27	7.7

[a] Activity was measured with 4.6 mM of chloroactealdehyde

Fitting the integrated Michaelis-Menten equation to such progress curves yielded K_m values in the range of 0.8–4.0 µM for non-halogenated aldehydes (Fig. 3A, Table 3). The K_m values for chloroacetaldehyde and bromoacetaldehyde were 3 and 4 orders of magnitude higher, respectively, and were calculated from initial velocities measured at varying substrate concentrations (Fig. 3B, Table 3). The V_{max} values for the halogenated and non-halogenated substrates were not very different.

Inactivation of the enzyme by bromoacetaldehyde in vivo

The difference between chloroacetaldehyde dehydrogenase activities of cells of strain GJ1 grown on 2-chloroethanol and of cells of strain GJ1M9 grown on 2-bromoethanol (Table 1) was much smaller than the difference in protein band intensities of these extracts as observed on SDS-PAGE. This suggested that a fraction of the enzyme from strain GJ1M9 grown on 2-bromoethanol might be inactive. To determine whether this inactivation might be caused by substrates, strain GJ1M9 was cultivated in 400 ml MMY medium with 5 mM 2-chloroethanol as the carbon and energy source. When the chloride concentration in the medium was 4 mM, 2-bromoethanol (3 mM) and chloroamphenicol (150 µg/ml) were added, and chloroacetaldehyde dehydrogenase activity was measured in crude extracts at different times (Fig. 4). Within 4.5 hours more than 85% of the enzyme activity was lost, and after 22 hours only 3% of the initial activity was left. When only 2-bromoethanol was added, a similar initial decrease was observed after 4.5 hours, but the activity was gradually restored. When only chloroamphenicol was added without any addition of bromoethanol, a much slower decrease in enzyme activity was observed, indicating that the presence of 2-bromoethanol indeed caused inactivation of the aldehyde dehydrogenase. SDS-PAGE showed that the relative amount of the

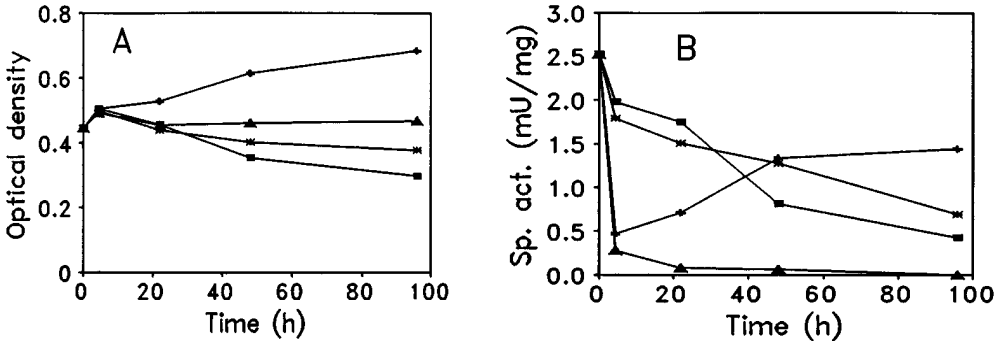

Fig. 4A, B Effect of 2-bromoethanol on aldehyde dehydrogenase activity of strain GJ1M9. Cells were precultivated on 2-chloroethanol. After dividing the culture in four parts, the effect of different additions on **A** growth and on **B** chloroacetaldehyde dehydrogenase activity was determined. *Squares* Control, no addition; *crosses* bromoethanol added; *triangles* bromoethanol plus chloramphenicol added; *asterisks* chloramphenicol added

Table 3 Kinetic constants of purified aldehyde dehydrogenase

Substrate	K_m (μM)	V_{max} (U/mg)
Acetaldehyde	4.0	8.8
Propionaldehyde	1.0	6.6
Butanal	0.8	5.7
Pentanal	1.2	6.9
Hexanal	1.7	4.9
Chloroacetaldehyde	1.7×0^3	7.7
Bromoacetaldehyde	10.5×10^3	6.3

aldehyde dehydrogenase protein in crude extracts did not change upon addition of chloroamphenicol or 2-bromoethanol and chloroamphenicol, but increased after addition of 2-bromoethanol (data not shown). This indicates that the increase in enzyme activity was due to newly synthesized enzyme and that the loss of activity caused by 2-bromoethanol was due to inactivation without degradation of the protein.

Discussion

In this paper we show that adaptation of the 2-chloroethanol-utilizing *Pseudomonas* sp. strain GJ1 to the toxic compound 2-bromoethanol is possible and that it is accompanied by the overexpression of an NAD-dependent aldehyde dehydrogenase. The level of this enzyme is already high in the wild-type strain, where it is the most abundant protein in cells grown on 2-chloroethanol (Fig. 1A), but amounts to more than half of the total cellular protein in the mutant strain grown on 2-bromoethanol. The subunit composition, cofactor dependence, and N-terminal amino acid sequence of this aldehyde dehydrogenase suggest that it is a normal NAD-coupled aldehyde dehydrogenase belonging to a large group of evolutionarily related enzymes. This includes cytoplasmic and mitochondrial aldehyde dehydrogenases from various eukaryotic organisms, and several fungal and bacterial enzymes. Overexpression of an enzyme of this class has also been observed in a mutant of *A. eutrophus* that utilizes ethanol (Jendrossek et al. 1988).

An NAD-dependent aldehyde dehydrogenase with elevated activity for chloroacetaldehyde is also necessary for growth with 2-chloroethanol in *X. autotrophicus* strain GJ10 (Tardif et al. 1991; Van der Ploeg et al. 1994). This strain seems to have acquired the chloroacetaldehyde dehydrogenase during adaptation to 1,2-dichloroethane and 2-chloroethanol, as indicated by the localization of the haloalkane dehalogenase and haloacetaldehyde dehydrogenase genes on a plasmid. The aldehyde dehydrogenase was the most abundant enzyme in crude extracts of cells grown on 1,2-dichloroethane in this organism. Strain GJ10, however, is not able to grow with 2-bromoethanol, and attempts to adapt strain GJ10 to 2-bromoethanol have not been successful.

2-Bromoethanol is a possible intermediate in mammalian 1,2-dibromoethane metabolism (Rannug 1980) that can be further converted to bromoacetaldehyde, which may be the main cause of the mutagenicity of 1,2-dibromoethane and 2-bromoethanol (Banerjee et al. 1979; McLean et al. 1987). Microorganisms that are able to grow with 2-bromoethanol have not been described before, but some hydrolytic dehalogenases can cleave the carbon-bromine bond of 2-bromoethanol (Janssen et al. 1987; Van den Wijngaard et al. 1992). The toxicity of bromoacetaldehyde also seems to be an important cause of the inability of these organisms to grow with 2-bromoethanol. Mutants of haloalkane-utilizing bacteria that are resistant to bromoethanol or 1,2-dibromoethane have been previously found to lose alcohol dehydrogenase or haloalkane dehalogenase, respectively (Janssen et al. 1987; Van der Ploeg et al. 1994).

The adaptation of strain GJ1 to 2-bromoethanol is thus related to increased expression of aldehyde dehydrogenase. A similar mechanism of adaptation has been observed in mutants of *X. autotrophicus* strain GJ10 resistant to toxic levels of bromoacetate. These mutants showed increased expression of haloacetate dehalogenase (Van der Ploeg et al. 1995). It remains to be determined which mutations caused increased expression of aldehyde dehydrogenase in strain GJ1M9 and if this overexpression is the only cause of 2-bromoethanol resistance. Additional mutations that allow for better growth may have occurred during the adaptation period. For example, PMS-linked

alcohol dehydrogenase activities in the mutant strain were also higher than in the wild-type, but only when the growth substrate was ethanol or 2-chloroethanol. The relatively low level of PMS-linked alcohol dehydrogenase in cells grown with 2-bromoethanol could also prevent the formation of high levels of toxic bromoacetaldehyde.

The observed overexpression of aldehyde dehydrogenase in the mutant may have two effects facilitating growth on 2-bromoethanol. First, it helps to overcome the low affinity of the enzyme for bromoacetaldehyde. Such a low affinity makes it necessary to maintain high intracellular concentrations of the aldehyde in order to obtain the rate of substrate metabolism required for growth. In principle, the intracellular concentration of a toxic intermediate that is needed for a certain growth rate can be decreased by increasing the cellular content of the enzyme that converts it, by increasing the V_{max} of the enzyme, or by increasing the affinity of the enzyme for the substrate. The first is what we observed in the bromoethanol-resistant mutants. The other reason for overexpression of the aldehyde dehalogenase is to compensate for the loss of enzyme by irreversible substrate inactivation in vivo. What causes the inactivation during bromoethanol conversion has not been determined, but it is likely that bromoacetaldehyde alkylates the conserved cysteine residue that is involved in activity (Blatter et al. 1992). This cysteine is sensitive to alkylating sulfhydryl reagents (Hempel et al. 1982), and the electrophilic bromine-bound carbon atom of bromoacetaldehyde may undergo nucleophilic attack by this cysteine.

In view of the high toxicity and reactivity of both chloroacetaldehyde and bromoacetaldehyde, the high K_m values of the aldehyde dehydrogenases for both substrates are remarkable, especially when they are compared to the much lower K_m values for non-halogenated aldehydes. The K_m value of the purified chloroacetaldehyde dehydrogenase from *X. autotrophicus* strain GJ10 for chloroacetaldehyde was about 10-fold lower than that of strain GJ1M9, but was still higher than that reported for an enzyme purified from horse liver (Eckfeldt and Yonetani 1982) and for that from *Pseudomonas putida* strain US2 (Strotmann et al. 1990), which had a K_m value of 27 μM for chloroacetaldehyde. The high K_m value of the enzymes that convert haloacetaldehydes might not be fortuitous. The rate-limiting step in aldehyde dehydrogenases was proposed to be product (NADH) release from the enzyme (MacGibbon et al. 1977). A low affinity of the enzyme for the aldehyde would lower the steady-state concentration of an enzyme · aldehyde or an enzyme · NAD · aldehyde Michaelis complex, which might help to prevent side reactions that cause inactivation. Thus, the enzyme may be optimized in the sense of slow binding and rapid turnover of the substrate.

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