Utilization of Trihalogenated Propanes by Agrobacterium radiobacter AD1 through Heterologous Expression of the Haloalkane Dehalogenase from Rhodococcus sp. Strain m15-3

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Trihalogenated propanes are toxic and volatile organic compounds. Attempts to obtain pure bacterial cultures able to use these compounds as sole carbon and energy sources were unsuccessful. Both the haloalkane dehalogenase from Xanthobacter autotrophicus GJ10 (DhIA) and that from Rhodococcus sp. strain m15-3 (DhaA) were found to dehalogenate trihalopropanes to 2,3-dihalogenated propanols, but the kinetic properties of the latter enzyme are much better. Broad-host-range dehalogenase expression plasmids, based on RSF1010 derivatives, were constructed with the haloalkane dehalogenase from Rhodococcus sp. strain m15-3 under the control of the heterologous promoters P

The resulting plasmids yielded functional expression in several gram-negative bacteria. A catabolic pathway for trihalopropanes was designed by introducing these broad-host-range dehalogenase expression plasmids into Agrobacterium radiobacter AD1, which has the ability to utilize dihalogenated propanols for growth. The recombinant strain AD1(pTB3), expressing the haloalkane dehalogenase gene under the control of the dhlA promoter, was able to utilize both 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane as sole carbon sources. Moreover, increased expression of the haloalkane dehalogenase resulted in elevated resistance to trihalopropanes.

In the present study, we describe the steady-state kinetics of the conversion of trihalogenated propanes by the haloalkane dehalogenases from Xanthobacter autotrophicus GJ10 (DhIA) and Rhodococcus sp. strain m15-3 (DhaA). The gene encoding the latter dehalogenase was engineered to be under the control of different heterologous promoters and was functionally expressed in several gram-negative bacteria. High expression of the haloalkane dehalogenase was obtained in A. radiobacter AD1, allowing this strain to grow on 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. T. Omori kindly provided Rhodococcus sp. strain m15-3, formerly Corynebacterium sp. strain m15-3 (39). Based on 16S rRNA gene sequence analysis, strain m15-3 was identified as a Rhodococcus sp. (25a).

Escherichia coli strains were grown in Luria-Bertani (LB) medium (30) at 30°C. The synthetic mineral (MMY) medium used in all growth experiments with A. radiobacter AD1, derivatives thereof, and Pseudomonas strains contained (per liter) 5.4 g of NaHPO₄ · 12H₂O, 1.4 g of KH₂PO₄, 0.5 g of (NH₄)₂SO₄, 0.2 g of MgSO₄ · 7H₂O, 5 ml of trace element metal solutions (15), and 5 mg of yeast extract. For the preparation of crude extracts for enzyme assays, A. radiobacter...
AD1 and the Pseudomonas strains were grown in MMY medium containing 5 mM citrate. For plates, 1.5% agar was added. Liquid cultures were cultivated at 30°C if not stated otherwise, with rotary shaking (200 to 250 rpm). When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/mL; kanamycin, 50 μg/mL, and chloramphenicol, 10 μg/mL. Utilization of halogenated compounds by strain AD1 or its recombinant derivatives was determined in batch cultures to which substrates were added at the concentrations indicated. Growth was followed by measuring the turbidity at 450 nm. For the toxicity experiments, strains AD1, AD1(pTB1), and AD1(pTB3) were grown in MMY medium containing 5 mM 1,3-dichloro-2-propanol and increasing concentrations of 1,2,3-trichloropropane, 1,2,3-tribromo-propane, or 1,2-dibromo-3-chloropropane. The optical density at 450 nm (OD450) was determined after 7 days of incubation.

Preparation of crude extracts. Crude extracts were prepared by sonication of cells grown to the stationary phase as described by van den Wijngaard et al. (34).

Isolation and manipulation of DNA. Plasmid isolation, DNA amplification, and restriction enzyme digestions, ligations, and transformations were performed as described by Sambrook et al. (30) or according to the specifications of the manufacturer of the materials used. Total DNA from bacteria is described by Poelarends et al. (26).

Construction of the broad-host-range expression plasmids. The dhaA gene was amplified by PCR using total DNA of \textit{Pseudomonas sp. strain GJ1} and \textit{Rhodococcus sp. strain m15-3} as template. PCR amplification was performed with Taq polymerase (Boehringer Mannheim, Mannheim, Germany) using a PCR program of 25 to 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s, followed by a final extension step of 72°C for 5 min. Oligonucleotides used for the amplification were \begin{verbatim}
5' Ap* - CGTTGGGGTGTCAGGTTTGGCATTG (reverse), where the
and 3' NcoI restriction sites are underlined. The PCR
product was ligated with BamHI and ligated into pBB-HI vector pRD215, resulting in pTB5.

For the construction of pKKdhaA, the dhaA gene was PCR amplified with primer p10, 5'AAAATCCGCACTGCAAGAATCGTTG (forward), and p11, 5'TGCAACTGCGTACCAGATGAAC (reverse), where the NcoI site is underlined and the start codon is in boldface. After restriction with NcoI, the dhaA gene was ligated into \\

Pseudomonas putida US2 and GJ31. After incubation for 16 to 20 h, the mating mixtures were replicated on separate nonselective plates previously spread with the recipient strains \textit{A. radiobacter AD1, Pseudomonas sp. strain GJ1, and Pseudomonas putida US2 and GJ31}. After incubation for 16 to 20 h, the mating mixtures were replicated on MMY agar plates supplemented with 5 mM citrate and kanamycin. Transconjugants were selected and tested for dehalogenase activity.

Purification of haloalkane dehalogenase. The haloalkane dehalogenases were expressed with a T7 promoter-based expression system and were purified by DEAE-cellulose and hydroxylapatite chromatography according to the method of Schimanski et al. (31).

Enzyme assays. Haloalkane dehalogenase assays were routinely carried out at 30°C by incubating appropriate amounts of purified enzyme solution or crude extracts in 50 mM NaHCO₃-NaOH buffer, pH 9.4, for DhaA, or in 50 mM Tris-SO₄ buffer, pH 8.2, for DhlA. Dehalogenase activities were measured by determining levels of halide released from halogenated substrates as described previously (20). Samples were taken at different times (5 to 45 min), and halide concentrations (0.1 to 1 mM) were measured colorimetrically at 460 nm after the addition of mercuric thiocyanate and ferric ammonium sulfate. Substrates were added to the following concentrations: 1,2-dibromoethane, 10 mM; 1,2,3-tribromopropane, 2.5 mM; 1,2-dibromo-3-chloropropane, 3 mM; and 1,2,3-trichloropropane, 10 mM. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of halide per min.

The \textit{K}_{m} values for trichloroethene were determined by measuring the initial rate of haloalcohol or halide production at various concentrations of halogenated substrate. The \textit{K}_{m} values were estimated by nonlinear regression analysis of the initial rate of haloalcohol and halide production as described by Schanstra et al. (32). Transformants of \textit{E. coli HB101}, containing dehalogenase expression plasmids, were screened for dehalogenase activity in 96-well plates or

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics\textsuperscript{a}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli} HB101</td>
<td>recA mutant, used for transformation of \textit{RSF1010} derivatives</td>
<td>6</td>
</tr>
<tr>
<td>\textit{A. radiobacter AD1}</td>
<td>Utilizes dehalogenated propanols</td>
<td>34</td>
</tr>
<tr>
<td>\textit{Pseudomonas sp. strain GJ1}</td>
<td>Utilizes 2-chloroethanol</td>
<td>14</td>
</tr>
<tr>
<td>\textit{P. putida US2}</td>
<td>Utilizes 2-chloroethanol</td>
<td>33</td>
</tr>
<tr>
<td>\textit{P. putida GJ31}</td>
<td>Utilizes long-chain alcohols</td>
<td>25</td>
</tr>
<tr>
<td>\textit{Rhodococcus sp. strain m15-3}</td>
<td>DhaA-producing strain</td>
<td>39</td>
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<tr>
<td>pKK233-2</td>
<td>\textit{Ap}\textsuperscript{+}, \textit{P}\textsuperscript{lac}</td>
<td>1</td>
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<td>pGEF\textsuperscript{+}</td>
<td>\textit{Ap}\textsuperscript{+}, \textit{P}\textsuperscript{lac}</td>
<td>28</td>
</tr>
<tr>
<td>pKKdhaA</td>
<td>\textit{dhaA} gene cloned into the \textit{NcoI} site of pKK233-2</td>
<td>This work</td>
</tr>
<tr>
<td>pDSK519</td>
<td>\textit{RSF1010} derivative, Km\textsuperscript{2}, \textit{P}\textsuperscript{lac}</td>
<td>19</td>
</tr>
<tr>
<td>pJRD215</td>
<td>\textit{RSF1010} derivative, Km\textsuperscript{2}</td>
<td>9</td>
</tr>
<tr>
<td>pKK600</td>
<td>Km\textsuperscript{2}, transfer functions for mobilization of \textit{RSF1010} derivatives</td>
<td>11</td>
</tr>
<tr>
<td>pTB1</td>
<td>\textit{dhaA} inserted as a 0.9-kb \textit{BamHI} fragment in pDSK519</td>
<td>This work</td>
</tr>
<tr>
<td>pTB3</td>
<td>1.5-kb \textit{BamHI-BamHI} fragment of \textit{P}\textsuperscript{lac}-\textit{dhaA} inserted in pJRD215</td>
<td>This work</td>
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<tr>
<td>pTB5</td>
<td>1.4-kb \textit{BamHI} fragment of \textit{P}\textsuperscript{lac}-\textit{dhaA-rmb} transcription terminator inserted in pJRD215</td>
<td>This work</td>
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\textsuperscript{a} \textit{Ap}, ampicillin; Km, kanamycin; Cm, chloramphenicol.
The steady-state parameters and products of trihalogenated propane conversion of haloalkane dehalogenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DhaA (kcat (s⁻¹))</th>
<th>DhlA (kcat (s⁻¹))</th>
<th>Halogenated product formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,3-Trichloropropane</td>
<td>3.6</td>
<td>2.3</td>
<td>2,3-Dibromo-1-propanol</td>
</tr>
<tr>
<td>1,2,3-Trimethoxane</td>
<td>14.3</td>
<td>13.9</td>
<td>2-Bromo-1-propanol</td>
</tr>
</tbody>
</table>

*See reference 32.

**Highest concentration checked. The Km values had errors ±25% and the kcat values had errors ±15%.

**RESULTS**

Specificity of haloalkane dehalogenase for trihalogenated propanes. To evaluate the activity of the haloalkane dehalogenases from *Rhodococcus rhodochrous* GJ10 (DhaA) and *Rhodococcus diobacter* sp. strain m15-3 (DhaA) on trihalogenated propanes, we used the recombinant enzymes expressed in *E. coli*. The quantity of both enzymes comprised up to 50% of the total soluble protein of *E. coli* BL21(DE3) when the expression was under the control of the T7 promoter using the expression vector pGEF (28). Recent work in our laboratory (25a) showed that the haloalkane dehalogenase gene from strain m15-3 is identical to that of the corresponding gene from *Rhodococcus rhodochrous* NCIMB 13064 (21). Based on sequence comparisons between DhIA and DhaA, these proteins are expected to have similar structures and catalytic mechanisms, but the substrate ranges are different (20, 39).

The steady-state kinetics of the conversion of trihalogenated propanes were studied using purified DhIA and DhaA. The halogenated products of these reactions were identified by gas chromatography and gas-chromatographic mass spectrometry analyses (Table 2). Both enzymes hydrolyzed trihalogenated propanes with a concomitant and stoichiometric accumulation of 2,3-dihalogenated propanol and halide. However, the kinetic properties of DhaA for trihalogenated propanes are much better (Table 2). For the trihalogenated propanes tested, DhaA had higher kcat values and lower Km values than DhIA. The enzyme specificity (kcat/Km) of DhaA for these chemicals is at least ninefold higher. Therefore, this enzyme was selected for the construction of a catabolic pathway for trihalogenated propanes. The kcat and Km values of DhaA were comparable for 1,2,3-trimethoxane and 1,2-dibromo-3-chloropropane. However, its affinity for 1,2-dibromo-3-chloropropane was somewhat lower. The chlorinated analog 1,2,3-trichloropropane was a poor substrate for the dehalogenase, as the enzyme had a high Km and a low kcat for this compound.

The activity of both enzymes towards 1,2-dibromomethane was striking. This compound is the best known substrate for DhIA in terms of kcat and Km. The kcat and Km values for DhaA were much higher than the values for DhIA.

Utilization of haloalcohols by *A. radiobacter* AD1. In previous studies, it was shown that *A. radiobacter* AD1 exhibited good growth kinetics for the conversion of 1,3-dichloro-2-propanol. The organism had a high affinity for this compound, which was correlated with a low Km value of the haloalcohol dehalogenase, the first catalytic enzyme in the pathway, for 1,3-dichloro-2-propanol (34, 35). Therefore, we used *A. radiobacter* AD1 as a possible host organism for the construction of a degradation pathway for trihalogeno propanes. Utilization of 2,3-dichloro- and 2,3-dibromo-1-propanol by strain AD1 was tested, since these compounds were produced during hydrolytic conversion of trihalogeno propanes by haloalkane dehalogenase. The results showed that strain AD1 was able to grow on both 2,3-dichloro- and 2,3-dibromo-1-propanol with similar generation times (Table 3). The highest growth rate was obtained on 1,3-dichloro-2-propanol with a generation time of 4 h. Strain AD1 completely utilizes both 2,3-dichloro-1-propanol and 1,3-dichloro-2-propanol. In contrast, approximately 50% conversion was found for 2,3-dichloro-1-propanol. Chiral gas chromatography of the culture fluid with a Chiraldex B-TA capillary column (30 m) (Astec) showed that only the (S)-enantiomer of 2,3-dichloro-1-propanol remained after growth had ceased, indicating that the conversion of 2,3-dichloro-1-propanol was stereospecific (32a), whereas both enantiomers of the brominated analog supported growth.

Construction of a broad-host-range dehalogenase expression plasmid. Expanding the substrate range of *A. radiobacter* AD1 by introducing the dhIA gene requires an efficient vector system and sufficient expression of the dehalogenase, since both the kinetic properties of the first catalytic enzyme and the dehalogenase content are important (35). We therefore constructed three different broad-host-range expression vectors.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Generation time (h)</th>
<th>Halide produced (mM)</th>
<th>Substrate remaining (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Dichloro-1-propanol</td>
<td>7</td>
<td>5.2</td>
<td>2.5</td>
</tr>
<tr>
<td>2,3-Dibromo-1-propanol</td>
<td>6</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1,3-Dichloro-2-propanol</td>
<td>4</td>
<td>11</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

TABLE 3. Utilization of dihalogenated propano

by *A. radiobacter* AD1

The steady-state parameters and products of trihalogenated propane conversion of haloalkane dehalogenase.
containing different promoters to direct expression of DhaA. The resulting expression vectors, pTB1, pTB3, and pTB5, are all based on the RSF1010 replicon, because the mobilization and replication of RSF1010 derivatives have been reported for Agrobacterium (3, 22). Figure 1 shows schematically the promoter dehalogenase regions of the constructed expression vectors.

In plasmid pTB1, the dhaA gene was fused to the 5' part of the lacZa gene and placed under the control of the lac promoter. This resulted in the formation of a fusion protein composed of the first 18 amino acids of LacZa followed by DhaA.

Plasmid pTB3 contained the dhaA gene under the control of the dhlA promoter from X. autotrophicus GJ10, which was previously shown to operate efficiently in several gram-negative bacteria (16). The dhaA gene, including its own transcription terminator (21), was attached by PCR fusion to a fragment containing both promoter sequences of the dhlA gene (16). Overlap between primers p4 and p5 resulted in the precise attachment of the promoter to the dehalogenase gene. The dhlA promoter fragment provided a strong ribosome binding site four nucleotides upstream from the start codon (16).

The E. coli hybrid trp-lac promoter, P_trc, was used to direct the expression of DhaA in plasmid pTB5. The vector carries the dhaA gene translationally fused to the trc promoter, the lacZ ribosome binding site, and the rrnB transcription terminators.

Kanamycin-resistant transformants of E. coli HB101 containing the different broad-host-range expression vectors were identified by screening for dehalogenase activity.

PCR-amplified segments of all constructs were sequenced. Only in plasmid pTB3 was a base substitution (C to T) at position –13 found in the spacer region between the –35 and –10 promoter sequences of the first dhlA promoter.

Expression of the dhaA gene in different gram-negative bacteria. To evaluate the use of the constructs for the expression of the dehalogenase, the recombinant plasmids were introduced by trip parental mating into different gram-negative bacteria. A. radiobacter AD1, Pseudomonas sp. strain GJ1, and P. putida US2 and GJ31 were used as recipients because these organisms could grow on various halogenated alcohols. Dehalogenase activities were measured in cell-free extracts with 1,2-dibromoethane as the assay substrate (Table 4). The broad-host-range plasmids are all based on the same replicon, RSF1010, so the efficiency of the different promoters can be compared without regard to copy number effects.

Low dehalogenase activities were obtained in strains containing plasmid pTB1, where the dhaA gene is under the control of the lac promoter. In all of these cases, the expression level of the dehalogenase was approximately 1% or less of the total soluble cellular protein. Higher specific activities were found in strains containing plasmid pTB3 or pTB5. The expression levels of the dehalogenase were comparable for these constructs, but levels varied with the host organism. Only small differences were observed between the efficiencies of the dhlA and trc promoters in various Pseudomonas species. P. putida US2 (pTB3) exhibited the highest dehalogenase activity. The enzyme amounted to almost 20% of the total soluble cellular protein in crude extracts. In A. radiobacter AD1, the dhlA and trc promoters generated similar dehalogenase levels. The haloalkane dehalogenase was present at approximately 7 to 8% of the total soluble cellular protein. The strength of the dhlA promoter is similar to that of the trc promoter, which is one of the strongest hybrid E. coli promoters (29). Introducing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Sp act (mU/mg of protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pTB1</td>
<td>pTB3</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>LB medium</td>
<td>150</td>
</tr>
<tr>
<td>A. radiobacter AD1</td>
<td>Citrate</td>
<td>320</td>
</tr>
<tr>
<td>Pseudomonas sp. strain GJ1</td>
<td>Citrate</td>
<td>80</td>
</tr>
<tr>
<td>P. putida GJ31</td>
<td>Citrate</td>
<td>190</td>
</tr>
<tr>
<td>P. putida US2</td>
<td>Citrate</td>
<td>240</td>
</tr>
</tbody>
</table>

* The specific activities are the means of two experiments with differences of ≤15%.
a NdeI restriction site at the ATG translation initiation codon of dhaA in pTB3 would facilitate the exchange of dhaA with other dehalogenase genes. However, the expression level of the dehalogenase in E. coli HB101 was almost fivefold lower (data not shown). Due to the introduction of this NdeI site, the triplet preceding the start codon has been changed from TCT to CAT. This may explain the decrease in dehalogenase activity, since it is known that this triplet can affect the efficiency of translation (13). The recombinant strain A. radiobacter AD1(pTB3) was further characterized with respect to utilization of trihalogenated propanes.

Utilization of trihalopropanes by A. radiobacter AD1(pTB3).

The introduction of halogen substituents into organic compounds may increase toxicity. Therefore we tested which concentrations of trihalogenated propanes were tolerated by strain AD1 and its derivatives (Fig. 2). The addition of increasing concentrations of trihalogenated propanes inhibited the growth of strains AD1, AD1(pTB1), and AD1(pTB3). Both 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane exhibited a high toxicity. Addition of these chemicals to a growing culture of strain AD1, AD1(pTB1), or AD1(pTB3) caused strong inhibition of growth and even cell death. No viable cells were obtained on nutrient broth plates from cultures that were previously incubated with the highest concentrations of 1,2,3-tribromopropane (1.5 mM) and 1,2-dibromo-3-chloropropane (1.6 mM). However, for the recombinant strain AD1(pTB3), the tolerance towards these compounds was increased due to a high expression level of DhaA. Of the trihalogenated propanes tested, 1,2,3-trichloropropane was least toxic. Moreover, the presence of DhaA did not increase the tolerance towards 1,2,3-trichloropropane, due to the low conversion rates of the dehalogenase for this compound.

Growth of the recombinant strain AD1(pTB3) with 1,2,3-tribromopropane, 1,2-dibromo-3-chloropropane, or 1,2,3-trichloropropane.

FIG. 2. Effect of increasing concentrations of trihalogenated propanes on growth of A. radiobacter strains AD1 (●), AD1(pTB1) (▲), and AD1(pTB3) (■) growing on 5 mM 1,3-dichloro-2-propanol. The turbidity (OD450) of the cultures was measured after 7 days of cultivation at 30°C. Initial OD450 values ranged from 0.5 to 0.7. (A) 1,2,3-tribromopropane; (B) 1,2-dibromo-3-chloropropane; (C) 1,2,3-trichloropropane.

FIG. 3. Growth and degradation of trihalogenated propanes by A. radiobacter AD1(pTB3). Cultures were grown aerobically at room temperature in MMY medium supplemented with 1 mM 1,2,3-tribromopropane (4 pulses), 1.2 mM 1,2-dibromo-3-chloropropane (2 pulses), or 1.6 mM 1,2,3-trichloropropane as carbon source. (A) 1,2,3-Tribromopropane (●), 2,3-dibromo-1-propanol (▲), OD450 (▲); (B) 1,2-dibromo-3-chloropropane (●), 2-bromo-3-chloro-1-propanol (▲), OD450 (▲); (C) 1,2,3-trichloropropane (●), 2,3-dichloro-1-propanol (▲), OD450 (▲); (D) (sterile controls) 1,2,3-tribromopropane (●), 1,2-dibromo-3-chloropropane (▲), 1,2,3-trichloropropane (▲).
chloropropane was monitored in batch cultures (Fig. 3). Because of their toxicity, pulses of 1 mM 1,2,3-trichloropropane and 1.2 mM 1,2-dibromo-3-chloropropane were added, while 1,2,3-trichloropropane was added at an initial concentration of 1.6 mM. The concentrations of 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane rapidly decreased immediately after the addition of these substrates. The subsequent addition of these compounds resulted in an increase of biomass.

The conversion of 1,2,3-trichloropropane proceeded at a much lower rate. After 25 days of incubation, approximately 0.7 mM of 1,2,3-trichloropropane was converted, yielding a very small increase in OD. Transformation of the trihalogenated propanes was due to enzymatic activity, since its concentration decreased much more slowly in sterile controls (Fig. 3). During conversion of the trihalogenated propanes, the corresponding halopropanols accumulated. The brominated propanols were completely converted, while a low amount of 2,3-dichloro-1-propanol remained present in the culture. The results thus showed that the recombinant strain AD1(pTB3) was able to rapidly dehalogenate 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane to the corresponding haloalcohols, which were subsequently utilized. The chlorinated analog 1,2,3-trichloropropane, which is a poor substrate for DhaA, was converted slower and stimulated growth to a much lower extent.

DISCUSSION

To obtain bacterial growth on trihalogenated propanes, we introduced the haloalkane dehalogenase from Rhodococcus sp. strain m15-3 (DhaA) into A. radiobacter AD1. The resulting strain could use the environmental chemicals 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane as sole carbon and energy sources. The proposed degradation pathway of strain AD1(pTB3) is shown in Fig. 4. The complete dehalogenation of trihalogenated propanes involves the combined activities of haloalkane dehalogenase, haloalcohol dehalogenase, and epoxide hydrolase, finally yielding glycerol, which is further metabolized by the organism (34). The conversion of 2,3-dihalogenated propanols by the host strain AD1 is similar to that of a Flavobacterium sp. utilizing 2,3-dibromo-1-propanol (7).

For growth on trihalogenated propanes, the kinetic properties ($K_a$ and $K_m$) of DhaA for trihalopropanes are of major importance. The best substrate for DhaA, 1,2,3-tribromopropane, was rapidly degraded by strain AD1(pTB3), whereas degradation of 1,2-dibromo-3-chloropropane by AD1(pTB3), for which the dehalogenase has a lower affinity, was somewhat slower. This is probably due to a lower conversion rate of 1,2-dibromo-3-chloropropane by DhaA, since the concentration used was approximately the $K_m$ value of the dehalogenase for this compound. Although 1,2,3-trichloropropane is a poor substrate for DhaA and did not support growth, strain AD1 (pTB3) was still able to convert about 46% of the 1,2,3-trichloropropane added in 25 days, yielding a small increase in OD. Furthermore, the haloalcohol dehalogenase produced by strain AD1 converted only the (R)-enantiomer of 2,3-dichloro-1-propanol, which also limited utilization of 1,2,3-trichloropropane. The stereospecific degradation of 2,3-dichloro-1-propanol by strain AD1 was similar to that of Pseudomonas sp. strain OS-K-29 (17).

A high expression level of DhaA in strain AD1 increased the conversion rates of trihalogenated propanes. Due to the low expression level of DhaA in strain AD1(pTB1), growth was only observed on 1,2,3-tribromopropane, and the maximum concentration tolerated was about 0.7 mM (data not shown). The low expression levels of DhaA in strains containing plasmid pTB1 are probably caused by the production of a fusion protein. In this construct, the dhaA gene was translationally fused to the 5' end of the lacZ gene. This could result in reduced stability of the mRNA, which depends on its sequence, secondary structure, and association with ribosomes (4), or increased degradation of the fusion protein. Because of the higher DhaA content, strain AD1(pTB3) was capable of growth on 1,2,3-tribromopropane to a concentration of 1.2 mM and could also grow on 1,2-dibromo-3-chloropropane.

Both the dhlA and trc promoters directed high-level constitutive expression of DhaA in several gram-negative bacteria. Because contaminated sites and industrial waste streams often contain mixtures of halogenated compounds, constitutive expression could be an advantage, since degradation of a wide range of haloalkanes could be enhanced due to the broad substrate range of DhaA.

Toxicity of halogenated aliphatics or the formation of toxic intermediates during conversion of these chemicals may inhibit bacterial growth. For example, the formation of bromoacetaldehyde is probably the cause of the inability of strain GJ10 to grow on 1,2-dibromoethane. The loss of the haloalkane dehalogenase causes resistance towards the latter compound (36). The 1,2-dibromoethane-utilizing strain Mycobacterium sp. strain GP1 circumvents the formation of bromoacetaldehyde as an intermediate (27). On the other hand, trihalogenated propanes are toxic themselves, since strain AD1 could efficiently grow on dihalogenated propanols, at least up to 5 mM. Here, the presence of a haloalkane dehalogenase causes resistance towards trihalogenated propanes.
The fact that a catabolic route can be easily established indicates that the absence of organisms that utilize trihalogenated propanes in the environment is due to the absence of dehalogenases for haloalcohols and for haloalkanes in a single organism. This could be due to the relatively recent entry of these compounds into the environment, giving microorganisms insufficient time to evolve appropriate pathways. The only example of an isolate which can produce both enzymes is a recently described isolate of *Mycobacterium sp*. strain GP1 which grows on 1,2-dibromomethane. This organism was obtained after prolonged adaptation and selection in batch culture (27).

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