APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2000, p. 219–222 Vol. 66, No. 1
0099-2240/00/$04.00+0

Dehalogenation of Haloalkanes by *Mycobacterium tuberculosis* H37Rv and Other Mycobacteria

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Received 3 June 1999/Accepted 18 October 1999

Haloalkane dehalogenases convert haloalkanes to their corresponding alcohols by a hydrolytic mechanism. To date, various haloalkane dehalogenases have been isolated from bacteria colonizing environments that are contaminated with halogenated compounds. A search of current databases with the sequences of these known haloalkane dehalogenases revealed the presence of three different genes encoding putative haloalkane dehalogenases in the genome of the human parasite *Mycobacterium tuberculosis* H37Rv. The ability of *M. tuberculosis* and several other mycobacterial strains to dehalogenate haloaliphatic compounds was therefore studied. Intact cells of *M. tuberculosis* H37Rv were found to dehalogenate 1-chlorobutane, 1-chlorodecane, 1-bromobutane, and 1,2-dibromoethane. Nine isolates of mycobacteria from clinical material and four strains from a collection of microorganisms were found to be capable of dehalogenating 1,2-dibromoethane. Crude extracts prepared from two of these strains, *Mycobacterium avium* MU1 and *Mycobacterium smegmatis* CCM 4622, showed broad substrate specificity toward a number of halogenated substrates. Dehalogenase activity in the absence of oxygen and the identification of primary alcohols as the products of the reaction suggest a hydrolytic dehalogenation mechanism. The presence of dehalogenases in bacterial isolates from clinical material, including the species colonizing both animal tissues and free environment, indicates a possible role of parasitic microorganisms in the distribution of degradation genes in the environment.

Hydrolytic dehalogenation by haloalkane dehalogenases is commonly observed as the first step in the aerobic degradation of synthetic haloalkanes that occur as soil pollutants (16). Haloalkane dehalogenases have been identified in organisms growing on 1-chloro-*n*-alkanes, 1-bromo-*n*-alkanes, or α,ω-dihalo-*n*-alkanes (13) and are not considered to be generally present in uncontaminated environments, since they were isolated above all from bacteria colonizing contaminated environments (8, 9, 13, 15).

The amino acid sequence of haloalkane dehalogenase is known for at least three bacteria: *Xanthobacter autotrophicus* GJ10 (dehalogenase DhlA) (12), *Sphingomonas paucimobilis* UT26 (dehalogenase LinB) (19, 20), and *Rhodococcus rhodochrous* NCIMB 13064 (dehalogenase DhaA) (14). These three sequences were compared with the sequences deposited in the genetic databases. This comparison revealed the presence of three different genes encoding for putative haloalkane dehalogenases on the chromosome of *Mycobacterium tuberculosis* H37Rv, whose complete genome has been sequenced recently (3).

The objectives of this study were to confirm activity of *M. tuberculosis* H37Rv toward halogenated aliphatic substrates and to study if other representatives of the genus *Mycobacterium* also show the ability to dehalogenate haloalkanes.

MATERIALS AND METHODS

Homology search, sequence alignment, solvent accessibility, and secondary structure prediction. The BLAST program (1) was used to screen protein and DNA databases for sequences that share similarity with the sequences of the haloalkane dehalogenases of *X. autotrophicus* GJ10 (accession no. M26950), *S. paucimobilis* UT26 (D14594), and *R. rhodochrous* NCIMB 13064 (L49435). The initial alignment of protein sequences was made by using multiple alignment algorithm CLUSTALW 1.7 (25) and further modified manually in the program Cameleon 3.14a (Oxford Molecular, Oxford, United Kingdom). Manual realignment was done in an iterative way: a three-dimensional model was constructed from each new alignment by using the program Modeller (22), and incorrectly modelled regions were identified by means of stereochemical validation and realigned. This procedure was repeated until no further improvements in the quality of the three-dimensional models could be obtained. Solvent accessibility and secondary structure were predicted by using the modelling servers PredAcc 1.0 (18) and JPred (4), respectively.

Bacteria, growth conditions, and chemicals. Ten *Mycobacterium* strains were isolated from clinical material at the Teaching Hospital Bohunice in Brno, Czech Republic: *M. avium* MU1, *M. parafortuitum* MU2, *M. triviale* MU3, *M. cheloneae* MU4, *M. xenopi* MU5, *M. flavescens* MU6, *M. kansasii* MU7, *M. fortuitum* MU8, *M. gordonae* MU9, and *M. bovis* BCG MU10. Four *Mycobacterium* strains were obtained from the Czech Collection of Microorganisms: *M. smegmatis* CCM 4622, *M. smegmatis* CCM 2300, *M. smegmatis* CCM 1693, and *M. phlei* CCM 5639. These fourteen tested organisms can be divided into three groups: (i) saprophytes (*M. parafortuitum*, *M. smegmatis*, and *M. phlei*), (ii) common facultative parasites (*M. avium*, *M. cheloneae*, *M. xenopi*, *M. kansasii*, and *M. fortuitum*), and (iii) less common facultative parasites (*M. triviale*, *M. flavescens*, *M. gordonae*, and *M. bovis* BCG). Mycobacterial strains were grown aerobically at 37°C on a medium containing (per liter of distilled water): 2 g of yeast extract, 2 g of Proteose Pepton no. 3, 2 g of Casitone, 2.5 g of $Na_2HPO_4 \cdot 12H_2O$, 1 g of KH_2PO_4, 1.5 g of sodium citrate, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of Tween 80, 50 ml of glycerol, and 20 g of agar. The pH of the medium was adjusted to 7.0. *M. tuberculosis* H37Rv (CNCTC My 331/80^T) was obtained from the Czechoslovak National Collection of Type Cultures in Prague, Czech Republic. This strain was grown on solid Löwenstein-Jensen's and Ogawa's media (17) under aerobic conditions. Biomass was harvested in glycine buffer-NaOH (pH 8.6), and resting cells were used for determination of dehalogenating activity by spectrophotometric measurements. *Rhodococcus erythropolis* Y2 (CCM 4426) and *Lactobacillus lactis* (CCM 1877^T) were obtained from the Czech Collection of Microorganisms and were used as the positive and negative controls in dehalogenation experiments, respectively. Halogenated compounds as well as other chemicals were obtained from Sigma-Aldrich.

Preparation of crude extracts. The cells grown on solid medium were harvested in glycine buffer-NaOH (pH 8.6), washed twice in the same buffer, frozen overnight, and disrupted by sonication (12 times for 1 min each). Intact cells were removed by centrifugation (300,000 × *g*, 4°C, 30 min). Prepared crude extracts were stored at −20°C for later use in the dehalogenation measurements by gas chromatography.

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Dehalogenation assay: colorimetry. To minimize manipulation with the viable cells of this human pathogen, a qualitative colorimetric assay was employed for initial screening of *M. tuberculosis* for the presence of dehalogenating activity. The buffer for the colorimetric assay consisted of 1 mM HEPES, 20 mM sodium sulfate, and 1 mM EDTA. The pH of the buffer was adjusted to 8.2 by adding NaOH. Phenol red was added to a final concentration of 0.02 mg/ml. The various substrates were consecutively dissolved in the buffer to a concentration of 10 mM. The assay was performed in 2-ml vials. Each vial received 1.47 ml of the substrate buffer solution and 30 μl of the cell suspension. The progress of the reaction was followed by the change in the buffer color from red through orange to yellow, due to a decrease of the pH in the reaction mixture, as described by Holloway et al. (10).

Dehalogenation assay: spectrophotometry. Dehalogenation experiments were performed in 25-ml Erlenmeyer flasks that were closed gas tight by headspace caps. Five milliliters of cell suspension (optical density at 600 nm, 0.8 to 1.0) in glycine buffer (pH 8.6) was incubated with a 10 mM concentration of substrate. The reaction mixture was incubated on a shaking water bath at 37°C. Samples (1 ml) were taken at 15, 30, and 45 min to monitor the progress of the reaction. The enzymatic reaction in a sample was terminated by adding 0.1 ml of 30% HNO_3, the reaction mixture was centrifuged, and the supernatant was mixed with mercuric thiocyanate and ferric amonium sulfate. Halide production was monitored spectrophotometrically at 460 nm (11). Dehalogenating activity was related to the biomass used in the assay and corrected for abiotic dehalogenation. The dehalogenation assay has been conducted in parallel with cells exposed to 5 mM 1-chlorobutane to test the inducibility of the dehalogenating enzymes. Abiotic dehalogenation was tested in glycine buffer without adding the cell suspension.

Dehalogenation assay: gas chromatography. Cultivation conditions were the same as those for the spectrophotometric assay. The progress of the reaction was monitored by taking 0.5-ml samples at 0.5, 3, and 10 h (assay with intact cells) and 15, 30, and 45 min (assay with crude extracts). The enzymatic reaction in the samples was terminated by addition of 0.5 ml of methanol. In tests with *M. tuberculosis*, the reaction mixture was sterilized for 25 min at 120 kPa to kill the cells and destroy their pathogenicity. Teflon caps were used to prevent evaporation of the product during autoclaving. The reaction mixture was directly applied on the gas chromatograph equipped with a flame ionization detector (Hewlett Packard 6890). A capillary column DB-FFAP (30 m by 0.25 mm by 0.00025 ml; J&W Scientific) was used for separation. Samples were injected by using split technique. The analyses were run isothermally at 30, 40, 60, or 100°C. The product was identified and quantified by using standards. The dehalogenation reaction rate was quantified by the amount of product formed within time.

Dehalogenation assay under anaerobic conditions. The headspace of 5 ml of crude extract was replaced by a mixture of CO_2:NO_2 (20:80). Trace elements of oxygen in solution were removed by the addition of a reduced form of glutathione to a concentration of 10 mM. Resazurin (0.00213 mM) was used as an indicator of anoxic conditions in the assay. The dehalogenating activity of the crude extracts toward three compounds was monitored by using gas chromatography.

RESULTS

Identification of three genes encoding putative haloalkane dehalogenases in *M. tuberculosis* H37Rv. The sequences of the haloalkane dehalogenases from *X. autotrophicus* GJ10 (DhlA), *S. paucimobilis* UT26 (LinB), and *R. rhodochrous* NCIMB 13064 (DhaA) were compared with DNA and protein sequences available in the current databases. This comparison revealed the presence of three putative haloalkane dehalogenase genes (*rv1833c*, *rv2579*, and *rv2296*) in *M. tuberculosis* H37Rv, which are spread along its chromosome. The extent of sequence homology between the different putative haloalkane dehalogenases is summarized in Table 1, and an alignment of the deduced amino acid sequences is shown in Fig. 1. The sequence identities suggest that three sequentially homologous dehalogenases from at least two different substrate specificity classes could be present in strain H37Rv. The sequence alignment revealed the presence of a putative catalytic triad and nucleophilic elbow in Rv1833c, Rv2579, and Rv2296, suggesting a hydrolytic reaction mechanism for these enzymes (6, 7). These observations lead to the hypothesis that *M. tuberculosis* and possibly also other members of the genus *Mycobacterium* contain hydrolytic haloalkane dehalogenases. Further experiments were performed to test this hypothesis.

Screening for dehalogenating activity with *M. tuberculosis* H37Rv. In order to determine whether *M. tuberculosis* H37Rv expresses dehalogenating enzymes, whole cells of strain H37Rv were analyzed for the presence of dehalogenase activity toward several haloaliphatic compounds. Whole cells of *M. tuberculosis* H37Rv showed dehalogenating activity with 1-chlorodecane, 1-bromobutane, and 1,2-dibromoethane, low activity toward 1-chlorobutane, and no activity toward 1,2-dichloroethane and 2-chloropropane. 1-Bromobutane, 1,2-dibromoethane, and 1-chlorobutane were converted to the corresponding monoalcohols, which indicates that dehalogenation of haloalkanes is a hydrolytic reaction in this organism.

TABLE 1. Sequence identity of haloalkane dehalogenases with putative H37Rv dehalogenases

Dehalogenase	% Sequence identity[a] with:					
	DhlA	LinB	DhaA	Rv1833c	Rv2296	Rv2579
DhlA						
LinB	28.3					
DhaA	30.1	49.0				
Rv1833c	27.0	31.7	30.3			
Rv2296	38.5	28.2	31.1	31.9		
Rv2579	26.8	68.3	43.6	29.4	28.9	

[a] Percent sequence identity calculated for the global alignment by using the ALIGN routine of FASTA.

Screening for dehalogenating activity with different isolates of mycobacteria. Fourteen strains of mycobacteria of different origins were screened for dehalogenating activity toward 1,2-dibromoethane. This compound was chosen for the screening because it is a good substrate for haloalkane dehalogenases belonging to different substrate specificity classes. Thirteen of 14 isolates showed dehalogenating activity with 1,2-dibromoethane, even without induction of the dehalogenases by halogenated substrate (Table 2). Induction of dehalogenating enzymes by 1-chlorobutane did not have any apparent effect on the activity.

Characterization of dehalogenating properties of *M. avium* MU1 and *M. smegmatis* CCM 4622. Crude extracts of two isolates with good activity toward 1,2-dibromoethane (DBE), i.e., *M. avium* MU1 and *M. smegmatis* CCM 4622, were further analyzed to determine the substrate specificity and to identify whether an oxygenase or a hydrolytic dehalogenase is responsible for dehalogenation. Dehalogenating activities toward a selected set of chlorinated and brominated aliphatic compounds were determined by using gas chromatography (Table 3). Crude extracts of strains MU1 and CCM 4622 converted haloalkanes to the corresponding alcohols, indicating that dehalogenation of haloalkanes is hydrolytic in both organisms. Dehalogenation in the presence of oxygen and dehalogenation in the absence of oxygen were compared to further confirm the hydrolytic mechanism of the dehalogenation reaction. This experiment was conducted with three different substrates, i.e., 1-iodohexane, 1-bromobutane, and 1-chlorohexane. Crude extracts of *M. avium* MU1 and *M. smegmatis* CCM 4622 were active toward these three compounds under both aerobic and anaerobic conditions. There was no significant difference between dehalogenase activity in the presence or in the absence of oxygen in the reaction mixture, indicating that dehalogenation of haloalkanes is indeed a hydrolytic reaction.

DISCUSSION

The identification of genes encoding putative haloalkane dehalogenases in the genome of *M. tuberculosis* H37Rv motivated this study. Dehalogenating activity of strain H37Rv toward at least four halogenated alkanes, 1-chlorobutane, 1-chlorodecane, 1-bromobutane, and 1,2-dibromoethane, has been

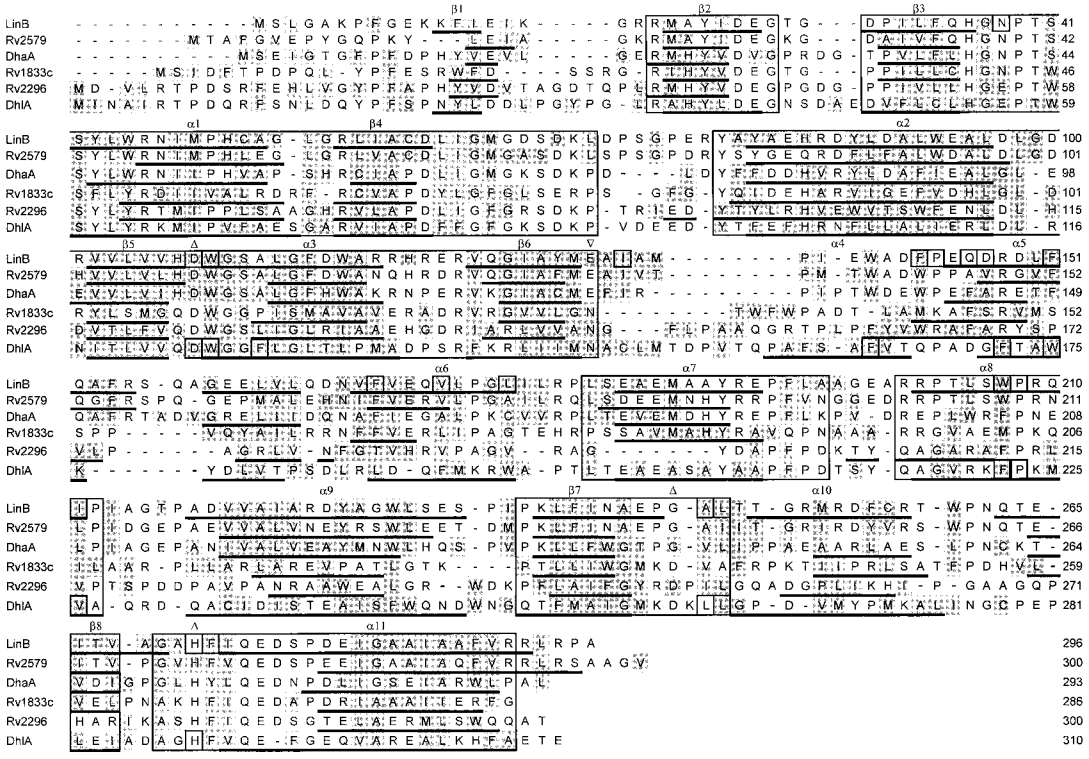

FIG. 1. Alignment of the protein sequences of the haloalkane dehalogenases from *S. paucimobilis* UT26 (LinB), *R. rhodochrous* NCIMB 13064 (DhaA), and *X. autotrophicus* GJ10 (DhlA) and the putative haloalkane dehalogenases from *M. tuberculosis* H37Rv (Rv1833c, Rv2579, and Rv2296). Solvent-unaccessible (buried) residues are shaded. Conserved regions and the active site residues are boxed. The catalytic triads Asp-His-Asp or -Glu are indicated by the triangles. Experimentally observed secondary structure elements (DhlA and LinB) and predicted elements (all other) are indicated by the lines under the sequences. Secondary structure elements are numbered according to DhlA.

found. The corresponding primary alcohols were identified as the reaction products, suggesting the involvement of a hydrolytic dehalogenase in the dehalogenation reaction. Normally, organisms that contain hydrolytic dehalogenases primarily originate from environments contaminated with halogenated pollutants (8, 9). The presence of dehalogenating activity in parasites colonizing human tissue, like strain H37Rv, evokes the question about the origin of dehalogenating enzymes and their possible function in the metabolism of these parasitic organisms. We speculate that dehalogenases were part of the enzymatic equipment of the progenitor of *M. tuberculosis*, which presumably arose from a soil bacterium. It is believed that the human bacillus may have been derived from the bovine form following the domestication of cattle (3). The genome of *M. tuberculosis* complex is highly conserved (21), which could explain the persistence of the dehalogenase genes on its chromosome even after its colonization of mammal tissue. Another possible explanation for the presence of haloal-

TABLE 2. Dehalogenating activity of 14 strains of mycobacteria toward 1,2-dibromoethane

Tested strain	Sp act (mU/mg of protein)[a]
M. avium MU1	36
M. parafortuitum MU2	22
M. triviale MU3	61
M. chelonae MU4	20
M. xenopi MU5	15
M. flavescens MU6	NA[b]
M. kansasii MU7	2
M. fortuitum MU8	76
M. gordonae MU9	11
M. bovis BCG MU10	99
M. smegmatis CCM 1693	7
M. smegmatis CCM 2300	2
M. smegmatis CCM 4622	49
M. phlei CCM 5639	22

[a] Micromoles of bromide produced per minute per milligram of protein.
[b] NA, not active. The detection limit of the spectrophotometric assay for Br^- was 0.01 mM.

TABLE 3. Dehalogenating activity of crude extracts of *M. avium* MU1 and *M. smegmatis* CCM 4622 toward several halogenated aliphatic compounds

Substrate	Product	Sp act (mU/mg of protein)[a]	
		M. avium MU1	*M. smegmatis* CCM 4622
1-Bromopropane	1-Propanol	0.87	3.58
1,3-Dibromopropane	3-Bromo-1-propanol	1.23	2.50
1-Bromo-2-methylpropane		NA	NA
1-Chlorobutane	1-Butanol	0.11	0.42
1-Bromobutane	1-Butanol	0.66	1.12
1-Chlorohexane	1-Hexanol	1.02	2.62
1-Bromohexane	1-Hexanol	0.77	0.91
1-Iodohexane	1-Hexanol	1.38	3.32
1-Chloroheptane		NA	NA

[a] Micromoles of primary alcohol produced per minute per milligram of protein. NA,- not active. The detection limit of the gas chromatographic assay for primary alcohols was 0.007 to 0.015 mM.

kane dehalogenases in parasitic bacteria is that these enzymes have some other function besides the catalysis of the carbon-halogen bond cleavage. Assuming that the different dehalogenases in *M. tuberculosis* have the same evolutionary progenitor, they might be created by gene duplication. It should be noticed that haloalkane dehalogenases have previously been isolated from *Rhodococcus* spp. (5, 23, 24, 27) and *Corynebacterium* spp. (27), both being genera which are phylogenetically closely related to *Mycobacterium*.

Thirteen of 14 isolates of mycobacteria from different sources were shown to release bromide ions from 1,2-dibromoethane. No apparent difference in the activity of the cells with and without induction of the dehalogenating enzymes by 1-chlorobutane was observed, suggesting that these enzymes might be expressed constitutively. It was confirmed that a hydrolytic dehalogenase is involved in the dehalogenation of haloalkanes by *M. avium* MU1 and *M. smegmatis* CCM 4622, since the crude extracts of these bacteria showed good dehalogenating activity under anaerobic conditions and primary alcohols were identified as the reaction products. Crude extracts of both strains preferred short alkyl-chain substrates over long-chain substrates and brominated substrates over chlorinated ones. It has been described for *R. erythropolis* Y2, containing two dehalogenating enzymes (2), that the halidohydrolase-type dehalogenase prefers short-chain substrates (optimum C_4-C_6 1-haloalkanes), while the oxygenase-type dehalogenase prefers long-chain substrates (optimum C_{14} 1-haloalkane). The higher activities of *M. avium* MU1 and *M. smegmatis* CCM 4622 toward short-chain substrates thus correspond with the observed hydrolytic mechanism of the dehalogenation. Better dehalogenation of brominated substrates is not surprising since bromine is generally a better leaving group than chlorine (26). Specific dehalogenase activities of the mycobacterial crude extracts were two orders of magnitude lower than those of crude extracts of most other haloalkane-degrading organisms (e.g., *X. autotrophicus* GJ10, *R. erythropolis* Y2, *Rhodococcus* sp. m15-3, and *Acinetobacter* sp. GJ70). Lower activity could be attributed either to a lower expression level of the dehalogenases in mycobacteria or to a lower catalytic performance.

This study suggests that haloalkane dehalogenating enzymes are possibly not so rare in the environment as previously believed and even parasitic organisms may contain enzymes for the biotransformation of haloalkanes. It appears that the ability of mycobacteria to dehalogenate haloalkanes might be a more general phenotypic property of this genus, but additional experiments are needed to confirm this proposal. The presence of the dehalogenases in bacterial isolates from clinical material, including the species colonizing animal tissues and free environment, lets us speculate that animals and humans can mediate the transport of dehalogenase genes in the biosphere.

ACKNOWLEDGMENTS

We thank M. D. Milan Šlosárek (National Institute of Public Health, Prague, Czech Republic) and M. D. Lev Mezenský (Teaching Hospital in Bohunice, Brno, Czech Republic) for providing us with the isolates of mycobacteria. A.J. thanks Alena Ansorgová and Kamila Hynková (Masaryk University, Brno, Czech Republic) for help with the gas chromatography analyses. Emiel Rorije (BASF, Ludwigshafen, Germany) and Gerrit J. Poelarends (University of Groningen, Groningen, The Netherlands) are acknowledged for many useful comments on the contents of the manuscript.

This project was financially supported by the Czech Grant Agency and by the Czech Ministry of Education through the grants (Postdoc 203/97/P149 and ME276/1998) awarded to J.D. Financial support is gratefully acknowledged.

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