Isolation and Characterization of a Novel Haloacid Permease from *Burkholderia cepacia* MBA4[▽]

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Burkholderia cepacia **MBA4 is a bacterium that can utilize 2-haloacids as carbon and energy sources for growth. It has been proposed that dehalogenase-associated permease mediates the uptake of haloacid. In this paper, we report the first cloning and characterization of such a haloacid permease. The structural gene, designated *deh4p*, was found 353 bases downstream of the dehalogenase gene *deh4a*. Quantitative analysis of the expression of *deh4p* showed that it was induced by monochloroacetate (MCA), to a level similar to the MCA-induced level of *deh4a*. The nucleotide sequence of *deh4p* was determined, and an open reading frame of 1,656 bp encoding a putative peptide of 552 amino acids was identified. Deh4p has a putative molecular weight of 59,414 and an isoelectric point of 9.88. Deh4p has the signatures of sugar transport proteins and integral membrane proteins of the major facilitator superfamily. Uptake of [^{14}C]MCA into the cell was Deh4p dependent. Deh4p has apparent K_ms of 5.5 and 8.9 μM and V_{max}s of 9.1 and 23.1 nmol mg^{-1} min^{-1} for acetate and MCA, respectively. A mutant with a transposon-inactivated haloacid operon failed to grow on MCA even when *deh4a* was provided in *trans*.**

Burkholderia spp. are gram-negative bacteria with versatile capability. They have been isolated in various niches including soil, water, plants, the rhizosphere, and animals including humans. *Burkholderia cepacia* strains have been shown to be plant and/or animal pathogens, and yet they have also been found to possess antibacterial and antifungal activity. Moreover, they have also been shown to be involved in the bioremediation of many types of environmental pollutants (2, 34, 44). *Burkholderia cepacia* MBA4 was isolated from soil by an enrichment method using monobromoacetic acid (MBA) as the sole carbon and energy source. *B. cepacia* MBA4 is also able to grow on monochloroacetate (MCA), 2-monochloropropionate (2MCPA), and 2-monobromopropionate (47). This bacterium produces a haloacid-induced dehalogenase (renamed Deh4a) in batch culture conditions. 2-Haloacid dehalogenases or halidohydrolases are hydrolytic enzymes that cleave the halogen-carbon bond(s) in halogenated aliphatic acids, yielding hydroxy- or oxoalkanoic acids from mono- or disubstituted substrate, respectively (12). Deh4a has been purified and characterized (47). Its structural gene (renamed *deh4a*) has also been isolated and characterized and encodes 231 residues. Deh4a exists as a dimeric protein of 45 kDa, composed of two identical subunits of 23 kDa each (27, 45). The regions involved in the dimerization and the activity functions of the enzyme have also been elucidated (30, 46).

Deh4a is mainly found in the cytoplasm of *B. cepacia* MBA4, and no signature of any signal sequence has been identified. In order for the cell to utilize haloacid as a growth substrate, the availability of a transporter protein specialized in carrying the substrate into the cell would be necessary. Previous study on the toxicity of 2-haloacids to *Pseudomonas putida* suggested the presence of a dehalogenase-associated permease. Cells expressing inducible haloacid dehalogenase were more sensitive to haloacid than mutant cells having a decreased level of expression (53). While the presence of a haloacid permease has been suggested for a long time, the isolation and characterization of this haloacid transporter have never been achieved. Dehalogenases have also been isolated in other microorganisms (17, 22), and other enzymes that acted on haloalkanes or haloalcohols have also been identified (4, 7, 43). Nonetheless, no transport system for these halogenated compounds has been identified and characterized.

It is well known that permeases have overlapping specificity. Permease LldP of the *E. coli* lactate operon and transporter GlcA of the glycolate operon are both involved in the uptake of 2-hydroxymonocarboxylic acids (28, 29). An acetyl-coenzyme A synthetase gene (*acs*)-contranscribed gene, *yjcG* (or *actP*), which was initially classified as encoding a member of the sodium-solute symporter family, has been shown to be an acetate permease (10). MctP of *Rhizobium leguminosarum* is able to transport alanine and other monocarboxylates (18). These studies revealed that the transport of monocarboxylic acids can be managed by specific transporters of versatile capability. It is therefore possible that the transport of haloacids is also mediated by a specific permease.

In prokaryotes, genes coding for products that perform related functions were usually grouped together and controlled under the same regulatory sequence. This operon type organization provides a convenient means of detecting genes involved in a metabolic pathway. By means of chromosome walking, we aimed to identify the permease gene involved in the uptake of haloacids. Here we report the first cloning and characterization of a dehalogenase-associated permease in *B. cepacia* MBA4. The corresponding gene, *deh4p*, is located 353 bases downstream of *deh4a* and encodes a transmembrane protein of 552 residues. This protein is a member of the major facilitator superfamily (MFS) and is responsible for the uptake

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of haloacetate. Our results showed that *deh4a* and *deh4p* form an operon that mediates the utilization of haloacetate in *B. cepacia* MBA4.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. cepacia* MBA4 is a soil isolate that can utilize 2-haloacids as the carbon and energy source for growth (47). *Escherichia coli* TOP10 (Invitrogen) was used as the host for genetic manipulation. *B. cepacia* MBA4 was grown at 30°C in Luria broth without NaCl (1% tryptone, 0.5% yeast extract) or in defined medium with an appropriate carbon source (0.5 g carbon liter^{-1}) as described previously (47). *E. coli* TOP10 was grown at 30°C or 37°C in Luria broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with antibiotics.

RNA isolation and analytical RT-PCR. Total RNA was isolated from monobromoacetate-grown *B. cepacia* MBA4 cells with the High Pure RNA isolation kit (Boehringer-Mannheim) according to the supplier's protocol. Oligonucleotides deh4aC (5′-CCGCT GCTAG CGAAG AACGT TACA-3′) and deh4pN (5′-CGCAC GTCCT TCAAT AGTCG CCAT-3′) were used to amplify the intergenic region between *deh4a* and *deh4p*. Primers deh4a5 (5′-ATTAA GGTCA TGGTG GATTC ACTCC GC-3′) and deh4p3 (5′-AAGCT TGTCA GAACT GGAGA CGATT AGTCC-3′) were used to amplify the region between the 5′ end of *deh4a* and the 3′ end of *deh4p*. Reverse transcriptase PCR (RT-PCR) was performed with SuperScript one-step RT-PCR system with Platinum *Taq* (Invitrogen). The reaction was carried out in 1× reaction mixture (0.2 mM of each of the deoxynucleoside triphosphates and 1.2 mM MgSO$_4$), 1.6 ng total RNA, 0.5 μl RT-*Taq* mixture, and 0.1 μM of each of the forward and reverse primers in a final volume of 25 μl. A similar reaction with *Taq* DNA polymerase (Invitrogen) instead of RT-*Taq* mixture was also set up. Reverse transcription and predenaturation were performed at 50°C for 30 min and 94°C for 2 min, respectively. The conditions for 35 cycles of PCR were as follows: 94°C for 2 min, 60°C for 30 s, and 72°C for 35 s. The PCR products were analyzed on a 1% agarose gel.

Quantitative real-time RT-PCR. The reverse transcription was performed with Maloney murine leukemia virus RT (Promega) in 1× reaction mixture with 1 μg total RNA, 20 pmol reverse primer, 1 μl of RNasin (Promega), and water to 20 μl. The reaction mixture was incubated at 42°C for 1 h. The quantitative PCR was set up with 4 μl of reaction mixture from a LightCycler FastStart DNA Master Plus SYBR green I kit (Roche), 2 μl of RT product, 1 μl each of forward and reverse primers (10 μM), and water to a final volume of 20 μl. The quantitative PCR was carried out in a LightCycler (Roche) at 94°C (10 s), 63°C (5 s), and 72°C (11 s) for 45 cycles. The initial amount of the specific transcript was quantified mathematically by a relative method (33). The fit point method in the LightCycler software 3.3 (Roche) was used, and the crossing point (CP) was determined at a fluorescence value of 0.5. The CP is the point where the fluorescence rises above the background fluorescence. The amounts of *deh4a* and *deh4p* were estimated with reference to the 16S rRNA level in each sample. The relative expression ratio is expressed as follows: ratio = $(E_{\text{target}})^{\Delta\text{CPtarget(control} - \text{sample})}/(E_{\text{ref}})^{\Delta\text{CPref(control} - \text{sample})}$. ΔCPtarget and ΔCPref are the CP differences of the relative fluorescence levels of target (*deh4a* or *deh4p*) and reference (16S rRNA), respectively. Control and sample are preparations from cells grown in pyruvate or MCA. *E* stands for the efficiency of amplification, which is determined separately for each primer pair used in this study. The efficiency was calculated by the equation $E = 10^{(-1/\text{slope})}$ (35). The slopes were obtained from real-time PCR runs by using serial dilutions of the DNA template. The cycle numbers of CP were plotted against the input in log concentration. The *E* value obtained for primers 16SF (5′-GGTAA TACGT AGGGT GCAAG CGTT-3′) and 16SR (5′-CACCA ATGCA GTTCC CAGGT TAAG-3′) was 1.965. The *E* value for primers deh4aF (5′-GGCTG ATGAG TGCGT ACAAG GA-3′) and deh4aR (5′-CTCAT CGTTA CCGTT CGAAA GGA-3′) was 1.752, and the *E* value for primers deh4pF (5′-CTGCA GATGG CGACT ATTGA AGGAC GTGCG-3′) and deh4pR (5′-ATATC GCAAG TGAAC CAGCG AGAT-3′) was 1.741. Mean CPs were obtained from triplicates of three experiments.

Transport assays. *B. cepacia* MBA4 cells were cultured in minimal medium with pyruvate, acetate, or MCA to late logarithmic phase with an optical density at 600 nm (OD$_{600}$) of 0.5 to 0.7. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS; Fluka) and resuspended in PBS to an OD$_{600}$ of 0.5. [2-^{14}C]MCA (Sigma-Aldrich) or [2-^{14}C]acetate (Moravek) was diluted to 1 mM in PBS. The diluted radiolabeled substrate (100 μl) was mixed with washed cells (400 μl), and 100-μl samples were taken immediately and at 1 min, 2 min, 4 min, and 6 min. The samples were filtered

through 0.45-μm-pore-size nitrocellulose filter disks (Millipore) and washed with 2 ml PBS under continuous suction (QiaVac 24 Plus vacuum manifold; QIAGEN). Filter disks with trapped cells were put into vials with 2 ml scintillation fluid (Ready-Solv EP; Beckman), and the amounts of carbon 14 were measured by a scintillation counter (LS6500; Beckman). Total protein in each sample was measured with protein assay reagent (Bio-Rad). All uptake assays were performed at room temperature.

For the competition study diluted radiolabeled substrate was mixed with 10× competing substrate (10 mM in PBS) at a 1:1 ratio before mixture with prepared cells. Percent inhibition was calculated as [1 − (uptake rate with competing substrate/uptake rate without competing substrate)] × 100%. The kinetic constants of MCA and acetate uptake by *B. cepacia* MBA4 were determined using various [2-^{14}C]MCA or [2-^{14}C]acetate concentrations in a standard uptake assay.

Transposon mutagenesis. The haloacid operon was inactivated by insertion of the Tn5 transposon. Tn5 carrying plasmid pOT182 (26) was delivered into *B. cepacia* MBA4 via electroporation (8). Briefly, late-logarithmic growing cells (100 ml) were harvested by centrifugation at 1,600 × *g* for 15 min at 4°C, washed twice with 20 ml of ice-cold electroporation buffer (1 mM MgCl$_2$, 1 mM HEPES, pH 7.0), resuspended in 2 ml buffer, and stored on ice until use. Plasmid DNA was gently mixed with 100 μl cell, and the mixture was incubated at 4°C for at least 5 min before electroporation. The cell-DNA mixture was transferred to a prechilled (4°C) sterile electroporation cuvette with a 0.2-cm electrode gap. A Gene Pulser with a pulse controller unit (Bio-Rad) was used for the electroporation. The settings were 12.5 kV cm^{-1}, 25 μF, and 200 Ω. After the discharge through the cells, 900 μl of SOC medium (tryptone, 2%; yeast extract, 0.5%; NaCl, 0.06%; KCl, 0.02%; MgCl$_2$ · 6H$_2$O, 0.2%; MgSO$_4$, 0.12%; glucose, 0.18%; all percentages are wt/vol) was immediately added and the mixture was mixed gently. A volume (100 μl) of this transformation mixture was plated on an LB$^-$ agar plate containing the appropriate antibiotics. The plate was incubated at 30°C for 2 to 3 days. Tetracycline-resistant transformants were screened for their ability to grow on MCA. Clones that failed to grow on MCA were further analyzed for the site of integration (26).

Construction of Deh4a- and Deh4a-Deh4p-producing plasmids. DNA fragments containing *deh4a* or *deh4a* plus *deh4p* were amplified from genomic DNA by PCR using primers PstI-4a-F (5′-CTGCA GTATC GATCG AGCCT CGTGA CCGAC C-3′) and KpnI-4a-R (5′-GGTAC CGCGT TGTCT CCTGA TCTAA TCGTA TAAGT C-3′) or primers PstI-4a-F and KpnI-4ap-R (5′-GG TACC GACGC GCTTG CTGTC CGGCT C-3′), respectively. The DNA fragments were cloned into PstI- and KpnI-cut pUCP28T (41) to form plasmids p28T4A (containing *deh4a* only) and p28T4AP (containing *deh4a* and *deh4p*). These plasmids were transformed into mutant 15D02 by electroporation, and transformants were selected on the basis of their resistance to trimethoprim and tetracycline.

Nucleotide and protein sequence accession numbers. The nucleotide sequence of the *deh4p* open reading frame (ORF) has been deposited with GenBank under accession number AF439266, and the associated protein sequence has been deposited under UniProt accession number Q7X4L6.

RESULTS

Identification of haloacid permease activity in *B. cepacia* MBA4. Expression of the haloacid-metabolizing enzyme, dehalogenase, in *B. cepacia* MBA4 is regulated. The gene *deh4a* (previously *hdlIVa*) is induced only when haloacid is the sole carbon source (47). If an associated permease is present in *B. cepacia* MBA4, the expression of this permease is most likely also regulated. *B. cepacia* MBA4 cells were therefore grown in defined medium with pyruvate, acetate, or MCA as the carbon and energy source, and [2-^{14}C]MCA transport was examined. Figure 1 shows that cells grown on pyruvate transport MCA into the cell at a rate of 3.9 nmol mg protein^{-1} min^{-1}. Cells grown on acetate have an initial transport rate of 17.5 nmol mg protein^{-1} min^{-1}, but this rate was reduced to a rate similar to that of pyruvate-grown cells after 2 min. MCA-grown cells, on the other hand, have an uptake rate of 35.5 nmol mg protein^{-1} min^{-1}, and this rate was maintained throughout the assay period of 6 min. This suggested that MCA induced a trans-
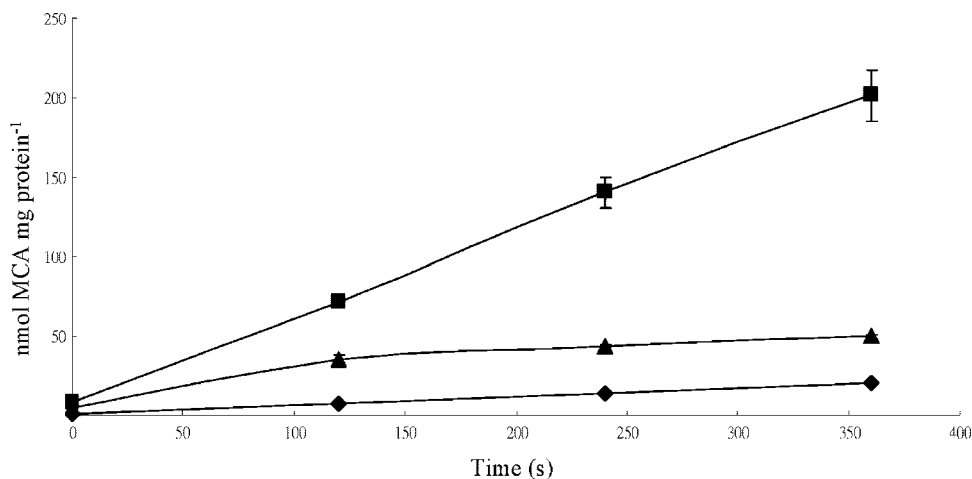
FIG. 1. Uptake of monochloroacetate by *B. cepacia* MBA4. Uptake of 200 μM [2-^{14}C]MCA was assayed by a filtration method for cells grown in minimal medium containing pyruvate (◆), acetate (▲), or MCA (■). Data shown are the means of three independent experiments with error bars.

porter protein or proteins that facilitated the specific uptake of MCA.

Property of the haloacid permease activity. In order to characterize the substrate specificity of the induced permease, MCA uptake assays were carried out in the presence of a 10-fold excess of a number of solutes. Competing substrates selected were 2-haloacids (monofluoroacetate [MFA], MCA, MBA, and 2MCPA), monocarboxylates (acetate, glycolate, pyruvate, and lactate), dicarboxylate (succinate), monosaccharide (glucose), and disaccharide (lactose). Figure 2 shows the inhibition of [2-^{14}C]MCA uptake by MCA-grown cells in presence of competing substrate. The results showed that only acetate and derivatives (MFA, MCA, and MBA) are strong inhibitors of MCA uptake (between 80 and 93% inhibition). Other solutes tested did not inhibit MCA uptake (glucose and lactose) or inhibited MCA uptake weakly (2MCPA, glycolate, pyruvate, succinate, and lactose), between 17 and 39%. The

inhibition by acetates seems to be dependent on molecular size, with the smaller compounds showing more inhibitory effect (acetate > MFA > MCA > MBA). These results revealed that the MCA-induced permease is a specific transporter for acetates.

Since the MCA-induced permease activity was able to transport both MCA and acetate, it is necessary to characterize its properties in more detail. MCA-grown cells were used, and different concentrations (3 to 100 μM) of [2-^{14}C]acetate or [2-^{14}C]MCA were used to determine the K_m and V_{max} of the permease activity. The K_ms for acetate and MCA were 5.5 and 8.9 μM, respectively, and the V_{max}s were 9.1 and 23.1 nmol mg^{-1} min^{-1}, respectively.

Identification of a putative transporter gene downstream of *deh4a* of *B. cepacia* MBA4. The discovery of MCA-induced haloacetate permease activity prompted us to look for the permease gene. Since genes of related functions are usually
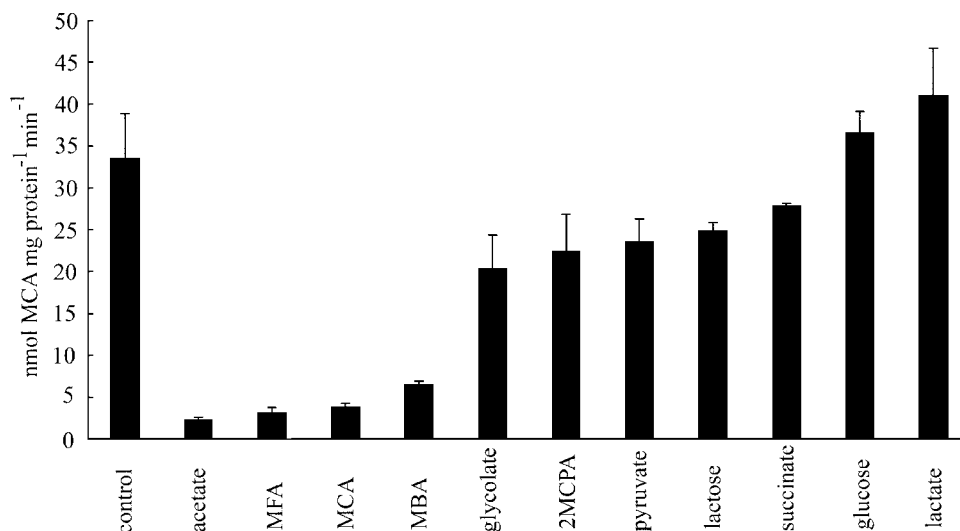

FIG. 2. Inhibition of MCA uptake by other solutes. Uptake of 200 μM [2-^{14}C]MCA by MCA-grown MBA4 was assayed by a filtration method. Competing solutes were added to a final concentration of 2 mM. Data shown are the means of two independent experiments with error bars.

clustered together in bacteria as an operon, we strived to look for a related gene(s) involved in the degradation of 2-haloacids. The *deh4a* gene (GenBank accession no. X66249) was isolated on a 1.6-kb EcoRI fragment, and *E. coli* cells harboring this fragment were able to produce Deh4a (45). This suggested that a promoter present on this fragment probably drove the expression of Deh4a in *E. coli*. Computational analysis of the sequences upstream of *deh4a* by the Neural Network Promoter Prediction program (36) has revealed the presence of a putative bacterial promoter. The transcription initiation site has recently been identified by primer extension to start at an A at −26 upstream of the initiation codon of *deh4a* (J. S. H. Tsang and L. Sam, unpublished data). Oligonucleotides were synthesized and used to unravel the sequence downstream of the *deh4a* gene. An ORF of 1,659 bp, designated *deh4p* for permease, was identified 353 bp downstream of the *deh4a* gene. No terminator sequence was identified between *deh4a* and this ORF. On the other hand, sequences that exhibit structural properties of intrinsic transcriptional terminators (11, 40) were identified downstream of this putative gene.

A postulated Shine-Dalgarno region (GGAGA) was found at the right proximity close to the ATG of the gene at position 307. Analysis of the putative amino acid sequence of Deh4p revealed that it is probably a member of the MFS transporters (31). It has the signature of family 1: sugar transporter family proteins. The signature [LIVMF]-X-G-[LIVMFA]-X(2)-G-X(8)-[LIFY]-X(2)-[EQ]-X(6)-[RK] is found between residues 130 and 155. The putative gene product Deh4p has an apparent molecular weight of 59,414 and an isoelectric point of 9.88. The nucleotide sequence of *deh4p* exhibits up to 74% similarity with putative MFS transporter genes of many *Proteobacteria*. Analysis of the predicted amino acid sequence with SOUSI (16) shows that Deh4p is a putative integral membrane protein containing 12 transmembrane spanners. Moreover, residues 81 to 90, GRLGD MIGRK, are also consistent with the consensus region connecting helices 2 and 3 of a typical MFS member (15).

***deh4p* is the second member of a haloacid operon.** The identification of a putative transporter protein gene downstream of *deh4a* prompted us to consider the possibility of an operon containing *deh4a* and *deh4p*, responsible for the utilization of haloacids in *B. cepacia* MBA4. Quantitative RT-PCR analysis was used to check the abundance of the transcripts in different growth conditions. The results showed that both genes were concomitantly expressed in the presence of MCA and turned off in its absence (growth in pyruvate). Moreover, the transcript amounts in the induced condition for both genes were roughly the same (260-fold for *deh4a* and 280-fold for *deh4p*). This suggested that *deh4a* and *deh4p* were probably expressed in series in a haloacid operon.

In order to confirm that a single transcript was made for both genes, RT-PCR was performed. A pair of specific primers was used to anneal to the 3′ end of *deh4a* and 5′ end of *deh4p*. An expected RT-PCR product of about 400 bp was amplified (Fig. 3A, lane 1), while the negative control (no reverse transcription) showed no observable band (Fig. 3A, lane 2). Oligonucleotide primers were also designed to amplify transcripts from the 5′ end of *deh4a* to the 3′ end of *deh4p*. The results showed that an RT-PCR product of around 2.7 kb was detected (Fig. 3B, lane 3). The size of this RT-PCR product
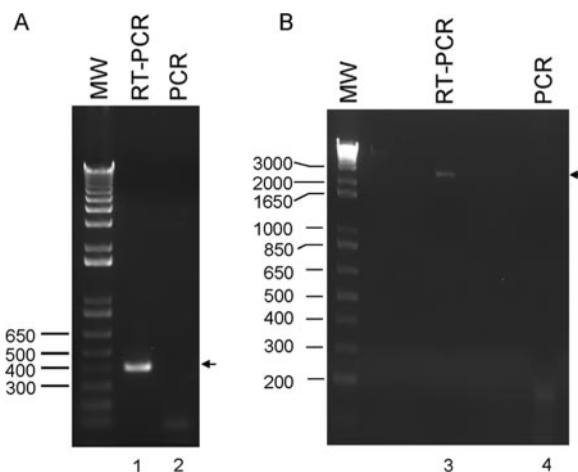

FIG. 3. Agarose gel electrophoresis of RT-PCR products. Total RNAs were isolated from MCA-grown MBA4, and primers deh4pN (for lane 1) and deh4p3 (for lane 3) were used for first-strand synthesis. Primers deh4aC and deh4a5, respectively, were used for the PCR. PCR of the corresponding RNA samples without reverse transcription (lanes 2 and 4) was performed with similar primer pairs.

matches the sum of the *deh4a* and the *deh4p* genes and some 5′ and 3′ untranslated regions. Similarly, no PCR product was detected in the RNA preparation when reverse transcription was omitted (Fig. 3B, lane 4). These results suggested that the RT-PCR products were not the consequence of contaminated genomic DNA and that *deh4a* and *deh4p* were expressed as a single transcript.

Complementation of mutant defective in the haloacid operon. To show that Deh4p is essential for the cell to transport and for the uptake of haloacids, it is necessary to confirm its function by complementation of a Deh4p-negative mutant. While the generation of gene-specific deletion or disruption has not been successful for *B. cepacia* MBA4, we used transposon mutagenesis (42) to generate a mutant with a defective *deh4p*. Transposon Tn5 was introduced into *B. cepacia* MBA4 by electroporation, and transformants were screened for their inability to grow on MCA. A transformant, 15D02, able to grow on pyruvate but not on MCA was isolated. This mutant was characterized by cloning the DNA fragment containing the transposon and sequences flanking the insertion site. The transposon was found to insert at nucleotide 300 of the *deh4a* gene, resulting in a truncated nonfunctional protein. This truncated protein is unlikely to be functional because D181 of Deh4a is essential for dehalogenase activity (30). Since *deh4p* is the second gene of the haloacid operon, it is likely that its expression was also disrupted due to a polar effect.

The failure of the mutant to grow on MCA could be explained by the lack of a functional dehalogenase rather than the requirement for a coexpressed permease. Strain 15D02 was transformed with plasmid p28T4A, which contains a wild-type *deh4a*. Figure 4 shows that this cell failed to grow on MCA. This suggested that the provision of a functional dehalogenase in *trans* was not sufficient to complement the haloacid utilization phenotype. This suggested that the expression of *deh4p* was also inhibited by the insertion of the transposon. Conversely, a cell transformed with plasmid p28T4AP, containing
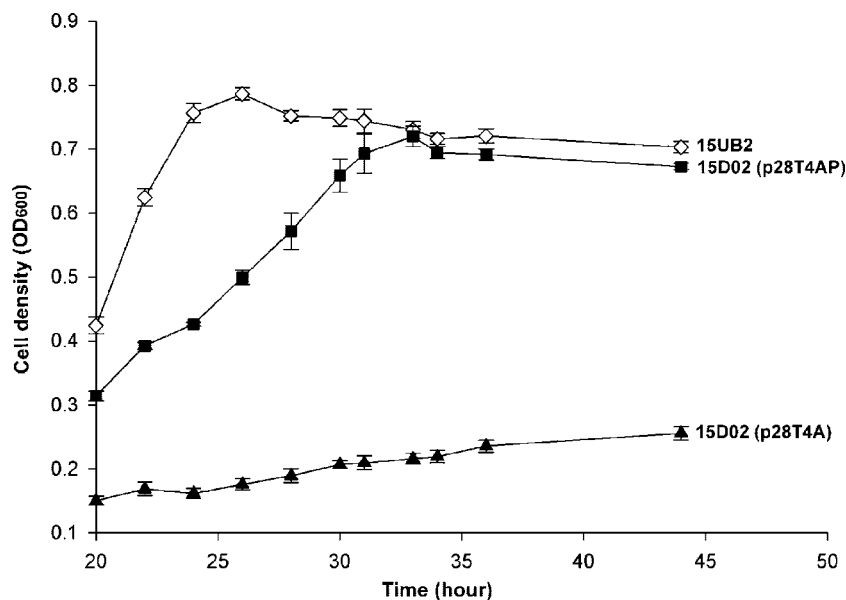
FIG. 4. Growth of mutant 15D02 carrying various constructs. Mutant 15D02, containing plasmid p28T4A (▲) or p28T4AP (■), was grown at 30°C in a defined medium containing MCA, tetracycline, and trimethoprim. A randomly selected Tn5-generated mutant, 15UB2 (◇), with wild-type dehalogenase activity was used as the dehalogenase-positive control. Data shown are the means of at least three independent experiments with the error bars indicated.

both *deh4a* and *deh4p*, was able to utilize MCA as the carbon and energy source. This indicated the importance of Deh4p in MCA utilization and that *deh4a* and *deh4p* are organized as an operon. Tn5 insertional mutants with wild-type *deh* activities have higher MCA growth rates than 15D02(p28T4AP), and a typical growth curve for one of these strains, 15UB2, carrying a pUCP28T-derived plasmid is shown in Fig. 4.

DISCUSSION

In this study, we have demonstrated that the uptake of MCA for *B. cepacia* MBA4 was induced when MCA was the sole carbon source. This property was in line with the expression of the *deh4p* gene located immediately downstream of the dehalogenase IVa gene, *deh4a*. The expression of *deh4a* is inducible and is essential for MBA4 to degrade MCA. Structural analysis of a dehalogenase-producing *Pseudomonas putida* strain (strain PP3) has also identified a putative heavy-metal-associated cation efflux transporter gene adjacent to a DehI dehalogenase gene (52). This transporter, however, is truncated, and its haloacid uptake function has not been demonstrated. A similar genomic structure has also been found in *Agrobacterium* sp. strain NHG3, where putative transporter gene *dehP* (GenBank/EBI Data Bank accession number CAD32751) was found 66 bp upstream of D-specific and L-specific 2-chloropropionic acid dehalogenases (13). The function of this putative transporter, however, has not been investigated. The current study is the first to report the presence of a haloacid operon that mediated the uptake and degradation of haloacid in bacteria. Haloacetate or 2-haloacid dehalogenase genes have been identified in a wide variety of bacteria (14). Comparative analysis of the genomes (24) of these bacteria showed that, while a putative dehalogenase gene can be found in many *Proteobacteria* such as *Burkholderia cenocepacia* (http://img.jgi.doe.gov/; Integrated Microbial Genome [IMG] gene object

accession no. 636436945, 636439065, 636442040, and 636446170), *Burkholderia mallei* (IMG accession no. 5771450 and 5789940), *Agrobacterium tumefaciens* (IMG accession no. 922710, 955800, 957310, 957340, and 972890), *Ralstonia* spp. (IMG accession no. 623760310, 623760340, 623782300, 3498150, and 3509420), and *Rhizobium* spp. (IMG accession no. 635185875, 635188675, 635188740, 635193460, 635211910, 635211950, 636299245, 636315300, and 636319835), none of these genes were found next to a gene encoding an MFS transporter.

Comparison of the protein sequence of Deh4p with the translated genomic sequences of various *Proteobacteria* has identified many putative proteins of the MFS. These putative MFS proteins, found in *Burkholderia* sp. strain 383 (NCBI accession number ABB09576), *Burkholderia xenovorans* LB400 (NCBI accession number ABE32266), *Burkholderia pseudomallei* (NCBI accession number YP_332381), *B. mallei* (NCBI accession number AAU48731), *B. cepacia* (NCBI accession number EA043951), *Burkholderia vietnamiensis* (NCBI accession number EAM26890), and *Burkholderia dolosa* (NCBI accession number ZP_00987315), exhibit around 80% identities with Deh4p. Inspection of the locations of the corresponding genes failed to discover any putative dehalogenase gene in juxtaposed positions. It is likely that the haloacid operon, containing dehalogenase and permease genes, is not common in *Proteobacteria*. It is also possible that the haloacid operon was evolved fairly recently and that those related transporters have a different natural substrate.

Haloacetic acids are disinfection by-products (6, 20) and have been shown to possess toxic and mutagenic properties (21, 38). It is thus reasonable to identify haloacetate dehalogenase genes in various bacteria because the acquisition of the gene helps the bacterium to detoxify and survive (50). To further eliminate the unwelcome chemical, the presence of an

efflux transporter that pumps the compound out of the cell would be favorable. On the other hand the acquisition of a transporter that enhanced the uptake of haloacid will damage and impair the growth of the organism. The evolution of a haloacid operon containing a dehalogenase gene and a permease gene suggests that *B. cepacia* MBA4 has adapted to an environment that allows the cell not just to tolerate the presence of haloacids but to utilize these mutagenic compounds as a growth substrate. The regulated expression of these genes by haloacetate further confirms their role in substrate utilization and metabolism. The genome of MBA4 has the cryptic dehalogenase gene *chd1*, which was not normally expressed (48). This dehalogenase has a leader sequence that targeted the protein to the periplasm but the affinity for monobromoacetate of this protein is only 1/10 that of Deh4a.

The suggestion that there is an operon containing *deh4a* and *deh4p* is supported by (i) the concomitant induced expression of these genes to similar levels; (ii) detection of reverse transcription-PCR products spanning the two genes from mRNA and not from DNA; (iii) the presence of a terminator sequence at the end of *deh4p* and not at the end of *deh4a*; and (iv) the polar effect of Tn*5* insertion in *deh4a* on *deh4p* expression, as shown by the lack of growth of strain 15D02(p28T4A). The possession of a haloacid operon containing *deh4a*, encoding a haloacid dehalogenase, and *deh4p*, encoding a haloacetate transporter, helps the cell to grow on haloacetates. The regulated expression of *deh4a* and *deh4p* in response to haloacids implied that they are involved in the metabolism of the halogenated compound. Figure 4 shows that strain 15D02(p28T4AP) was able to grow on MCA, but its growth was not as efficient as other Tn*5* insertion mutants with the wild-type dehalogenase phenotype (e.g., strain 15UB2). This can be explained by the fact that the regulatory proteins required for the expression of the haloacid operon bound to the *deh* promoters on the chromosome and on the plasmids. The binding of the regulatory factor to the promoter located on the chromosome will produce only a truncated transcript and nonfunctional peptide. This may have quenched the activities of the promoters on the plasmids, and full-length transcripts and functional proteins were not produced to their utmost levels.

In order to confirm that the MCA-induced haloacid permease activity was due to the expression of Deh4p, we have cloned the structural gene of Deh4p and expressed it in *E. coli* cells. The *deh4p* gene was expressed in *E. coli* TOP10 using a ribosomal *s12* promoter. *E. coli* cells harboring *deh4p* (on plasmid p28tS12-4p) have a higher (threefold) MCA uptake rate than cells harboring the vector (p28tS12-E) only (54). One would expect a more dramatic difference in uptake rate by using stronger promoters. We in fact used an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *trc* promoter initially, but the growth of the cell was severely hindered even under a repressed condition. Moreover, in the presence of IPTG *E. coli* cells produced transporter proteins such as LacY (3) that transported nonspecific substrates (23, 39) including monochloroacetate (54).

Comparative analysis of the primary sequence of Deh4p with the transport classification database (TCDB; http://www.tcdb.org/) has placed it in MFS 2.A.1. Uptake inhibition studies for MCA-grown MBA4 cells suggested that the induced permease has narrow substrate specificity for short-chain aliphatic monocarboxylates. This is similar to the acetate per-

mease ActP of *E. coli* (10). The apparent K_m for acetate for ActP is 5.4 μM, which is comparable to that for Deh4p. However, the V_{max} for ActP is 19.6 nmol mg^{-1} min^{-1}, which is much higher than that for Deh4p. Preliminary results suggest the presence of 12 transmembrane helices, as predicted by the majority of topology programs (5, 9, 16, 19, 25, 32, 37, 49, 51, 55). Most of these predictions suggested that the N terminus is located in the cytoplasm. By tagging PhoA-LacZ dual reporters (1) at various positions of Deh4p and expressing them in *E. coli* we have confirmed that the N terminus is indeed located in the cytoplasm (Y. M. Tse and J. S. H. Tsang, unpublished data). The topology of Deh4p, however, still awaits further analysis.

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