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Adaptation of *Xanthobacter autotrophicus* GJ10 to Bromoacetate due to Activation and Mobilization of the Haloacetate Dehalogenase Gene by Insertion Element IS*1247*

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Monobromoacetate (MBA) is toxic for the 1,2-dichloroethane-degrading bacterium *Xanthobacter autotrophicus* GJ10 at concentrations higher than 5 mM. Mutants which are able to grow on higher concentrations of MBA were isolated and found to overexpress haloacid dehalogenase, which is encoded by the *dhlB* gene. In mutant GJ10M50, a DNA fragment (designated IS*1247*) had copied itself from a position on the chromosome that was not linked to the *dhlB* region to a site immediately upstream of *dhlB*, resulting in a 1,672-bp insertion. IS*1247* was found to encode an open reading frame corresponding to 464 amino acids which showed similarity to putative transposases from two other insertion elements. In most of the other MBA-resistant mutants of GJ10, IS*1247* was also present in one more copy than in the wild type, which had two copies located within 20 kb. After insertion to a site proximal to *dhlB*, IS*1247* was able to transpose itself together with the *dhlB* gene to a plasmid, without the requirement of a second insertion element being present downstream of *dhlB*. The results show that IS*1247* causes bromoacetate resistance by overexpression and mobilization of the haloacid dehalogenase gene, which mimics steps during the evolution of a catabolic transposon and plasmid during adaptation to a toxic growth substrate.

Xanthobacter autotrophicus GJ10 was isolated on 1,2-dichloroethane as the sole carbon and energy source and is able to grow on a variety of short-chain halogenated aliphatic compounds (13). The organism produces two different dehalogenases which hydrolyze carbon-halogen bonds. The haloalkane dehalogenase encoded by *dhlA* has activity with halogenated alkanes (16), whereas the haloacid dehalogenase encoded by *dhlB* is active with halogenated short-chain carboxylic acids (38). Both enzymes have been purified, and the amino acid sequences have been derived from the sequences of the cloned genes (12, 16, 38).

Not all of the compounds which are converted by the two dehalogenases of *X. autotrophicus* GJ10 are growth substrates (16). Especially the brominated compounds 1,2-dibromoethane, 2-bromoethanol, and bromoacetic acid (monobromoacetate [MBA]) do not support growth, although enzymes necessary for their conversion to glycolate seem to be present (13). There may be different reasons for this: there may be a lack of uptake of the substrates into the cell, or substrates or intermediates produced during degradation may be toxic. Both 1,2-dichloroethane and 2-chloroethanol are readily used, and the entrance of these substrates into the cell is not a rate-limiting step (36). There is no indication for the requirement of an active uptake system for 1,2-dibromoethane or 2-bromoethanol. We have previously postulated that the inability to use 2-bromoethanol and 1,2-dibromoethane can be attributed to the toxicity of the intermediate bromoacetaldehyde (39).

Of the halogenated carboxylic acids, only 2-monochloropropionic acid was found to serve as a good growth substrate for *X. autotrophicus* GJ10 (13). Chloroacetate (MCA), which is an intermediate in the degradation of 1,2-dichloroethane, is utilized poorly, while MBA is not utilized at all (13). Moreover, MBA is toxic at concentrations of 10 mM when citrate is used as a growth substrate. We have previously described an MBA-resistant mutant of GJ10, GJ10M50, in which haloacid dehalogenase was overexpressed (38). On the other hand, mutants of *Pseudomonas putida* PP3 impaired in uptake of MCA were resistant to normally toxic concentrations of halogenated carboxylic acids (25). The toxicities of these compounds therefore seem to be related to the ratio between their rates of uptake and the velocity of conversion.

The mechanism of overexpression of haloacid dehalogenase in *X. autotrophicus* GJ10M50 was not elucidated. We now report the genetic characterization of strain GJ10M50 and other MBA-resistant mutants and the identification of an insertion element involved in the adaptation of *X. autotrophicus* GJ10 to high concentrations of MBA.

MATERIALS AND METHODS

Organisms and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *X. autotrophicus* strains and mutants were grown in nutrient broth or in a mineral medium supplemented with 10 mg of yeast extract per liter (MMY [35]). *Escherichia coli* strains were grown in Luria-Bertani medium at 30 or 37°C. For plates, 1.5% agar was added. For maintenance of plasmids, ampicillin was added to 100 μg/ml and tetracycline was added to 12.5 μg/ml. When necessary, IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added to concentrations of 0.4 mM and 10 μg/ml, respectively.

Primers. The following primers (5′ to 3′) were used for DNA sequencing: B, TCTCGCCGCGGGATCCGGTTTCCGA (positions 71 to 95 of the *dhlB* sequence [38]; a *Bam*HI site was introduced by replacing G with C [underlined]); H, TTCCGGCAATCAGTCTTTGG (positions 343 to 362 of the sequence shown in Fig. 3); I, ACGGTTGGCGTCGTTGACAT (positions 402 to 391 in Fig. 3); G, CAGCATCCCGAACTGGTTGA (positions 714 to 695 in Fig. 3); D, ACCTCATTGTCGCGGAACTT (positions 1235 to 1216 in Fig. 3); E, TCGAA CACGACTGCCTTGAT (positions 1764 to 1745 in Fig. 3); and T, CGAGA AAGGGTGACGATTGA (positions 572 to 591 of the sequence of the tetracycline resistance region from RP1 [40]).

Isolation of MBA-resistant mutants. Mutants of strain GJ10 that were resistant to MBA were obtained as described previously (38) by spreading cells on MMY plates containing 5 mM citrate and 10 mM MBA. Mutants that appeared were restreaked and purified on plates with the same composition. A different approach was used to isolate mutants of GJ10M30(pPJ20)M1. Cells (0.1 ml of a

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
X. autotrophicus		
GJ10	Wild type, $DhlB^+$, pXAU1	12
GJ10M50	GJ10Ω[*dhlC*::IS*1247*], MBA resistant	38
GJ10M30	Lacks pXAU1	39
XD	Wild type, $DhlB^-$	12
E. coli		
JM101	Δ(*lac-proAB*)[F′ *lacI*^{q}ZΔM15]	44
JM109	*endA1 recA1* Δ(*lac-proAB*)[F′ *lacI*^{q}ZΔM15]	44
Plasmids		
pGEM-5Zf(−)	Ap^r ColE1	Promega
pGEM-7Zf(−)	Ap^r ColE1	Promega
pBluescript II SK(−)	Ap^r ColE1	Stratagene
pPJ20	pLAFR1 Tc^r $DhlA^+$ $DhlB^-$	12
pM1A	pPJ20, insertion of IS*1247* + 8.8 kb, $DhlB^+$	This study
pM1F	pPJ20, insertion of IS*1247* + 4.1 kb, $DhlB^+$	This study
pPM50	pBluescript II SK(−), *Pst*I-*Not*I fragment of GJ10M50 containing IS*1247*	This study
pA4	pBluescript II SK(−), 7.8-kb *Pst*I fragment of pM1A containing IS*1247*	This study
pA45	pGEM-5Zf(−), 2.0-kb *Sal*I fragment of pM1A	This study
pFHB	pGEM-7Zf(−), 7-kb *Hin*dIII fragment of pM1A containing IS*1247*	This study
pFHA	pGEM-7Zf(−), 7-kb *Hin*dIII fragment of pM1F in orientation opposite to that of pFHB	This study
pFHAS	*Sma*I deletion in pFHA	This study
pFHBB	*Bam*HI-*Bgl*II deletion in pFHB	This study
pRK2013	*tra*(RK2), ColE1 replicon, Km^r	5

nutrient broth preculture) were spread on plates containing citrate and a gradient of MBA. Colonies that appeared above the growth front were purified and tested for their resistance. Resistance of the wild-type strain and of the mutants to carboxylic acids was determined by replica plating on MMY with 5 mM citrate and different concentrations of a carboxylic acid.

Haloacid dehalogenase assays. Crude extracts were prepared as described before (38). Haloacid dehalogenase activity was routinely measured by monitoring chloride release from 5 mM MCA dissolved in glycine-NaOH buffer (pH 9.0), using a colorimetric assay (38). One unit is the amount of enzymes that catalyzes the liberation of 1 μmol of halide per min.

DNA manipulations. Standard DNA techniques were used for plasmid isolation, restriction enzyme digestion, ligation, and transformation (23). DNA fragments were isolated from agarose gels as described by Pai and Bird (19). Triparental mating was done as described before (12) with pRK2013 as a helper plasmid (5). Total DNA from strains of *X. autotrophicus* was isolated from 100-ml cultures grown in nutrient broth medium for 2 to 3 days. Cells were collected by centrifugation, washed in 10 mM Tris-Cl (pH 8.0)–1 mM EDTA–50 mM NaCl, and resuspended in the same buffer containing 10 mg of lysozyme. The mixture was incubated for 1 h at room temperature. After addition of 8 ml of 10% sodium dodecyl sulfate (SDS) and incubation at 55°C for 1 h, 6 ml of 3 M Na acetate (pH 7.0) and 45 ml of isopropanol were added and mixed. DNA was collected by centrifugation, dried, and further purified by CsCl density gradient centrifugation.

Southern blotting and hybridization experiments. For hybridization experiments, DNA was digested with appropriate restriction enzymes and separated by agarose gel electrophoresis. Capillary transfer of DNA to nylon membranes (Nytran NY13N; Schleicher and Schuell) was done as recommended by the manufacturer. After transfer, the membrane was illuminated by UV radiation for 5 min.

DNA probes were prepared by incorporation of digoxigenin-dUTP, either with Klenow polymerase with the nonradioactive labelling kit as described by the manufacturer (Boehringer Mannheim) or during PCR as described previously (35). Hybridization and detection of hybridized probe were performed at 68°C as described before (35). *Hin*dIII-digested and digoxigenin-labelled λ DNA (Boehringer Mannheim) was used as a molecular weight marker.

For colony blotting, fresh bacterial colonies were transferred by putting a membrane (Nytran NY13N) on top of the agar plate. The membrane was then placed on a Whatman no. 1 filter which had been saturated with 0.5 N NaOH for 15 min at room temperature. The membrane was neutralized by placing it on a Whatman no. 1 filter saturated with 1 M Tris-HCl (pH 7.0)–1 M NaCl for 15 min. Finally, the membrane was washed with 10 mM Tris-HCl (pH 7.0) containing 1% SDS and dried.

DNA sequencing and sequence analysis. Nucleotide sequence information was obtained either by sequencing of exonuclease III-generated deletion clones by the procedure of Henikoff (9) or by using synthesized primers deduced from the already-determined sequence. The dideoxy chain termination method (24) was used. For radioactive labelling, α-^{35}S-dATP (Amersham) was used, while fluorescein-dATP or fluorescein-labelled primers were used for sequencing reactions on an automated laser fluorescent sequencing system (Pharmacia). For preparation of plasmid DNA template for sequencing, *E. coli* JM109 appeared to be more suitable as a host than strain JM101.

Analysis of the nucleotide sequence was done with the program PC/GENE (Genofit, Geneva, Switzerland). Comparison of amino acid sequences and nucleotide sequences with sequences from the SWISS-PROT protein database release 29 and EMBL database release 39 (EMBL, Heidelberg, Germany) was done by using the program FASTA (20). Sequence alignments were done with the program CLUSTALV (10).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to EMBL under accession number X84038.

RESULTS

Toxicities of halogenated and nonhalogenated carboxylic acids. *X. autotrophicus* GJ10 does not grow with MBA and grows only poorly with MCA. To test whether the inability to utilize various halogenated carboxylic acids is caused by toxicity, the effects of several of these compounds and of nonhalogenated carboxylic acids on the growth of *X. autotrophicus* GJ10 were determined on plates containing 5 mM citrate and 5, 10, 20, or 50 mM acetic acid, glycolic acid, trichloroacetic acid, MBA, or 2-monochloropropionic acid. The toxic concentration was defined as the lowest concentration at which no growth was observed. The nonhalogenated carboxylic acids were not toxic at these concentrations. 2-Monochloropropionic acid was the least toxic halogenated substrate, since growth was completely impaired only at a concentration of 50 mM. Trichloroacetic acid was toxic at 20 mM, whereas MBA completely inhibited growth at concentrations of 5 mM and higher. This shows that the halogenated compounds are more toxic than the nonhalogenated compounds.

Characterization of GJ10M50. Because MBA was the most toxic of the compounds tested, this substrate was chosen to isolate mutants that are able to grow with citrate and MBA as cosubstrates. One such mutant, GJ10M50, has been described before as a mutant that overproduces haloacid dehalogenase (38). The growth rate of GJ10M50 with 5 mM MCA was 0.087 h^{-1}, compared with 0.050 h^{-1} for the wild-type strain. Strain GJ10M50 was also able to grow on plates with 5 mM MBA as the sole carbon and energy source but not on 2-bromoethanol

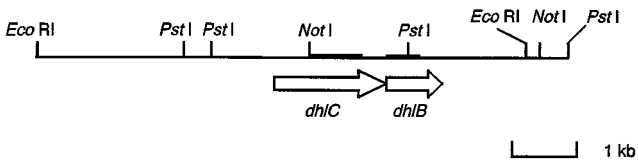

FIG. 1. Restriction site map of the *dhlB* region and positions of DNA probes A (*dhlB*) and B (*dhlC*).

or 1,2-dibromoethane. Haloacid dehalogenase expression appeared to be constitutive, since no significant difference in activity was found between cells grown on citrate (6.1 U/mg) and cells grown on citrate and MBA (5.7 U/mg).

To investigate the genetic changes that led to GJ10M50, and which could have occurred in the vicinity of the *dhlB* gene, hybridization experiments were carried out with two different DNA probes. Probe A was specific for a part of the *dhlB* gene, while probe B was homologous to DNA upstream of *dhlB* (Fig. 1) in the region containing *dhlC*, which encodes a putative transport protein (38a). Two *Pst*I fragments of GJ10M50 DNA of 4.8 and 2.3 kb hybridized with probe A, whereas only the 4.8-kb fragment hybridized with probe B (Fig. 2). For GJ10, probe A hybridized with *Pst*I fragments of 2.9 and 2.3 kb, and probe B hybridized with the 2.9-kb fragment only. A 5.5-kb *Not*I fragment of GJ10M50 DNA hybridized with both probes A and B, while in the wild type a 4.0-kb *Not*I fragment did so. From these results we concluded that a fragment of about 1.7 kb had inserted between the *Not*I site 1.7 kb upstream of *dhlB* and the *Pst*I site in *dhlB* (Fig. 1). Since the haloacid dehalogenase (DhlB) of mutant GJ10M50 appeared not to have changed (38), we presumed that the insertion had taken place upstream of the translational start site of *dhlB*.

Cloning of the inserted sequence. To analyze the inserted sequence in detail, the 3.0-kb *Pst*I-*Not*I fragment of strain GJ10M50 that hybridized with both probes was cloned. DNA of GJ10M50 was digested with *Pst*I and separated by agarose gel electrophoresis. Fragments of about 5.5 kb were isolated from the gel, digested with *Not*I, and ligated to *Not*I-*Pst*I-digested pBluescript II SK(−). *E. coli* JM101 was transformed with the ligation mixture, and the resulting transformants were screened for the correct insert by colony blotting with probe A. Isolation of a plasmid from one of the positive colonies and subsequent hybridization of this plasmid (designated pPM50) with probe B confirmed that the correct fragment had been cloned. Moreover, digestion of plasmid pPM50 with *Pst*I or *Pst*I-*Not*I followed by hybridization of the restriction fragments with probe B yielded the expected fragments of 6.0 and 1.9 kb, respectively.

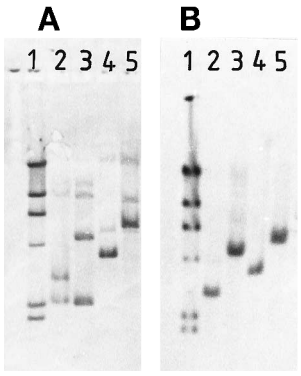

FIG. 2. Hybridization experiments with *dhlB* (A) and *dhlC* (B) probes with DNAs of strains GJ10 (lanes 2 and 4) and GJ10M50 (lanes 3 and 5) digested with *Pst*I (lanes 2 and 3) and *Not*I (lanes 4 and 5). Lanes 1, *Hin*dIII-digested and digoxigenin-labelled phage λ DNA (fragment sizes, 23.1, 9.4, 6.7, 4.4, 2.3, and 2.0 kb).

Sequence analysis of the inserted sequence. The nucleotide sequence of the inserted sequence and its bordering regions was determined in both directions (Fig. 3). The inserted fragment was designated IS*1247* since all the characteristics of an insertion sequence (IS) element (7) are present, as described below. The insertion had taken place at a site 34 bp upstream of the translation start codon of *dhlB* (38), in an open reading frame (ORF) of unidentified function designated *dhlC*. As a result of this insertion, the C-terminal sequence of *dhlC* had changed. A stop codon was present in the same reading frame 17 bp downstream of the insertion site. In this way, the last amino acid of the putative protein encoded by *dhlC* was replaced by seven other amino acids.

The insertion sequence is 1,672 bp in size and has terminal inverted repeats of 17 and 16 bp with three mismatches (Fig. 3). The GC content of IS*1247* is 62.3%, which is between that of the *Xanthobacter dhlB* gene (67% [38, 42]) and that of the plasmid-encoded *dhlA* gene (58% [12]). A search for ORFs revealed one ORF that could encode a protein of more than 150 amino acids. The ORF encodes a protein of 464 amino acids and has a codon usage that resembles the codon usage of *dhlB*. The hypothetical protein encoded by this ORF has a high percentage of positively charged amino acids and a calculated isoelectric point of 10.98. A potential ribosome binding site is present immediately upstream of the start of the ORF (Fig. 3). No consensus *E. coli* promoter could be identified according to the method of Staden (26), although a stretch of DNA with a high percentage of AT is found 50 to 30 bp upstream of the ORF (Fig. 3).

Sequence comparisons. A comparison of the hypothetical protein encoded by the ORF of IS*1247* with the SWISS-PROT protein sequence database revealed significant similarity with two putative transposases (Fig. 4). The highest degree of similarity (23.7% identity) was found with the putative transposase encoded on IS*1380* from *Acetobacter pasteurians* (28). The size of this transposase was 461 amino acids, which is close to that of the IS*1247* protein. The sizes of IS*1380* (1,665 bp) and its terminal inverted repeats (15 bp) are very similar to those of IS*1247*. Upon insertion of *IS1380*, a 4-bp duplication of the target site was generated (28). A lower degree of similarity (12.3% identity) was found with the putative transposase encoded on IS*940* from *Bacteroides fragilis* (21). This IS element is 1,598 bp in size, has 15-bp inverted repeats, and encodes a transposase of 430 amino acids.

A FASTA search of the EMBL DNA data bank with the sequence of IS*1247* revealed a very high degree of similarity (86.7% in 670 bp) with the region immediately upstream of the *aac*(*3*)-*Vb* gene from *Serratia marcescens* (22). This region contains an incompletely sequenced ORF. The C-terminal part of the protein encoded by this ORF has 98% identity with the putative transposase of IS*1247*. Most differences between the nucleotide sequences were located in the third position of each codon and did not result in differences in amino acid sequence. The similarity was 93.5% in the region encoding the ORF and 75.9% in the region downstream of the ORF. The GC content of this fragment was 65%.

Copy number of the insertion element. In order to determine the copy number of IS*1247* in *X. autotrophicus* GJ10 before the insertion in front of the *dhlB* gene occurred, total DNA of GJ10 was digested with different restriction enzymes, separated on an agarose gel, and hybridized with probe IS,

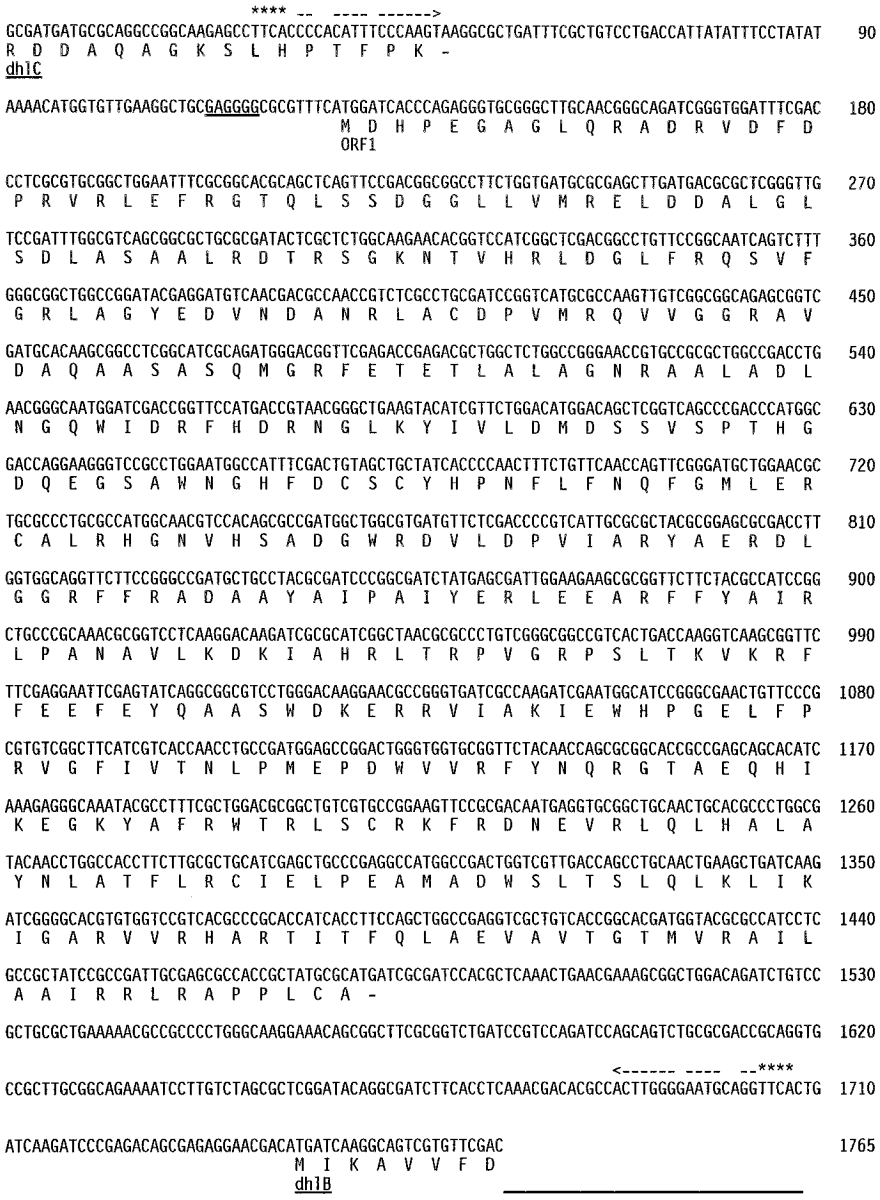

FIG. 3. DNA sequence of insertion element IS*1247*. The amino acid sequence of the putative transposase, the N-terminal sequence of *dhlB*, and the C-terminal sequence of *dhlC* are shown in the one-letter code. The imperfect inverted repeats are depicted by arrows; nucleotides of the target site of insertion are marked by asterisks. The putative ribosome binding site is underlined. Stop codons are shown by dashes.

which consisted of the internal 524-bp *Bgl*II-*Eco*RI fragment of IS*1247* (Fig. 5). Two copies of the insertion element were found to be present when the GJ10 DNA was digested with *Xho*I, *Bam*HI, *Eco*RI, or *Pst*I. When the DNA was digested with *Not*I, *Sal*I, or *Hin*dIII, only one fragment hybridized, the smallest being a *Hin*dIII fragment of about 20 kb. This indicates that the two copies of the insertion element are within a distance of 20 kb.

The insertion element could not be identified in *X. autotrophicus* XD, which does not utilize chlorinated compounds, but it was present in two copies in mutant GJ10M30, which is strain GJ10 lacking plasmid pXAU1. This plasmid encodes haloalkane dehalogenase and chloroacetaldehyde dehydrogenase (29, 39). Thus, both copies of IS*1247* are located on the chromosome of strain GJ10.

Isolation and characterization of other MBA-resistant mutants. Several new independent mutants resistant to MBA were isolated as described in Materials and Methods. Mutants were obtained after 1 to 2 weeks of incubation. All mutants were still able to grow with 1,2-dichloroethane and 2-chloroethanol but, like the wild-type strain and GJ10M50, not with 1,2-dibromoethane or 2-bromoethanol. The mutants showed better growth on plates with MCA and MBA as the sole carbon sources than the wild-type strain did. The mutants were tested for their resistance to MBA by replica plating on plates containing 5 mM citrate and different concentrations of MBA (Table 2). All mutants were able to grow with 10 mM MBA, but they showed different degrees of tolerance to higher MBA concentrations.

All mutants that showed increased MBA resistance had

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S1247      MDHPEGAGLQRADRVDFDPRVRLEFRGTQLSSDGGLLVMRELDDALGLSD   50
S1380      MQTECSAG-AYEFPASCGRRVVARFDGGRMSSDGGVILVKQADDILGLSR   49
S942       MA-------------------KVQIKSEKLTPFGGIFSIMEQFDAL-LAQ   30
                              . . .... **.. . .  * * *.

S1247      LASAALRDTRSGKNTVHRLDGLFRQSVFGRLAGYEDVND-ANRLACDPVM   99
S1380      RFAACFRDKRHPGFVEYRVEDLVRQRIMGLALGYEDLND-HDALRHDLIF   98
S942       TIDSTL-GLRCTMFG-YQYSEILRSLMCVYLCGGSCIEDVTTHLMKHLSL   78
              . . . *       .  . * .     *   ..*    * .

S1247      RQVVGGRAVDAQAASASQMGRFETETLALAGNRAA-----LAD---LNGQ  141
S1380      -GLASGRLSGGRANCAALAGKSTLNRLERSGHKADRYCRIIADHEALATL  147
S942       HPTLRTCSADTILRAIEELTCKNI-TYKSASASGNSY--DFNTADKMNCL  125
                      .                    ..  .     .    .

S1247      WIDRF---HDRNGLKYIVLDMDSSVSPTHGDQEGSAWNGHFDCSCYHPNF  188
S1380      FVTLFLDQHEHAPAR-IVLDVDATDDRIHGHQEGRAFHGYYGHNCYLPLY  196
S942       LIKALLATGQLKSGQEYDFDFDHQFIETEKYDAKPTYKKFLG---YSPGV  172
            .  .    .    .  .* *      .  .  .     .   * *

S1247      LF---NQFGMLERCALRHGNVHSADGWRDVLDPVIARYAERDLGGRFFRA  235
S1380      VFCGDHLLSATLRTADRDPGKEALADIRRIVEQIRSRWPRVRI---LVRG  243
S942       AVINDMIVGI----ENRDGNTNVRFNQKETLERIFKRLEASEIYISRARM  218
                      .      *.   .    .  .. .  *     .     *

S1247      DAAYAIPAIYERLEEARFFYAIRLPANAVLKDKIAHRLTRPV--GRPSLT  283
S1380      DSGFARDSLMTWCEDNHVDFLFGLAGNTRLYDRIA-SLSAEVRDEAATTG  292
S942       DCGSCSEEIVDMVEAHCRHFYIRANRCSSFYDSMFALTGWKTVEINGIEF  268
            * .    .   *    . .    . . * .

SSMT                                          WHPGELFPRVGFIVTNLP
S1247      KVKRFFEEFEYQA-ASW---DKERRVIAKIE-WHPGELFPRVGFIVTNLP  328
S1380      RAARGFASFDWITKDSW---TRRRRVVAKAE-WRHGNRYHR--FIVTTLP  336
S942       ELNSILVE-KWKGKPYRLVIQRQRRIDGDLDIWEGEYTYRCI--LTNDYK  315
                        .   . **. . . *     .     .

SSMT       MEPDWVVRFYNQ----RGTAEQHIKEGKYAFHWTRLSCRKFRDNEVRLQL
S1247      MEPDWVVRFYNQ----RGTAEQHIKEGKYAFRWTRLSCRKFRDNEVRLQL  374
S1380      QGMSDPRHLYEQIYCARGDMENRIKECQMDLFSDRTSSHTIRANQLRLWF  486
S942       SSARDIVEFYNL----RGGKERIFDDMNNDFGWNRLPKSFMAQNTVFLLM  361
                  .*.    **  *  . . .  . *.     * . *

SSMT       HALAYNLA-TFLRCIELPET-M-ADWSLTSLQLKLIKIGARVVRHARAIT
S1247      HALAYNLA-TFLRCIELPEA-M-ADWSLTSLQLKLIKIGARVVRHARTIT  421
S1380      SAAAYVLL-TALQRLALGQTSL-ETATCGTIRARLLKIATRVTLSVRRIV  434
S942       TALIRNFYKAIMQRLKTHEFGLRATSRIKTFVFKFISVPAKWIKTSRRHV  411
            *   . .. .  . .      ..  ... . ..     *

SSMT       FQL-AEVAVTGPMVRAILAAIRRLRAPPLCA
S1247      FQL-AEVAVTGTMVRAILAAIRRLRAPPLCA  451
S1380      LSM-PDMFPCQHEFALAHARLRRLRQA---I  461
S942       LNIYSDNNAYANLFKTDFG------------  430
           . . ..          .
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FIG. 4. CLUSTALV sequence alignment of putative transposases encoded on IS*1247* from *X. autotrophicus* GJ10M50, IS*1380* from *A. pasteurians* (28), and IS*942* from *B. fragilis* (21). The incomplete ORF (ISSMT) encoded upstream of the *aac*(*3*)-*Vb* gene from *S. marcescens* (22) was not used in the alignment but is shown above the putative transposase of IS*1247*. Symbols: ∗, identical residue; ., conserved residue; –, gap.

higher haloacid dehalogenase activities than the wild type, but there was no strict correlation between the level of dehalogenase activity and the degree of resistance to MBA, which indicates that resistance is also determined by other factors.

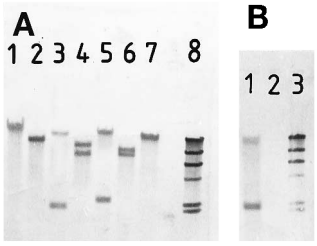

FIG. 5. Copy number determination for IS*1247* in *X. autotrophicus* GJ10, GJ10M30, and XD. (A) Hybridization with probe IS with DNA of GJ10 digested with *Sal*I (lane 1), *Hin*dIII (lane 2), *Eco*RI (lane 3), *Xho*I (lane 4), *Bam*HI (lane 5), *Pst*I (lane 6), or *Not*I (lane 7). (B) Hybridization with DNAs of GJ10M30 (lane 1) and XD (lane 2) digested with *Eco*RI. Lanes 8 and 3 in panels A and B, respectively, show *Hin*dIII-digested and digoxigenin-labelled λ DNA.

TABLE 2. Characterization of MBA-resistant mutants of *X. autotrophicus* GJ10

Strain	MBA resistance (mM)[a]	Haloacid dehalogenase activity (U/mg)
GJ10	<5	0.9
GJ10M50	20–25	3.2
GJ10M61	10–15	2.7
GJ10M62	15	ND[b]
GJ10M63	10	2.6
GJ10M66	15	2.9
GJ10M67	10–15	2.8
GJ10M68	20	3.6
GJ10M75	20–25	1.2

[a] MBA resistance was defined as the highest concentration of MBA that still allowed growth on plates containing 5 mM citrate.
[b] ND, not determined.

To determine whether insertion of IS*1247* upstream of *dhlB* had occurred in all MBA-resistant mutants, eight independent mutants were characterized by Southern blotting (Fig. 6). With probe A, it was found that in all the mutants except GJ10M50 and GJ10M75, the 2.9-kb *Pst*I fragment and the 1.8-kb *Eco*RI-*Pst*I fragment had not changed (Fig. 6A). This implies that insertions had taken place immediately upstream of *dhlB* in GJ10M50 and GJ10M75 but not in the other mutants. As shown in Fig. 6B, however, hybridization of the mutants with probe IS revealed that in all mutants one or two additional copies of the insertion element were present.

Analysis of mutants of GJ10M30(pPJ20) resistant to MBA. *X. autotrophicus* GJ10M30 is a derivative of GJ10 that is not able to grow with 1,2-dichloroethane because it lacks the pXAU1 plasmid, which encodes haloalkane dehalogenase and chloroacetaldehyde dehydrogenase. The strain still contains the chromosomally encoded haloacid dehalogenase activity (39). Strain GJ10M30(pPJ20) is a derivative of GJ10M30 that contains the *dhlA* gene cloned in the broad-host-range vector pLAFR1 (12). This strain was used to isolate the MBA-resistant mutant GJ10M30M1 in the same way that mutant GJ10 M50 was isolated, in order to check whether the dehalogenase gene could be mobilized to a plasmid. The resistance to MBA and the haloacid dehalogenase activity were higher than those in GJ10M30 (Table 3). Subsequently, mutant GJ10M30M1

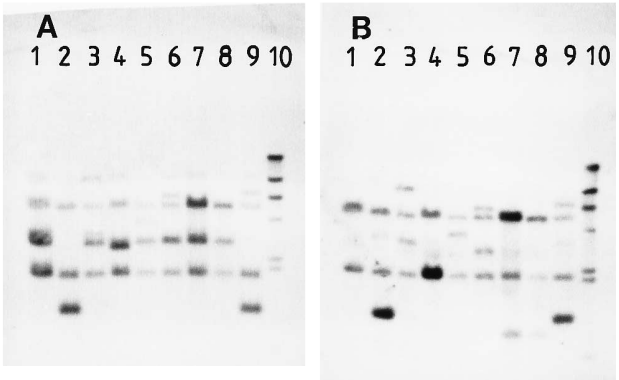

FIG. 6. Southern hybridization analysis of MBA-resistant mutants. DNAs from wild-type GJ10 (lanes 1) and mutants GJ10M50 (lanes 2), GJ10M61 (lanes 3), GJ10M62 (lanes 4), GJ10M63 (lanes 5), GJ10M66 (lanes 6), GJ10M67 (lanes 7), GJ10M68 (lanes 8), and GJ10M75 (lanes 9) were digested with *Pst*I and *Eco*RI and hybridized with probe A (A) or probe IS (B). Lanes 10, *Hin*dIII-digested and digoxigenin-labelled λ DNA.

TABLE 3. Activities and MBA resistance of mutants of GJ10M30(pPJ20)

Strain or mutant	MBA resistance (mM)[a]	Haloacid dehalogenase activity (U/mg)
GJ10M30(pPJ20)	5	0.6
GJ10M30M1	15	1.6
GJ10M30M1A	35	3.0
GJ10M30M1B	25	1.6
GJ10M30M1E	25	1.6
GJ10M30M1F	40	3.9
XD	<5	—[b]
XDM1	<5	—
XDM1A	15	3.0
XDM1B	<5	—
XDM1E	<5	—
XDM1F	20	1.8

[a] See Table 2, footnote *a*.
[b] —, no detectable haloacid dehalogenase activity.

was used to generate additional mutants which had even higher resistance to MBA. In this way, GJ10M30M1A, -M1B, -M1E, and -M1F were isolated (Table 3). Only two of these mutants produced a higher level of haloacid dehalogenase (Table 3).

Plasmids from mutants GJ10M30M1, -M1A, -M1B, -M1E, and -M1F were isolated and conjugated to *X. autotrophicus* XD. This strain does not possess haloacid dehalogenase activity and is sensitive to MBA at concentrations higher than 5 mM. Only transconjugants carrying the plasmids of GJ10M 30M1A and -M1F were found to be MBA resistant and to contain haloacid dehalogenase activity (Table 3). This implies that in these mutants *dhlB* had moved from the chromosome of GJ10M30 to plasmid pPJ20. Southern blotting analysis showed that in GJ10M30M1 and its derivatives, except GJ10M30M1F, *dhlB* was located on a 3.0-kb *Eco*RI restriction fragment (results not shown). In GJ10M30M1F, *dhlB* was located on a 4.5-kb *Eco*RI fragment, which suggests that in this mutant additional rearrangements had occurred.

Analysis of mobilized IS*1247-dhlB* regions. Mutants GJ10M 30M1, -M1B, and -M1E were not further characterized. The pPJ20-derived plasmids present in mutants GJ10M30M1A and -M1F (designated pM1A and pM1F, respectively) were analyzed by restriction site mapping and comparison with pPJ20 and by Southern blotting. Both plasmids contained one copy of IS*1247* as well as of *dhlB* (results not shown). This suggests that in these mutants IS*1247* had copied itself to a position close to *dhlB*, which was then followed by transposition to the plasmid. In pM1A, an insertion of about 10.5 kb had occurred between the tetracycline resistance region and the *cos* site of pLAFR1 DNA (6) (Fig. 7). In pM1F, an insertion of about 6 kb had taken place in the 1.7-kb *Hin*dIII fragment encoding *dhlA* (12) (Fig. 7).

To analyze both insertions further, appropriate restriction fragments were cloned and sequenced. A 7.8-kb *Pst*I fragment of pM1A containing the right end of the insertion (Fig. 7) was cloned in plasmid pBluescript II SK(−) to yield pA4. The right end of the insertion site was determined by DNA sequencing of pA4 with primer I. The sequence was identical to that of IS*1247*. Immediately to the right of IS*1247*, the sequence was found to be identical to the region downstream of the *tetR* gene of plasmid RP1, which is the same as that in pLAFR1, starting with nucleotide 524 of the published sequence (40). The left end of the insertion in pM1A was located on a 2.0-kb *Sal*I fragment of pM1A and cloned into the *Xho*I site of pGEM-

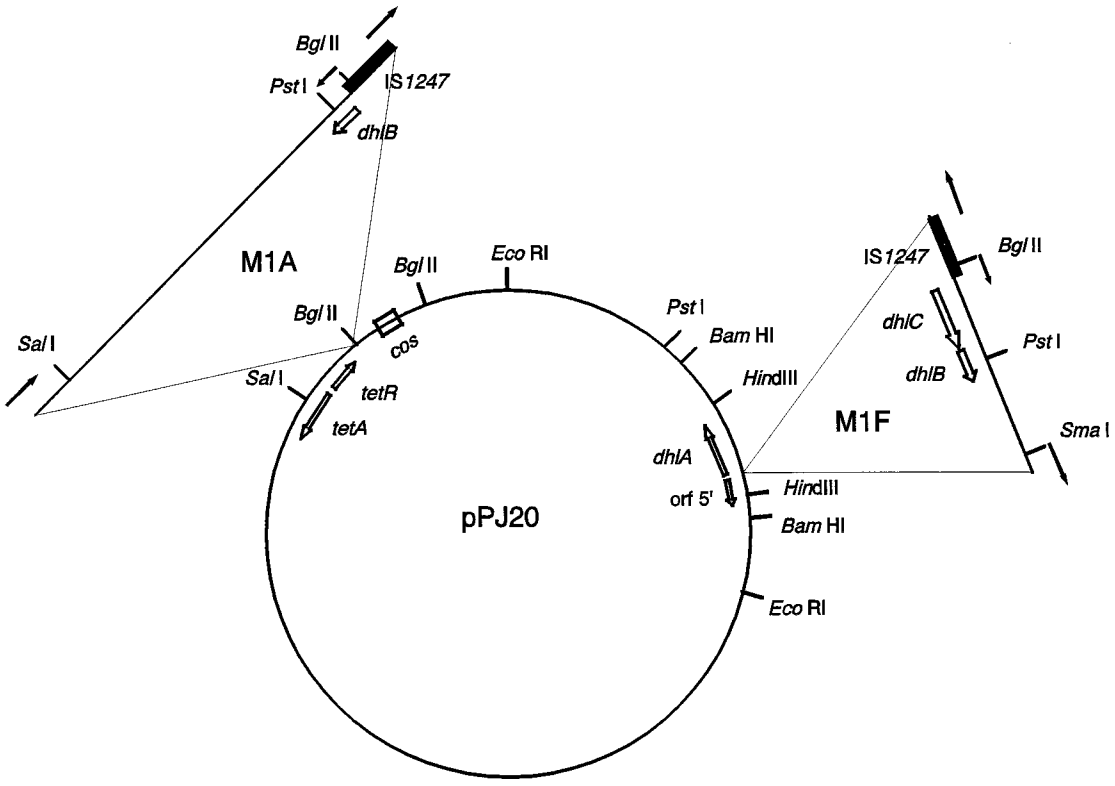

FIG. 7. Insertions in plasmid pPJ20. The insertion element is shown as a filled box; genes are shown as open arrows. Closed arrows represent the parts that were sequenced to determine the insertion sites. Only relevant restriction sites are shown.

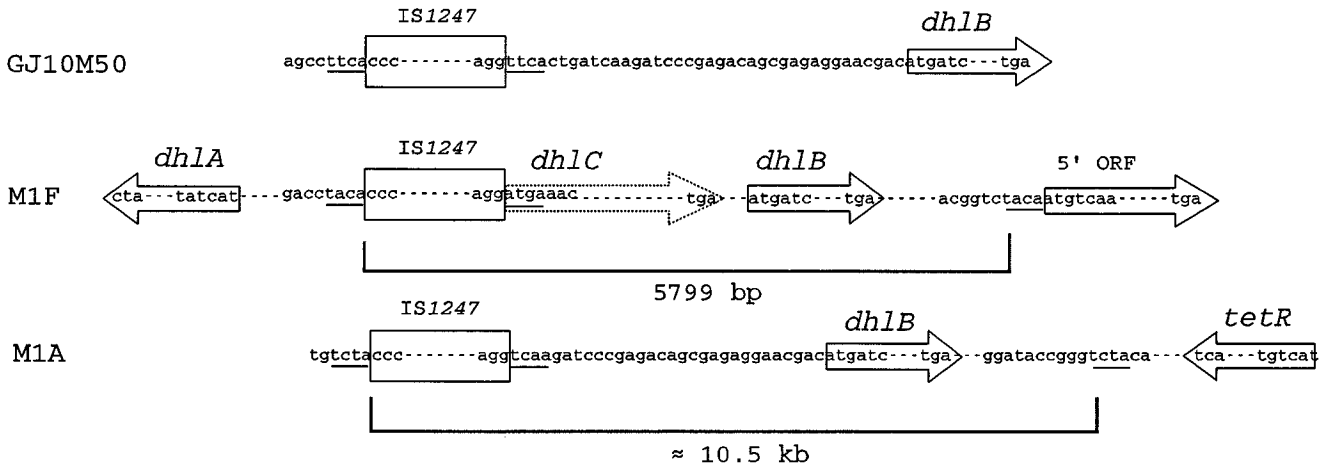

FIG. 8. Schematic representation of mutants GJ10M50, GJ10M30M1F, and GJ10M30M1A. Genes are shown as open arrows, IS*1247* is boxed, and the target sites of insertion of IS*1247* and of the one-ended transposition are underlined.

5Zf(−) to yield plasmid pA45. The sequence bordering the insertion was determined by using primer T, which is complementary to nucleotides 591 to 572 of the fragment encoding the tetracycline resistance genes (40). The same insertion site as at the right-end insertion site was found. The sequence originating from *X. autotrophicus* GJ10M30 DNA beyond the left-end insertion site did not have similarity to IS*1247*, nor was there substantial similarity to any other sequence from the EMBL database for the 457 bp that was determined. During insertion of the 10.5-kb fragment in the tetracycline resistance region of pPJ20, a 4-bp duplication (TCTA) occurred (Fig. 8). The position of IS*1247* upstream of *dhlB* in pM1A was also determined and was 26 bp upstream of the translational start site of *dhlB*, which is different from that in GJ10M50. The insertion element was in the same orientation relative to *dhlB* as in GJ10M50 (Fig. 8). These observations indicate that IS*1247* indeed had activated the *dhlB* gene and subsequently was copied with *dhlB* to the plasmid to further increase expression as a result of the higher copy number of the plasmid-harbored gene.

For analysis of pM1F, the 7-kb *Hin*dIII fragment containing the complete insertion was cloned in the *Hin*dIII site of pGEM-7Zf(−) in both orientations to yield plasmids pFHA and pFHB. The sequence of the right-hand border of the fragment that had inserted in pPJ20 (Fig. 7) was determined by using primer I. The left-end site was determined by making a *Sma*I deletion in pFHA and sequencing the resulting plasmid, pFHAS, with the universal M13 primer. The insertion had taken place between the gene encoding haloalkane dehalogenase (*dhlA*) and an ORF of unknown function, between positions 924 and 923 of the *dhlA* region (12). The region of the inserted sequence located closest to *dhlA* appeared to be IS*1247*. Upon insertion, a 4-bp duplication (TACA) had occurred. The left-hand inverted repeat (Fig. 3) of IS*1247* was different from that from GJ10M50 and pM1A, since the first C residue was missing. The position of IS*1247* upstream of *dhlB* was determined by making a *Bgl*II-*Bam*HI deletion in pFHB and sequencing the resulting plasmid with the reverse M13 primer. The insertion that activated the *dhlB* gene had taken place immediately upstream of the putative translation start codon of *dhlC* at the target site ATGA (Fig. 8). The total size of the insertion that occurred in plasmid pPJ20 was calculated to be 5,799 bp.

Target sites of IS*1247*. Inspection of the five different target sites for insertion of IS*1247* detected in this study (Fig. 8) reveals a preference for AT-rich sequences containing an A residue at the fourth position of the duplicated target.

DISCUSSION

In this paper we describe an experimental approach to study the genetic adaptation of a strain of *X. autotrophicus* to toxic concentrations of a halogenated aliphatic compound. We have shown that resistance of *X. autotrophicus* GJ10M50 to MBA is accompanied by the insertion of IS*1247* upstream of *dhlB*. The insertion caused increased expression of haloacid dehalogenase.

In GJ10, MBA is already toxic at 5 mM. The observation that GJ10M50 has an increased growth rate with MCA may indicate that this substrate is also toxic to some extent or that dehalogenation of MCA is a rate-limiting step. Other brominated compounds, such as 1,2-dibromoethane or 2-bromoethanol, also cannot be used by GJ10, whereas the chlorinated analogs are good growth substrates (13). Slater et al. (25) observed that MBA is more toxic than MCA for *P. putida* PP3. MBA was toxic even at the lowest concentration used (4 mM) to cultures in which dehalogenase was induced, as well as to uninduced cultures. Although brominated compounds are less stable than their chlorinated analogs, they also appear to be more toxic, probably because the carbon-bromine bond is more labile than the carbon-chlorine bond and thus is more reactive.

Toxicity of substrates or intermediates may be an important cause of recalcitrance of synthetic compounds to biodegradation. Although halogenated carboxylic acids are also produced in natural environments, it is of interest to obtain insight into the mechanism of adaptation to these compounds. The toxicity can be alleviated by various mechanisms. In the MBA-resistant mutants of GJ10, the resistance appears to be caused by the higher-level expression of haloacid dehalogenase, which reduces the concentration of MBA. This implies that the products have to be less toxic. Glycolate or acetate indeed was found not to be toxic at the same concentration as MBA.

Another mechanism to decrease the toxicity is to reduce the intracellular concentration of the toxic compound through decreased uptake or increased export. With *P. putida* PP3, mutants resistant to the nonmetabolizable and toxic substrate MCA showed both loss or decreased uptake of halogenated

carboxylic acids and a lower level of dehalogenase activity (41). It was concluded that the genes encoding the uptake system and dehalogenase were closely linked and that reduction of uptake decreased the intracellular concentration of MCA. Strotmann et al. (27) described a mutant of 2-chloroethanol-utilizing *P. putida* US2 that was resistant to high concentrations of 2-chloroethanol and that had lost chloroacetate dehalogenase activity. The resistance was accompanied by increased concentrations of chloroacetate in the medium, probably through increased export, although it was not clear why that would cause resistance to 2-chloroethanol.

The formation of toxic intermediates may also be prevented by the loss of enzyme activity. With *X. autotrophicus* GJ10, a mutant resistant to 1,2-dibromoethane had lost plasmid pXAU1, resulting in the loss of haloalkane dehalogenase and the inability to produce the toxic intermediate bromoacetaldehyde (39). Likewise, a mutant of the gram-positive 1,6-dichlorohexane-utilizer strain GJ70 that was resistant to 2-bromoethanol had lost alcohol dehydrogenase activity, which also prevented the formation of bromoacetaldehyde (11).

The increased expression of haloacid dehalogenase in GJ10M50 must be due to the insertion of IS*1247* upstream of *dhlB*. Many other insertion elements have been shown to be able to activate gene expression (7) and to be involved in the evolution of catabolic pathways for xenobiotic compounds (43). In *Pseudomonas cepacia* AC1100, IS elements were able to activate a promoterless stremptomycin gene (8). These IS elements were thought to play a role in the development of a 2,4,5-trichlorophenoxyacetic acid metabolic pathway in this strain.

Increased gene expression may be the result of transcription initiation from a different promoter, which can be located completely within the IS element (4), or from a hybrid promoter formed by a combination of a −35 region on the IS element and a −10 region on the adjacent element (1). Attempts to isolate sufficient mRNA from strain GJ10M50 to identify a promoter on IS*1247* by reverse transcriptase sequencing failed, possibly because of excess slime production and/or a low mRNA content in this slowly growing strain. In the wild-type strain GJ10, *dhlB* is probably expressed from a promoter located upstream of the *dhlC* gene (38a). This area is missing in mutants harboring pM1F and pM1A, in which the dehalogenase gene is mobilized to a plasmid without the region upstream of *dhlC*. Furthermore, in these mutants there are different sequences present upstream of IS*1247*, namely, *tetR* region DNA and DNA upstream of *dhlA*, both of which allow efficient expression of haloacid dehalogenase. Thus, it is unlikely that activation of sequences upstream of IS*1247* causes overexpression of *dhlB* in the mutants. Activation by IS*1247* of a regulatory sequence downstream of its insertion site is also unlikely to be the cause of increased expression, since there are only 30 and 26 nucleotides between the insertion element and the translational start of the *dhlB* gene in GJ10M50 and GJ10M30M1A, respectively. Thus, it is most likely that IS*1247* itself harbors the promoter sequence and transcription start site, as was proposed for the closely related IS*942* from *B. fragilis* (21). Since no consensus promoter sequences for *Xanthobacter* spp. are known, it is not yet possible to indicate what specific promoter site is causing *dhlB* overexpression in the mutants.

For most of the other mutants that were isolated and listed in Table 2, it is difficult to envisage that expression is increased by use of a stronger promoter, since these insertions were not closely linked to *dhlB*. These mutants are different in their resistance to MBA, and there appears to be no correlation between dehalogenase activity and resistance. Furthermore, the targets for IS*1247* appear to be different. The expression in these mutants may have been affected by mutations in regulatory genes.

The IS elements that are related to IS*1247* were reported to have different effects on gene expression. In *A. pasteurians*, insertion of IS*1380* in the gene encoding cytochrome *c* resulted in an inactive alcohol dehydrogenase and sensitivity to acetic acid (28). In *B. fragilis*, IS*942* was located upstream of the gene encoding a metallo-β-lactamase. It was proposed that expression of metallo-β-lactamase was driven from a promoter located on the IS element (21). The high level of sequence similarity between the sequence upstream of the *aac*(*3*)-*Vb* gene from *S. marcescens* and IS*1247* suggests that *aac*(*3*)-*Vb* is also an insertion element, although it has not yet been identified as such (22). This putative insertion element is probably involved in the expression of the acetyltransferase which determines resistance to certain aminoglycides. If the sizes of IS*1247* and the putative insertion element from *S. marcescens* are considered to be identical, then the right-end site of the insertion element from *S. marcescens* is located within 4 bp of the translational start site of the *aac*(*3*)-*Vb* gene, which suggests that the promoter is located on the element. There is also a reasonable ribosome binding site present on the IS element.

IS*1247* was not present in *X. autotrophicus* XD or on the plasmid pXAU1 of GJ10. This implies that it is not linked to the haloalkane dehalogenase gene or other plasmid-encoded genes. The presence of two closely linked copies of the insertion element may indicate that they are part of a transposon. Several other catabolic genes have also been found to reside on transposons (18, 33, 34, 37).

There are indications that haloacid dehalogenase genes also may be located on a transposon. In *P. putida* PP3, one of the two genes encoding haloacid dehalogenase (*dehI*) was found to reside on an element, designated *DEH*, which was unusual in that the size of the transposed fragment was not constant, contrary to the case for normal transposons (31, 32). Insertion elements in the *DEH* element have not yet been characterized. It has been suggested that, depending on the conditions, *dehI* can be switched off and on. Under unfavorable conditions, e.g., toxicity of substrate analogs, the element transposes in such a way that *dehI* becomes cryptic. Under other conditions, e.g., starvation, the element becomes decryptified again (30).

Kawasaki et al. (14) have suggested that *dehH2*, encoding a haloacetate dehalogenase which is similar to the enzyme encoded by *dhlB*, is also located on a transposable element. Frequent loss of a 5.4-kb element containing this gene and two repeated sequences from plasmid pUOH was observed (15). Sequence analysis showed that identical sequences were neighboring *dehH2*, although it is not known whether these repeated sequences are insertion elements (14).

A striking observation in our selection experiments with GJ10M30(pPJ20) is the transposition to plasmid pPJ20 of IS*1247* together with *dhlB* and downstream sequences that do not resemble IS-like elements and are not of identical size. This phenomenon has been termed one-ended transposition and has been observed before in transposons Tn*3*, Tn*21*, and Tn*1721*, which lack one end of the inverted repeat (2, 3, 17). It occurs at much lower frequencies than normal transposition. As in GJ10M50, the increased resistance of the mutants of GJ10M30(pPJ20) to MBA is related to the elevated expression of haloacid dehalogenase, which may also be caused by the copy number of the plasmid.

In conclusion, two important characteristics of an IS have been found during our selection experiments. A dehalogenase gene can become overexpressed by an insertion element, and the combination of the insertion element and dehalogenase

gene can transpose to another replicon. Insertion elements thus provide a mechanism that may be responsible for the frequently observed plasmid location of catabolic genes involved in the degradation of xenobiotic compounds. Under natural conditions, environmental stress, such as the presence of a toxic substrate, can lead to the selection of strains in which these events have occurred.

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