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Published in:
Journal of Bacteriology

DOI:
[10.1128/JB.182.8.2191-2199.2000](https://doi.org/10.1128/JB.182.8.2191-2199.2000)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2000

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Citation for published version (APA):

Poelarends, GJ., Kulakov, LA., Larkin, MJ., van Hylckama Vlieg, J. E. T., & Janssen, DB. (2000). Roles of horizontal gene transfer and gene integration in evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. *Journal of Bacteriology*, 182(8), 2191-2199. <https://doi.org/10.1128/JB.182.8.2191-2199.2000>

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Roles of Horizontal Gene Transfer and Gene Integration in Evolution of 1,3-Dichloropropene- and 1,2-Dibromoethane-Degradative Pathways

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Received 10 November 1999/Accepted 27 January 2000

The haloalkane-degrading bacteria *Rhodococcus rhodochrous* NCIMB13064, *Pseudomonas pavonaceae* 170, and *Mycobacterium* sp. strain GP1 share a highly conserved haloalkane dehalogenase gene (*dhaA*). Here, we describe the extent of the conserved *dhaA* segments in these three phylogenetically distinct bacteria and an analysis of their flanking sequences. The *dhaA* gene of the 1-chlorobutane-degrading strain NCIMB13064 was found to reside within a 1-chlorobutane catabolic gene cluster, which also encodes a putative invertase (*invA*), a regulatory protein (*dhaR*), an alcohol dehydrogenase (*adhA*), and an aldehyde dehydrogenase (*aldA*). The latter two enzymes may catalyze the oxidative conversion of *n*-butanol, the hydrolytic product of 1-chlorobutane, to *n*-butyric acid, a growth substrate for many bacteria. The activity of the *dhaR* gene product was analyzed in *Pseudomonas* sp. strain GJ1, in which it appeared to function as a repressor of *dhaA* expression. The 1,2-dibromoethane-degrading strain GP1 contained a conserved DNA segment of 2.7 kb, which included *dhaR*, *dhaA*, and part of *invA*. A 12-nucleotide deletion in *dhaR* led to constitutive expression of *dhaA* in strain GP1, in contrast to the inducible expression of *dhaA* in strain NCIMB13064. The 1,3-dichloropropene-degrading strain 170 possessed a conserved DNA segment of 1.3 kb harboring little more than the coding region of the *dhaA* gene. In strains 170 and GP1, a putative integrase gene was found next to the conserved *dhaA* segment, which suggests that integration events were responsible for the acquisition of these DNA segments. The data indicate that horizontal gene transfer and integrase-dependent gene acquisition were the key mechanisms for the evolution of catabolic pathways for the man-made chemicals 1,3-dichloropropene and 1,2-dibromoethane.

Synthetic haloalkanes form an important class of environmental pollutants because of their widespread use in industry and agriculture, persistence in the environment, and potential carcinogenicity. The poor biodegradability of these chemicals is mainly due to the inability of microorganisms to effectively metabolize these unnatural compounds. Nevertheless, microbial communities exposed to synthetic haloalkanes often respond by expressing specific pathways that degrade these molecules in order to exploit them as growth substrates. Since synthetic haloalkanes are xenobiotic compounds of recent origin, the way in which genes have been assembled to form functional catabolic pathways is an interesting subject for studying microbial evolution and gene transfer.

Rhodococcus rhodochrous NCIMB13064, isolated in the United Kingdom from a soil sample obtained from an industrial site which had previously been exposed to chlorinated alkanes, is capable of utilizing 1-chlorobutane and several other haloalkanes as the sole carbon and energy source (9). The cleavage of the carbon-halogen bond in 1-chlorobutane, which is the key step in its catabolism, is catalyzed by an inducible hydrolytic haloalkane dehalogenase (DhaA) and results in the formation of *n*-butanol. This intermediate is subsequently oxidized in two steps to *n*-butyric acid (Fig. 1), which

can serve as a growth substrate for many bacteria. The haloalkane dehalogenase gene (*dhaA*) was shown to be located on the autotransmissible plasmid pRTL1 (22) and was cloned and sequenced (23).

The gram-negative 1,3-dichloropropene-utilizing bacterium *Pseudomonas pavonaceae* 170, isolated in The Netherlands from soil that was repeatedly treated with the nematocidal soil fumigant 1,3-dichloropropene, was shown by PCR amplification to possess a haloalkane dehalogenase gene identical to the *dhaA* gene of the gram-positive strain NCIMB13064 (35). In contrast to the inducible production of DhaA in strain NCIMB13064, DhaA is constitutively produced in strain 170 and catalyzes the first step in the degradation of 1,3-dichloropropene (Fig. 1).

Recently, we demonstrated that the 1,2-dibromoethane-degrading organism *Mycobacterium* sp. strain GP1, which was isolated by prolonged batch enrichment from a mixed bacterial culture, also contains a haloalkane dehalogenase gene (*dhaA_f*) that is very similar to the *dhaA* gene found in strain NCIMB13064 (36). The haloalkane dehalogenase encoded by *dhaA_f* is identical to DhaA, except for three amino acid substitutions and a 14-amino-acid extension at the C terminus. Nucleotide sequence analysis indicated that the *dhaA_f* gene was formed by a fusion of a *dhaA* gene with the last 42 nucleotides of a *hheB* gene, which encodes a haloalcohol dehalogenase (50). The haloalkane dehalogenase (DhaA_f) is constitutively produced in strain GP1 and catalyzes the conversion of 1,2-dibromoethane to 2-bromoethanol, which is further metabolized via ethylene oxide (Fig. 1).

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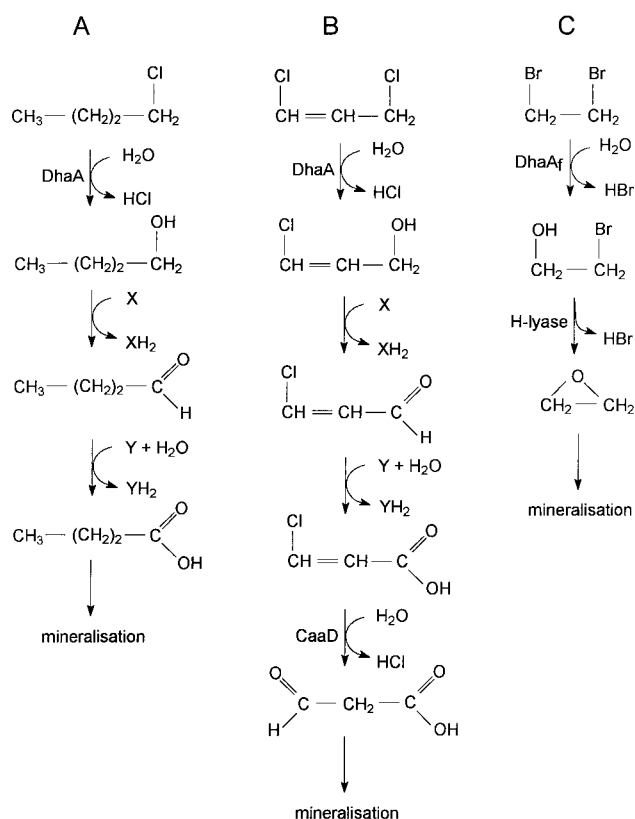


FIG. 1. Catabolic pathways for haloaliphatics. (A) 1-Chlorobutane in *R. rhodochrous* NCIMB13064. (B) 1,3-Dichloropropene in *P. pavonaceae* 170. (C) 1,2-Dibromoethane in *Mycobacterium* sp. strain GP1. Abbreviations: DhaA and DhaA_f, haloalkane dehalogenases; H-lyase, halohydrin halogen-halide lyase; CaaD, 3-chloroacrylic acid dehalogenase; X, alcohol dehydrogenase cofactor; Y, aldehyde dehydrogenase cofactor.

The presence of a highly conserved *dhaA* gene in the three phylogenetically different organisms *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 suggests that *dhaA* has been distributed among these organisms by horizontal transfer. To determine the size of the transferred DNA fragments and to identify the mechanisms that were involved in the distribution process, we have analyzed the DNA regions flanking the *dhaA* gene in these three haloalkane-utilizing strains. The results suggest that the haloalkane dehalogenase gene regions of strains 170 and GP1 originate from a 1-chlorobutane catabolic gene cluster similar to the one that is present on plasmid pRTL1 in strain NCIMB13064. Horizontal gene transfer and integrase-dependent acquisition of existing DNA fragments harboring the *dhaA* gene were probably the key steps during the evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. Furthermore, the constitutive expression of *dhaA* in strains 170 and GP1, in contrast to the inducible expression of *dhaA* in strain NCIMB13064, is explained by the absence or inactivation of the regulatory gene *dhaR*.

MATERIALS AND METHODS

Materials. Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase, the DNA-packaging kit, and materials used for Southern blot hybridization were purchased from Boehringer Mannheim (Mannheim, Germany). 1,2-Dibromoethane was supplied by Acros Organics (Geel, Belgium). The oligonucleotides used as primers were supplied by Eurosequence BV (Groningen, The Netherlands).

Bacterial strains, plasmids, and growth conditions. The characteristics of the 1,3-dichloropropene-degrading bacterium *P. pavonaceae* 170, formerly known as *Pseudomonas cichorii* 170 (44), and of the 1,2-dibromoethane-degrading organism *Mycobacterium* sp. strain GP1 are given elsewhere (35, 36). *R. rhodochrous* NCIMB13064 contains the large catabolic plasmid pRTL1 (22) and is able to use 1-chlorobutane as the sole carbon and energy source (9). *Escherichia coli* strains HB101 (7) and JM101 (49) and plasmid pBluescript SK(-) (Stratagene, Leusden, The Netherlands) were used for routine cloning experiments. *E. coli* HB101(pRK600) (13) was the helper strain used for mobilizing pLAFR3/5- and pDSK519-derived plasmids in triparental matings with the recipient *Pseudomonas* sp. strain GJ1 (17). Plasmid pDSK519 and cosmids pLAFR3 and pLAFR5 are mobilizable broad-host-range vectors (19, 39). Recombinant cosmid pLTL1k contains the 1-chlorobutane catabolic gene cluster of strain NCIMB13064 (23) and was used as template for DNA sequencing and as the source for some cloning and expression experiments described here. Recombinant cosmids pGP1-4B5, which contains the *dhaA_f* gene region of strain GP1 (36), and pPC33, which contains the *dhaA* gene region of strain 170 (this study), were used as templates for DNA sequencing.

R. rhodochrous NCIMB13064 and *Mycobacterium* sp. strain GP1 were grown at 30°C on nutrient broth or on mineral medium (17) supplemented with 5 mM *n*-propanol, respectively. *Pseudomonas* and *E. coli* strains were grown at 30°C on Luria-Bertani medium (38). When required, Difco agar (15 g/liter) was added to the medium. LBZ medium, used for qualitative dehalogenase activity determination, was solid Luria-Bertani medium without NaCl. Antibiotics were added in the following amounts: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 50 µg/ml; and tetracycline, 12.5 µg/ml. When necessary, media were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.4 mM).

DNA techniques. General procedures for cloning, transformation, and DNA manipulation were performed essentially as described by Sambrook et al. (38). Triparental matings were carried out as described by Janssen et al. (18). Isolation of total genomic DNA from strains NCIMB13064, 170, and GP1 was performed according to the phenol extraction procedure described previously (35). The IS2112- and IS1071-specific probes used in hybridization experiments were obtained by PCR amplification by using primers and conditions as previously described (21, 48). DNA fragments were purified by using the Qiaquick PCR purification kit (Qiagen). Southern blot hybridization experiments were performed as described previously (21).

Crude extracts and dehalogenase assays. *Pseudomonas* and *E. coli* cells were harvested in the late-exponential growth phase by centrifugation (10 min at 10,000 × g), were washed with 1 volume of 50 mM Tris-sulfate buffer (pH 8.2), and were disrupted at 4°C in an appropriate amount of this buffer by sonication (10 s per ml of suspension at a 70 W output in a Vibra cell sonicator). A crude extract was obtained by centrifugation (45 min at 16,000 × g).

Haloalkane dehalogenase activities were measured by incubating an appropriate amount of cell extract with 3 ml of 5 mM 1,2-dibromoethane in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (20). All dehalogenase activities are expressed as units per milligram; 1 U was defined as the amount of enzyme that catalyzes the production of 1 µmol of halide per min. Protein concentrations were estimated with Coomassie brilliant blue by using bovine serum albumin as the standard. Enzyme assays were carried out twice, and the differences in specific activities were less than 10%.

Cloning of the *dhaA* gene region from *P. pavonaceae* 170. A genomic library of *P. pavonaceae* 170 was constructed by using cosmid vector pLAFR3 according to a previously described procedure (39). Individual cosmid clones were screened for dehalogenase activity by monitoring halide production upon incubation with 1,2-dibromoethane. Out of 5,000 *E. coli* HB101 clones tested, two dehalogenase-positive clones were found. Recombinant cosmids pPC8 and pPC33 encoding haloalkane dehalogenase were isolated from these two HB101 clones and were digested with *Bam*HI. Both cosmids had a 3-kb *Bam*HI fragment in common. The 3-kb *Bam*HI fragment of pPC33 was ligated into the *Bam*HI site of pBluescript SK(-). The ligation mixture was used to transform cells of *E. coli* JM101, and transformants were plated on LBZ plates containing ampicillin, X-Gal, and IPTG. Ampicillin-resistant white colonies displaying haloalkane dehalogenase activity with 1,2-dibromoethane were selected. Plasmid DNA (pSK45) of one of these colonies was isolated and used as template for DNA sequencing. The nucleotide sequences of DNA regions flanking the 3-kb *Bam*HI fragment were determined by using cosmid pPC33 directly as a template for DNA sequencing.

Nucleotide sequencing and analysis. DNA sequencing was performed as previously described (21, 36). The nucleotide sequence data were analyzed by using the programs supplied in the DNASTAR software package (DNASTAR Inc., Madison, Wis.) or those supplied in the PC/GENE software package (Genofit, Geneva, Switzerland). Searches for nucleotide and amino acid sequence similarities were carried out by using the BLAST program (3) and the DDBJ, EMBL, and GenBank databases. Protein sequences were aligned by using CLUSTAL W (41), and alignments of nucleotide sequences were made by using LALIGN (Institut de Génétique Humaine, Montpellier, France).

Nucleotide sequence accession numbers. The nucleotide sequence data of the haloalkane dehalogenase gene regions from *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 have been submitted to the

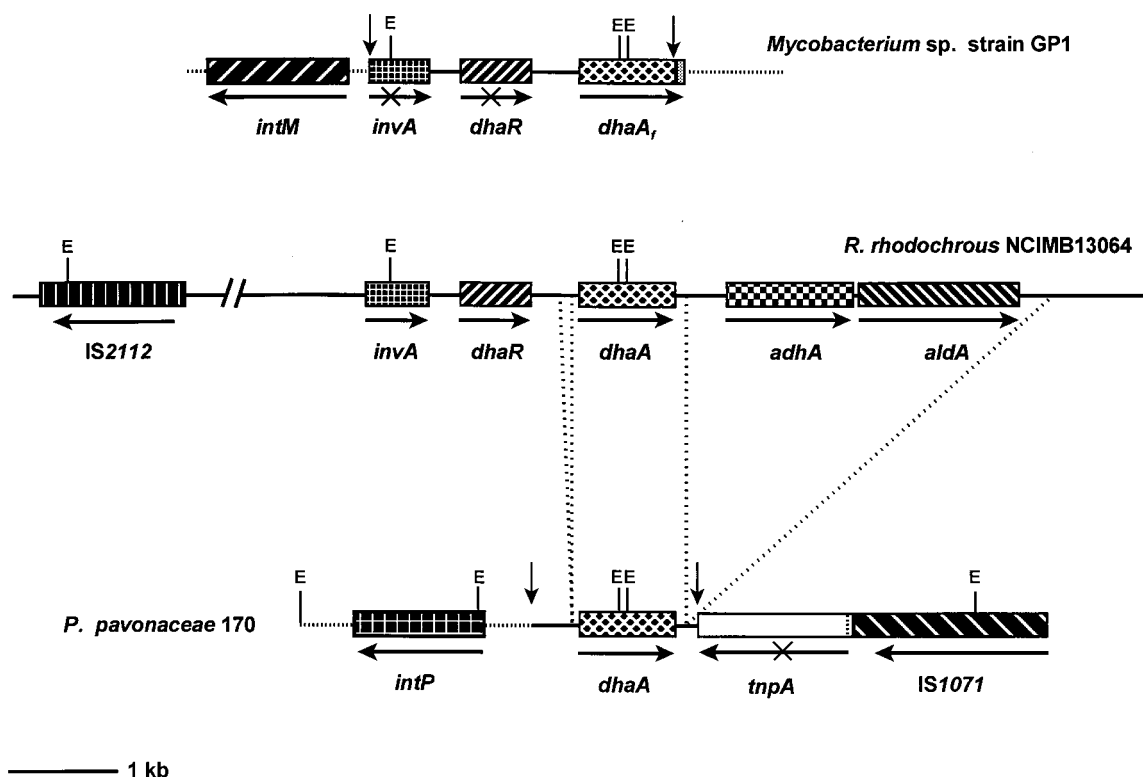


FIG. 2. Organization of the haloalkane dehalogenase gene regions of *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1. Genes are shown as hatched boxes, and arrows indicate the direction of transcription. Identical hatching indicates identical genes. Incomplete ORFs are indicated by a cross through the arrow. The borders of the conserved DNA segments in strains 170 and GP1, which are highly similar to segments of the 1-chlorobutane-catabolic gene cluster of strain NCIMB13064, are indicated by vertical arrows. The two deletions within the conserved region of strain 170 are indicated by vertical dotted lines. The 42-nucleotide extension of the *dhaA* ORF in strain GP1, which is the result of a fusion between the *dhaA* segment and haloalcohol dehalogenase HheB encoding DNA (36), is indicated by a shaded box. *EcoRI* restriction sites (E) are shown.

DDBJ, EMBL, and GenBank databases under accession no. L49435, AJ250371, and AJ250372, respectively.

RESULTS AND DISCUSSION

Sequence analysis of the *dhaA* gene region from *R. rhodochrous* NCIMB13064. The *dhaA* gene of the 1-chlorobutane-degrading bacterium *R. rhodochrous* NCIMB13064 is located on the 100-kb plasmid pRTL1 (23). A 13.1-kb DNA fragment of pRTL1, including a 8.2-kb region upstream and a 4-kb region downstream of *dhaA*, was sequenced (Fig. 2). The *dhaA* gene sequence and the analysis of insertion element IS2112, which is located approximately 6 kb upstream of *dhaA* (Fig. 2), were described previously (21, 23).

Two complete open reading frames (ORFs), designated *invA* and *dhaR*, were found upstream of *dhaA* (Fig. 2 and 3). *InvA* shares extensive similarity with proteins belonging to the invertase family of site-specific recombinases. The inversion reaction is a site-specific recombination between inverted repeat sequences which flank the invertible DNA fragment and is carried out by invertases. Several examples of invertible DNA that can serve as a genetic switch between the expression of alternative sets of genes have been described (14). *InvA* is most similar to the invertases Pin of *E. coli* (34) and Hin of *Salmonella enterica* serovar Typhimurium (51) (Table 1). The high amino acid sequence identity with these DNA invertases implies a common phylogenetic origin, although invertase action has yet to be demonstrated for *InvA*.

Database searches with the deduced amino acid sequence of

the *dhaR* gene revealed no proteins with significant similarity to the entire sequence of the *dhaR* product. However, DhaR contains a region near the N terminus which resembles the helix-turn-helix (HTH) DNA binding motifs of a number of transcriptional regulators (10, 30). Considerable similarity was found with the HTH motifs of the *Streptomyces* autoregulator receptors ArpA, BarA, and FarA (Fig. 4), which were proposed to act as repressor-type regulators for secondary metabolism and morphogenesis (28, 29, 45). The overall sequence similarity to these three *Streptomyces* regulators is low (Table 1).

Cells of strain NCIMB13064 grown in *n*-butanol do not possess DhaA activity, but growth on 1-chlorobutane induces the expression of the dehalogenase DhaA (9). The presence of a region resembling the HTH DNA binding motifs in DhaR and the localization of its encoding ORF directly upstream of *dhaA* suggest that DhaR modulates transcription of *dhaA* by binding directly to its promoter region in response to 1-chlorobutane. Sequence analysis of the *dhaR-dhaA* intergenic region revealed the presence of two identical directly repeated sequences of 13 bp (Fig. 3). The repeated sequences contain a 10-bp palindromic sequence (TGACCGGTCA) and are both part of a larger (imperfect) palindrome. The same 13-bp repeated sequence was also found upstream of *dhaR* (Fig. 3). The presence of these putative binding sites for DhaR upstream of both the *dhaR* and *dhaA* genes suggests that this protein regulates its own expression and that of DhaA. However, promoter sequences for *Rhodococcus* genes are not well

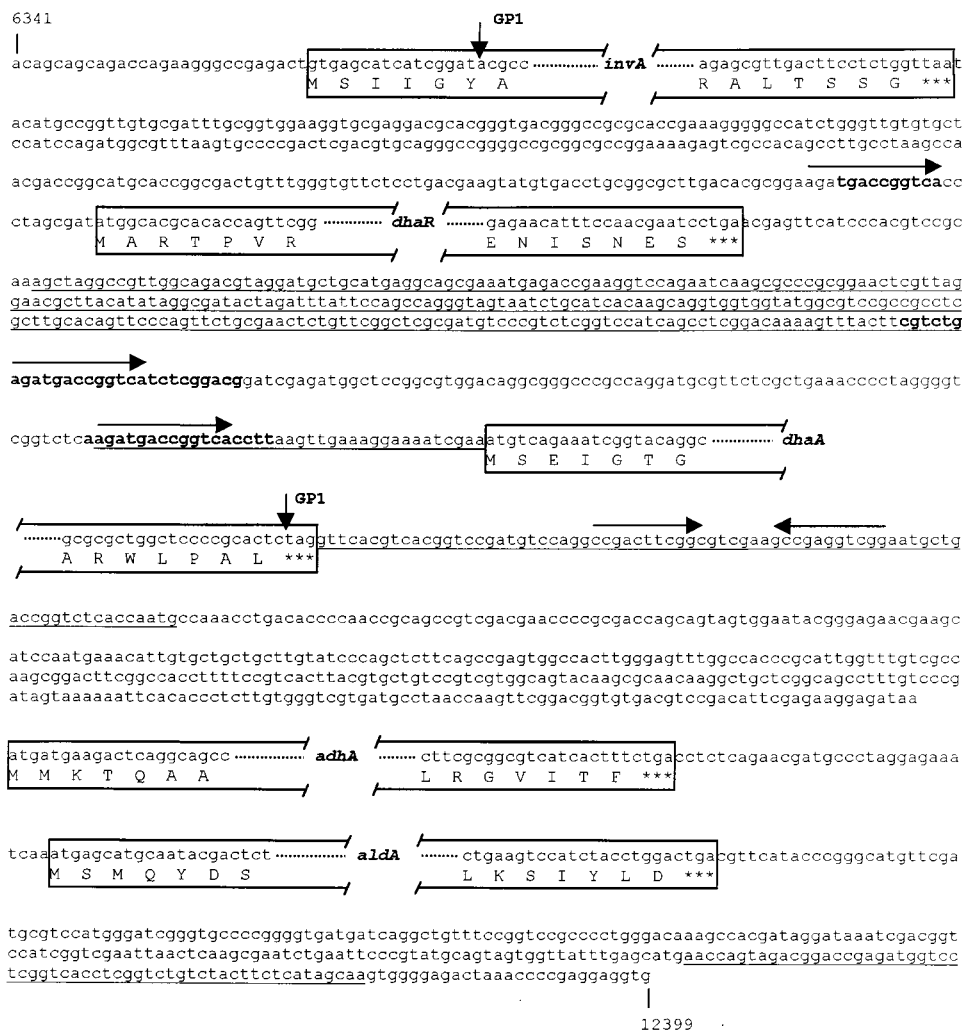


FIG. 3. Partial nucleotide sequence of the 1-chlorobutane-degradative gene cluster found in pRTL1 of *R. rhodochrous* NCIMB13064. The borders of the conserved DNA segment in *Mycobacterium* sp. strain GP1 are indicated by vertical arrows. DNA stretches identical to the nucleotide sequences flanking the *dhaA* gene in *P. pavonaceae* 170 are underlined. Inverted and directed repeats are indicated by horizontal arrows above the sequence. Palindromic sequences are shown in boldface.

characterized (24), and the promoter responsible for *dhaA* expression has not been identified.

Two ORFs, designated *adhA* and *aldA*, were found downstream of *dhaA* (Fig. 2 and 3). *AdhA* shares considerable similarity with several proteins that belong to the alcohol dehydrogenase subgroup which contains the NAD(P)- and zinc-dependent long chain alcohol dehydrogenases (Table 1). The highest similarity was found with putative alcohol dehydrogenases from *Mycobacterium tuberculosis* H37Rv (Table 1). Structural and catalytic residues that are conserved among almost all microbial alcohol dehydrogenases (37) are also present in *AdhA*. *AldA* shares considerable similarity with putative aldehyde dehydrogenases from *M. tuberculosis* H37Rv and with several known NAD(P)-dependent aldehyde dehydrogenases (Table 1). The cysteine residue (C302, family position) that is strictly conserved among all available sequences of the aldehyde dehydrogenase superfamily, and is proposed to be the active site nucleophile (6, 12), is also conserved in *AldA*.

Curragh and coworkers (9) showed that 1-chlorobutane was metabolized by strain NCIMB13064 via *n*-butanol and *n*-butyric acid (Fig. 1). 1-Chlorobutane is converted to *n*-butanol by *DhaA* (23). It therefore appears very likely that *adhA* and

aldA, which are located downstream of *dhaA* (Fig. 2), encode the alcohol and aldehyde dehydrogenases that are involved in the oxidative conversion of *n*-butanol to *n*-butyric acid. The genes for the initial steps in the degradation of 1-chlorobutane thus appear to be located in a cluster on plasmid pRTL1.

The extent of the conserved *dhaA* gene fragments in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1. When the haloalkane dehalogenase gene regions of *P. pavonaceae* 170 and *R. rhodochrous* NCIMB13064 are compared, the conserved DNA fragment in strain 170 can be seen to include a region of about 1.3 kb that harbors little more than the coding region of the *dhaA* gene (Fig. 2). The *dhaA* genes of strains 170 and NCIMB13064 are completely identical. The first 37 nucleotides upstream of the start codon of *dhaA* are also identical in both strains, after which there is a deletion of 98 nucleotides in the *Pseudomonas* sequence when compared to the *Rhodococcus* sequence (Fig. 2 and 3). Upstream of this deletion, the sequences continue to be identical for 268 nucleotides and then abruptly become completely unrelated. The 98-nucleotide deletion in the *Pseudomonas* sequence includes exactly the DNA sequence between the 13-bp directed repeat as well as one of the repeated sequences itself (Fig. 3). The formation of

TABLE 1. Localization of genes, the corresponding gene products, and identities with other proteins

Strain	Gene	Region of nucleotide sequence ^a	Gene product	Deduced mol wt (kDa)	% Identity with other proteins (references)
NCIMB13064	<i>invA</i>	6369–6929	Putative invertase	20.6	Pin, 50 (34); Hin, 48 (51)
	<i>dhaR</i>	7209–7815	Repressor-type regulator	22.7	ArpA, 20 (29); BarA, 20 (28); FarA, 20 (45)
	<i>dhaA</i>	8244–9125	Haloalkane dehalogenase	33.2	See reference 23
	<i>adhA</i>	9543–10655	Putative alcohol dehydrogenase	38.8	AdhD, 50 (8); AdhB, 48 (8); AdhI, 34 (33); AdhS, 33 (32)
	<i>aldA</i>	10687–12135	Putative aldehyde dehydrogenase	50.7	Rv0223C, 47 (8); CymC, 38 (11); DhaL, 38 (46); AldA, 35 (8)
170	<i>intP</i>	587–1726 ^b	Putative integrase	40.4	See Fig. 6A
	<i>dhaA</i>	2641–3522	Haloalkane dehalogenase	33.2	See reference 23
	<i>tnpAΔ</i>	3879–5024 ^b	Disrupted transposase		TnpA, 51 ^c
	<i>tnpA</i> of IS1071	5355–>6900 ^b	Transposase	108.4	See reference 26
GP1	<i>intM</i>	82–1437 ^b	Putative integrase	49.1	See Fig. 6B
	<i>dhaA_f</i>	3482–4405	Haloalkane dehalogenase	34.7	See reference 36

^a The nucleotide sequence numbers refer to the sequences of the haloalkane dehalogenase gene regions of strains NCIMB13064, 170, and GP1 that have been deposited at the DDBJ, EMBL, and GenBank databases under accession no. L49435, AJ250371, and AJ250372, respectively.

^b Located on the complementary DNA strand when compared to the haloalkane dehalogenase gene.

^c Accession no. AF028594.

this deletion may be explained by DNA strand slippage, which allows one repeated sequence to mispair with the complement of the other (2).

The two sequences are identical for only 77 nucleotides downstream of the *dhaA* gene. The following 60 nucleotides in the *Pseudomonas* sequence (nucleotides 3600 to 3659) are identical to a fragment downstream of *aldA* (nucleotides 12313 to 12372) in the *Rhodococcus* sequence (Fig. 3). This suggests that a large deletion of about 3.2 kb, including the alcohol and aldehyde dehydrogenase genes, has occurred in the *Pseudomonas* sequence when compared to the *Rhodococcus* sequence (Fig. 2). No similarity between the two sequences was found further downstream.

When the haloalkane dehalogenase gene regions of *Mycobacterium* sp. strain GP1 and *R. rhodochrous* NCIMB13064 are compared, the conserved DNA fragment in strain GP1 appears to include a region of about 2.7 kb which harbors *dhaA*, *dhaR*, and part of *invA* (Fig. 2). The similarity of the dehalogenase gene region of strain GP1 to that of strain NCIMB13064 starts at nucleotide 17 of the *invA* ORF and ends exactly before the stop codon of the *dhaA* ORF (Fig. 3). This DNA segment in strain GP1 is identical to the corresponding segment in strain NCIMB13064, except for three nucleotide substitutions in *dhaA* and a 12-nucleotide deletion in the *Rhodococcus* regulatory gene *dhaR*. No further similarity was found between the two sequences.

The haloalkane dehalogenase gene regions of the three phylogenetically different bacteria *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 thus

appear to have a common genetic origin. The globally distributed 1-chlorobutane catabolic gene cluster on plasmid pRTL1 (G. J. Poelarends, M. Zandstra, T. Bosma, L. A. Kulakov, M. J. Larkin, J. R. Marchesi, A. J. Weightman, and D. B. Janssen, submitted for publication) seems to be ancestral to the DNA fragments harboring the *dhaA* gene in strains 170 and GP1. These *dhaA* gene fragments probably originated from a catabolic gene cluster similar to the one present on pRTL1 in strain NCIMB13064 and were horizontally transferred to the present hosts. Since the structural genes are identical, we conclude that horizontal transfer of the *dhaA* gene fragment to strain 170 has occurred naturally and recently, resulting in the formation of a newly evolved pathway for 1,3-dichloropropene degradation (Fig. 1). The hydrolytic product of 1,3-dichloropropene, 3-chloroallyl alcohol, can be used as a carbon source by several gram-negative bacteria (5, 43). We also propose that the 1,2-dibromoethane-degrading pathway of strain GP1 arose by horizontal transfer of a DNA fragment harboring *dhaA* from a donor strain present in the original mixed enrichment culture to a 2-bromoethanol-degrading host. The host that obtained a complete degradation pathway for 1,2-dibromoethane in this way gained a selective advantage and became the predominant organism in the mixed culture.

The role of the *dhaR* gene in the regulation of *dhaA* expression. The sequence analysis indicated that expression of the *dhaA* gene in *R. rhodochrous* NCIMB13064 may be regulated by the *dhaR* gene product. If *dhaR* encodes a regulatory protein, its inactivation should lead to either constitutive expression or noninducibility of the *dhaA* gene, depending on the

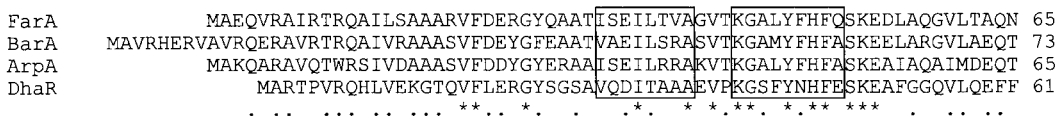


FIG. 4. Alignment of the N-terminal amino acid sequences of FarA, BarA, ArpA, and DhaR. Amino acids conserved in all four proteins are indicated by asterisks, whereas amino acids conserved in three out of four proteins are indicated by dots. The sequences corresponding to helix 2 and helix 3 in the HTH DNA binding motif are boxed.

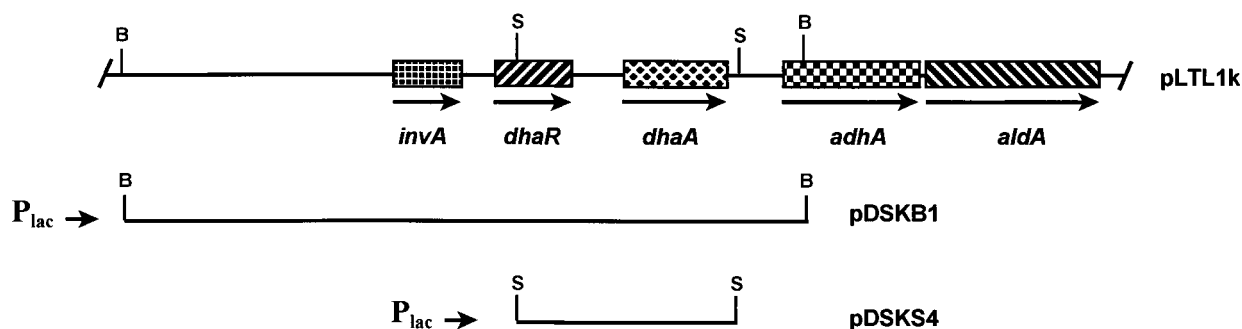


FIG. 5. Schematic overview of the 1-chlorobutane-degradative gene cluster in recombinant cosmid pLTL1k and of the two subclones used for *dhaA* expression studies in *Pseudomonas* sp. strain GJ1. Genes are indicated by hatched boxes, and arrows indicate the direction of transcription. Cosmid pLTL1k was cut at the indicated restriction sites, and the corresponding *dhaA* gene fragments were isolated and inserted into the *Bam*HI or *Sal*I site of pDSK519, yielding plasmids pDSKB1 and pDSKS4, respectively. The direction of the pDSK519-localized *lac* promoter is indicated. *Bam*HI and *Sal*I restriction sites are indicated by B and S, respectively.

negative or positive nature of the regulation, respectively. *E. coli* HB101 harboring recombinant cosmid pLTL1k, which carried intact *dhaR* and *dhaA* genes (Fig. 5), constitutively expressed *dhaA* (Table 2), suggesting that expression of *dhaA* is not regulated in *E. coli*. Introduction of pLTL1k into *Pseudomonas* sp. strain GJ1 did not lead to constitutive expression of *dhaA*. We therefore used strain GJ1 to analyze the role of the *dhaR* gene in the regulation of *dhaA* expression.

Plasmids that carried either *dhaA* and an intact *dhaR* gene (pDSKB1) or *dhaA* and the *dhaR* gene with a deletion in its proximal part (pDSKS4) were constructed (Fig. 5). In contrast to cell extracts prepared from strains GJ1(pLTL1k) and GJ1(pDSKB1), cell extract from strain GJ1(pDSKS4) displayed haloalkane dehalogenase activity (Table 2). Cell extract from strain GJ1 carrying pDSKS5, in which the *Sal*I insert that is present in pDSKS4 was placed in the direction opposite of that of the *lac* promoter of pDSK519, also displayed dehalogenase activity (Table 2), indicating that the constitutive expression of *dhaA* was controlled by its own promoter and not by the *lac* promoter of pDSK519. These results show that inactivation of *dhaR* leads to constitutive expression of the *dhaA* gene, indicating that the *dhaR* gene product putatively acts as a repressor of *dhaA* expression.

In contrast to the negatively regulated expression of *dhaA* in *R. rhodochrous* NCIMB13064, the haloalkane dehalogenase genes in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1 are constitutively expressed (35, 36). The constitutive expression of *dhaA* in strain 170 may be caused by the absence of a *dhaR* gene. Although strain GP1 possesses the *dhaR* gene in front of *dhaA_f*, the 12-nucleotide deletion present in *dhaR* may inactivate the regulatory protein (DhaR), leading to constitutive expression. To confirm that this deletion in *dhaR* inacti-

vated its gene product, recombinant cosmid pGP1-4B5, which carried the *dhaA_f* gene region of strain GP1 (Fig. 2), was introduced into *Pseudomonas* sp. strain GJ1. In contrast to cell extract prepared from strain GJ1 harboring cosmid pLTL1k, which carried the *dhaA* gene region of strain NCIMB13064 (Fig. 5), cell extract of strain GJ1(pGP1-4B5) displayed haloalkane dehalogenase activity (Table 2), indicating that the deletion in *dhaR* had inactivated its gene product. This further emphasizes the negative regulatory role of DhaR.

In strains 170 and GP1, a putative DNA integrase gene is present next to the conserved *dhaA* gene fragment. The sequence comparisons indicated that novel catabolic pathways for 1,3-dichloropropene and 1,2-dibromoethane were built by adding existing DNA fragments harboring *dhaA* to the genomes of *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1, respectively. This suggests the involvement of a mechanism for the acquisition of distinct DNA fragments. Gene acquisition by bacterial genomes could occur either by excisive-integrative recombination events mediated by insertion elements or by site-specific integration events mediated by DNA integrases (40, 42, 47).

Interestingly, an ORF encoding a putative DNA integrase (*intP*) was found upstream of the conserved *dhaA* gene fragment in strain 170 (Fig. 2 and Table 1). IntP shares significant similarity with proteins belonging to the integrase (Int) family of site-specific recombinases (Fig. 6A). Although the members of the Int family exhibit a large diversity in their sequences, they all harbor two regions of marked sequence similarity, called box I and box II (27). IntP shows 55% identity to its nearest neighbor, the integrase-like protein of *Selenomonas ruminantium*, in these two conserved domains (Fig. 6A). The tetrad R-H-R-Y, which includes the active-site tyrosine (31), is conserved in almost all members of the Int family (1, 4, 27) and is present as Arg-183, His-327, Arg-330, and Tyr-362 in IntP.

An ORF encoding a putative DNA integrase (*intM*) was also found upstream of the conserved *dhaA* gene fragment in strain GP1 (Fig. 2 and Table 1). The C-terminal region of IntM shares considerable similarity with proteins of the Int family (Fig. 6B). The conserved tetrad R-H-R-Y of the Int family (1, 4, 27) is present as Arg-260, His-352, Arg-355, and Tyr-415 in IntM (Fig. 6B).

The presence of an integrase gene in the vicinity of the conserved *dhaA* gene fragment both in strain 170 and in strain GP1 suggests acquisition of these DNA fragments by site-specific integration. Assuming that no additional recombinations have taken place after the primary insertion event, comparisons of the boundaries between the conserved *dhaA* gene

TABLE 2. Dehalogenase activities in crude extracts of *E. coli* HB101 and *Pseudomonas* sp. strain GJ1 harboring different constructs

Strain	Dehalogenase sp act (mU/mg of protein) ^a
HB101 (pLTL1k)	120
GJ1 (pLTL1k)	<20
GJ1 (pDSKB1)	<20
GJ1 (pDSKS4)	1,400
GJ1 (pDSKS5)	1,380
GJ1 (pGP1-4B5)	1,330

^a Specific activities with 1,2-dibromoethane (5 mM) were determined with extracts prepared from cells grown on Luria broth.

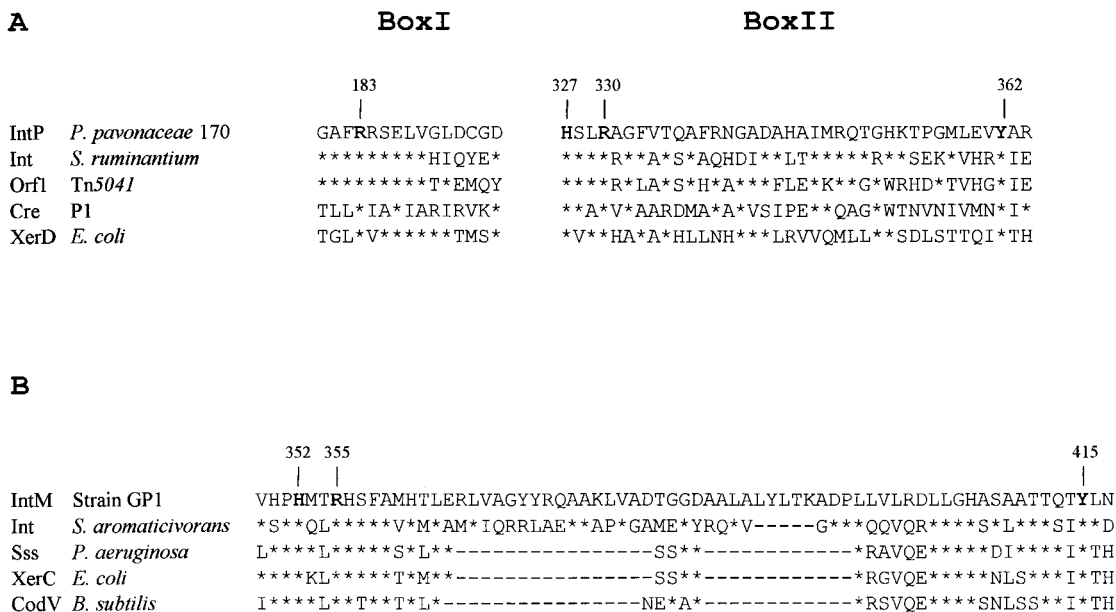


FIG. 6. (A) Amino acid sequence alignment of IntP with the conserved segments of the recombinases Cre of bacteriophage P1 (accession no. P06956) and XerD of *E. coli* (M54884) and the integrase-like proteins of *S. ruminantium* (AB011029) and Tn5041 (X98999). Asterisks represent bases identical to those in the upper sequence. Invariant amino acid residues which are believed to be involved in catalysis are numbered (IntP numbering) and shown in boldface. (B) Amino acid sequence alignment of IntM with the C termini of the recombinases Sss of *P. aeruginosa* (S61402), XerC of *E. coli* (P22885), and CodV of *Bacillus subtilis* (P39776) and the putative phage type integrase of *Sphingomonas aromaticovorans* (AF079317). Asterisks represent bases identical to those in the upper sequence. Dashes represent bases absent in other sequences. The conserved residues His-352, Arg-355, and Tyr-415 are numbered (IntM numbering) and shown in boldface.

fragments and the sequences unique for strain 170 or GP1 define the insertion site. No short duplication of bases on either side of the presumed point of insertion or regions of dyad symmetry that could serve as integrase binding sites were found when the flanking sequences were compared. The absence of these features could be due to an adjacent deletion removing the border segment on one side. Such a deletion could also explain the fusion of the *dhaA* gene to the 3' end of the haloalcohol dehalogenase gene *hheB* (Fig. 2).

The presence of insertion elements in the vicinity of the conserved *dhaA* gene fragments. Many catabolic genes are associated with transposons (42, 47), and transposition is clearly a major mechanism for the acquisition of catabolic genes by bacterial genomes. Sequence analysis of the regions (>4 kb) adjacent to the conserved *dhaA* gene fragment in *Mycobacterium* sp. strain GP1 showed that these regions do not encode any proteins related to known transposases. Distal to the *dhaA* gene fragment in *P. pavonaceae* 170, however, a large ORF (*tnpA*) was found (Fig. 2) of which the deduced amino acid sequence showed extensive similarity with the putative transposase of *Pseudomonas pseudoalcaligenes* JS45 (Table 1). The *tnpA* ORF, however, appeared to be interrupted by an insertion element, identified as *IS1071* (Fig. 2), causing a truncation of its product. Nucleotide sequencing of a 1.7-kb fragment of *IS1071* revealed no differences with the known sequence of *IS1071*, which is involved in transposition of the chlorobenzoate genes (26). PCR amplification, using a primer specific for both ends of *IS1071* (due to the inverted repeats at its termini) and recombinant cosmid pPC33 as template DNA, showed the formation of a 3.2-kb PCR product corresponding to the size of *IS1071* (26). Thus, a complete copy of *IS1071* was present as an insertion in the *tnpA* gene approximately 1.6 kb downstream of *dhaA*.

Southern hybridization analysis revealed five copies of *IS1071* in strain 170 but only two copies in strain 170M4, a

spontaneous mutant of strain 170 that has lost the *dhaA* gene (35) (results not shown). The 60-kb plasmid pPC170, that was previously identified in strain 170 (44), is still present in strain 170M4, showing that the *dhaA* gene is not plasmid localized. Assuming that the mutation in strain 170M4 was caused by a single deletion event, this could suggest that the *dhaA* gene region in strain 170 is flanked by several copies of *IS1071*, forming a composite class I element, and that a homologous recombination event between two insertion sequences was responsible for the loss of the *dhaA* gene and three copies of *IS1071*. Strain 170M4 also lacked the integrase gene *intP*, which is consistent with such a deletion event.

Many antibiotic resistance genes found on transposons in gram-negative bacteria are located within a conserved DNA sequence (15, 25, 40). These conserved elements, called integrons (16, 40), are formally distinct from other genetic elements in that they determine site-specific integration functions, a DNA integrase and a recombination site, and are thus able to acquire resistance genes at the specific site without the need for the presence of insertion sequences or integrase genes in the DNA segments that are acquired. If the integrase gene *intP* in *P. pavonaceae* 170 is flanked by *IS1071* sequences and confers on this transposon a capability of taking up individual and unrelated catabolic genes by integrase-mediated recombination, strain 170 may possess a mobile DNA element similar to the previously identified integrons which are capable of taking up antibiotic resistance genes (16, 40).

No sequences similar to *IS1071* were found in strains NCIMB13064 and GP1, indicating that this insertion element was not involved in distribution of the *dhaA* gene among these strains. Similarly, insertion sequence *IS2112*, which is located approximately 6 kb upstream of the *dhaA* gene in strain NCIMB13064 (Fig. 2) (21), is not present in strains 170 and GP1. These results are consistent with the hypothesis that site-specific integration events mediated by IntP and IntM

were responsible for the acquisition of the *dhaA* gene fragments, rather than excise-integrative recombination events mediated by insertion elements. However, the presence of multiple copies of an active mobile element (*IS1071*) around the *dhaA* gene in strain 170, combined with the fact that strain 170 was recently isolated from a 1,3-dichloropropene-contaminated environment in which natural genetic exchange is likely to be important (44), suggests that transposition has played a part in the mobilization of the *dhaA* gene among members of the microbial community present in this environment.

Concluding remarks. Our data provide further support for previous studies suggesting that horizontal transfer of genes involved in pollutant biodegradation may play an important role in the evolution of catabolic pathways and the adaptation of microbial communities to different environmental contaminants. Up to now, catabolic genes were thought to be transferred mainly by means of conjugative plasmids and transposons (42, 47). The results presented here suggest that genes specifying adaptation to xenobiotics can also spread as integrons, as has been proposed in the case of genes specifying antibiotic resistance (16, 40). The widespread use of antibiotics and the introduction of xenobiotics into the environment seem to lead to adaptation by similar molecular mechanisms.

ACKNOWLEDGMENTS

This study was supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO), and by the EC Environment and Climate Research Program contract ENV4-CT95-0086.

We thank P. Terpstra (BioMedical Technology Centre, University of Groningen, Groningen, The Netherlands) for his assistance in DNA sequencing and analysis.

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