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Organization of *lin* Genes and IS*6100* among Different Strains of Hexachlorocyclohexane-Degrading *Sphingomonas paucimobilis*: Evidence for Horizontal Gene Transfer

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The organization of *lin* genes and IS*6100* was studied in three strains of *Sphingomonas paucimobilis* (B90A, Sp+, and UT26) which degraded hexachlorocyclohexane (HCH) isomers but which had been isolated at different geographical locations. DNA-DNA hybridization data revealed that most of the *lin* genes in these strains were associated with IS*6100*, an insertion sequence classified in the IS*6* family and initially found in *Mycobacterium fortuitum*. Eleven, six, and five copies of IS*6100* were detected in B90A, Sp+, and UT26, respectively. IS*6100* elements in B90A were sequenced from five, one, and one regions of the genomes of B90A, Sp+, and UT26, respectively, and were found to be identical. DNA-DNA hybridization and DNA sequencing of cosmid clones also revealed that *S. paucimobilis* B90A contains three and two copies of *linX* and *linA*, respectively, compared to only one copy of these genes in strains Sp+ and UT26. Although the copy number and the sequence of the remaining genes of the HCH degradative pathway (*linB*, *linC*, *linD*, and *linE*) were nearly the same in all strains, there were striking differences in the organization of the *linA* genes as a result of replacement of portions of DNA sequences by IS*6100*, which gave them a strange mosaic configuration. Spontaneous deletion of *linD* and *linE* from B90A and of *linA* from Sp+ occurred and was associated either with deletion of a copy of IS*6100* or changes in IS*6100* profiles. The evidence gathered in this study, coupled with the observation that the G+C contents of the *linA* genes are lower than that of the remaining DNA sequence of *S. paucimobilis*, strongly suggests that all these strains acquired the *linA* gene through horizontal gene transfer mediated by IS*6100*. The association of IS*6100* with the rest of the *lin* genes further suggests that IS*6100* played a role in shaping the current *lin* gene organization.

Hexachlorocyclohexane (HCH) was introduced for the control of agricultural pests and of vector-borne diseases in early 1940s. While this compound was used extensively all over the world, several reports on the persistence of HCH isomers (α, β, γ, and δ) and their toxic effects on nontarget organisms appeared in the 1980s (11). These reports finally resulted in a ban on or restricted use of HCH in most countries. Neither the ban nor the restricted use has, however, reduced the levels of HCH residues in the environment (6, 25, 33), especially in soils that had a previous history of HCH application (2). One serious problem is the uptake of HCH residues from soil by crops, which then enter food products (1, 31). In addition to no further use of HCH, a decontamination program for HCH-polluted soils would diminish the risk posed by HCH residues to human, plant, and animal health. One possibility for decontamination is spontaneous or induced microbial degradation. Unfortunately, spontaneous microbial degradation of HCH isomers proceeds rather slowly (10, 15), although a number of bacteria which can degrade one or more isomers of HCH have been isolated. Thus, addition of naturally occurring microbes to contaminated soils could provide an alternative strategy. Such application could be assisted to a great extent by exploring the potential of such isolates, particularly in order to understand the physiology and genetics of HCH degradation in these strains.

As far as is known, aerobic degradation of HCH is carried out mostly by strains of *Sphingomonas paucimobilis* and *Rhodanobacter lindaniclasticus.* HCH-degrading strains have been isolated in different parts of the world; *S. paucimobilis* SS86 has been isolated in Japan (32, 38), *S. paucimobilis* B90A has been isolated in India (4, 29), and an *R. lindaniclasticus* strain has been isolated in France (24, 37). These three strains are remarkably similar and can all degrade α-, γ-, and δ-HCH (9, 14, 29, 37). In addition, *S. paucimobilis* B90A can also partially degrade β-HCH (10, 14, 29). The metabolic pathway and the genes involved in HCH degradation have been studied in great detail in *S. paucimobilis* strain UT26 (a mutant of SS86 resistant to nalidixic acid) and to a lesser extent in B90 (Fig. 1). The primary enzyme in γ-HCH degradation is HCH dehydrochlorinase encoded by the *linA* gene (9, 23). The remaining genes of the γ-HCH degradative pathway are *linB* (23), *linC* (20), *linD* (18), and *linE* (17, 19), which encode a halidohydrolase, a

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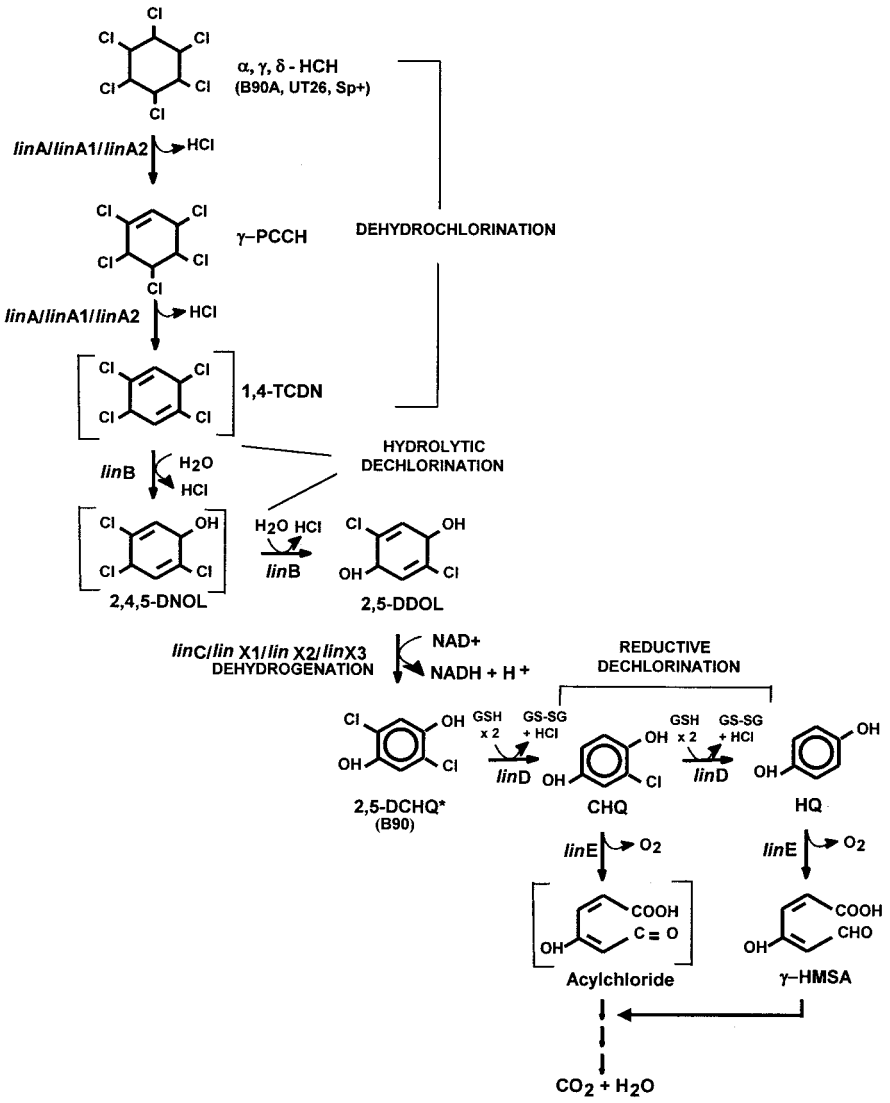

FIG. 1. Pathway for degradation of HCH isomers in *S. paucimobilis* strains (data adapted from references 14 and 20). γ-PCCH, gamma-pentachlorocyclohexene; 1,4-TCDN, 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 1,2,4-TCB, 1,2,4 trichlorobenzene; 2,4,5-DNOL, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; DCP, 2,5-dichlorophenol; 2,5-DDOL, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 2,5-DCHQ, 2,5-dichlorohydroquinone; 2-CHQ, 2-chlorohydroquinone; HQ, hydroquinone; γ-HMSA, gamma-hydroxymuconic semialdehyde. The asterisk indicates that in B90 the degradation of α-, γ-, and δ-HCH isomers stops at the level of 2,5-dichlorohydroquinone.

dehydrogenase, a reductive dechlorinase, and a dioxygenase, respectively. In addition, the *linX* gene, encoding a protein that has activity similar to that of LinC, was also cloned and characterized (20). A *linA* gene was also cloned and sequenced from *R. lindaniclasticus* (37) and was found to be identical to *linA* from strain UT26 (9) except for one 3-bp insertion, but the remaining genes of the HCH degradative pathway in this strain have still not been described (37). More recently, the *lin* genes of *S. paucimobilis* strain B90 were cloned and characterized (14, 21). In contrast to UT26 and *R. lindaniclasticus*, strain B90 contains two copies of *linA*, designated *linA1* and *linA2* (Fig. 1). Both copies of *linA* produce a functional HCH dehydrochlorinase when they are cloned in *Escherichia coli*. The amino acid sequences of the products encoded by the *linA1* and *linA2* genes are 92% identical to each other and 88% (LinA1) and 99% (LinA2) identical to the sequence of LinA of strain UT26 (9). The *linB*, *linC*, and *linX* genes were also cloned from strain B90 and were found to be 99% identical to the corresponding genes of *S. paucimobilis* UT26 (14). A closer look at the two copies of *linA* in strain B90 suggested that one of them contains an insertion of an IS element at the 3′ end. This results in a 22-bp difference at the 3′ end between *linA1* (462 bp) and *linA2* (468 bp). The inserted region exhibits complete sequence identity to IS*6100* of *Mycobacterium fortuitum* (16). In contrast to strain UT26, no *linD*, *linE*, and *linR* genes could be detected by PCR amplification and Southern hybridization in strain B90 (14).

Here we report on the mosaic character of the *lin* genes in the different *S. paucimobilis* strains and the close association of the IS*6100* element with these genes. By using Southern hy-

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source
E. coli DH5α	F⁻*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYA-argF)U169 deoR*	Lab stock
E. coli JM101	*supE thi*Δ(*lac-proAB*) F′[*traD36 proAB*⁺ *lacI*ᵠ *lacZ*ΔM15]	Lab stock
S. paucimobilis B90A	Aerobic, nonmotile, rod shaped, degrades all four isomers of HCH (α, β, γ, and δ)	N. Sethunathan, CRRI, Cuttack, India
S. paucimobilis B90	Mutant of *S. paucimobilis* B90A lacking *linD*, *linE*, and *linR*	Lab stock
S. paucimobilis UT26	Aerobic, nonmotile, rod shaped, degrades α-, γ-, and δ-HCH but not β-HCH	Y. Nagata, University of Tokyo, Tokyo, Japan
S. paucimobilis Sp+	Aerobic, nonmotile, rod shaped, degrades α-, γ-, and δ-HCH but not β-HCH	Tim Vogel, University of Lyon, Lyon, France
S. paucimobilis ATCC 29837^T	Aerobic, nonmotile, rod shaped, yellow pigmented	M. Hanspal, St. Elizabeth's Medical Center of Boston, Boston, Mass.
S. chlorophenolica DSM 7098^T	Aerobic, nonsporulating rods, yellow pigmented, degrades pentachlorophenol, and 2,4,6-trichlorophenol	J. Cullum, University of Kaiserslautern, Kaiserslautern, Germany
pUC 13/19	2.7-kb; Amp^r; multiple cloning site internal to *lacZ* gene	Lab stock
pWE15	8.2-kb cosmid vector; Amp^r; bacteriophage promoter sequences T3 and T7 flanking unique cloning site	Stratagene
pLINA57	pWE15 carrying 41-kb DNA fragment of B90A containing *linA1*, *linC*, *linX*, and IS*6100*	This study
pLIND33	pWE15 carrying 5.6-kb DNA fragment of B90A containing *linD*, *linE*, *linR*, and IS*6100*	This study
pLIND22	Subclone of pLIND33 in pWE15 (bp 1869 to 6700 of the contiguous ~8-kb sequence of pLIND33)	This study
pLIND22-12	Subclone of pLIND22 in pUC18 (bp 6701 to 7803 of the contiguous ~8-kb sequence of pLIND33)	This study
pLIND3R24	Subclone of pLIND33 in pUC18 (bp 1 to 1868 of the contiguous ~8-kb sequence of pLIND33)	This study
pLINB35	pWE15 carrying DNA fragment of B90A containing *linB* and IS*6100*	This study
pLINB23	Subclone of pLINB35 in pUC18 containing 2.2-kb fragment of pLINB35	This study
pLINUTIS	pUC 18 containing BamHI-digested 3.5-kb fragment of *S. paucimobilis* UT26 that hybridized with *IS6100*	This study
pLINSpIS	pUC18 containing HindIII-digested 2.2-kb Sp+ fragment that hybridized with IS*6100*	This study

bridization, cosmid cloning, and DNA sequencing of strain B90 and an older lab stock of strain B90 (designated B90A) and by comparing these strains with strains UT26 and Sp+, we discovered that multiple copies of IS*6100* are present in all of the strains, but they are in different configurations. The consequences of the activity of IS*6100* for the stability and distribution of the *lin* genes among these strains are discussed below.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Table 1 shows the bacterial strains and plasmids used in this study. *S. paucimobilis* B90A was obtained from N. Sethunathan (Central Rice Research Institute, Cuttuck, India) in 1992. *S. paucimobilis* B90, which lacks *linD*, *linE*, and *linR*, is a spontaneous mutant derived from *S. paucimobilis* B90A (14). *S. paucimobilis* UT26 and Sp+ were obtained from Y. Nagata (University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, Japan) and Tim Vogel (University of Lyon, Lyon, France), respectively. The non-HCH-degrading strains *S. paucimobilis* ATCC 29837^T and *Sphingomonas chlorophenolica* DSM 7098^T were also used for detecting any IS*6100* elements. All of the *S. paucimobilis* strains and *S. chlorophenolica* were grown in Luria broth (30) or in mineral salts (SM) medium containing glucose (1%, wt/vol) as described previously (14). *E. coli* strains were grown in Luria broth at 37°C. Antibiotics, when required, were added to a final concentration of 150 μg/ml (ampicillin) or 50 μg/ml (kanamycin).

DNA isolation and hybridization. Genomic DNA was isolated from *S. paucimobilis* strains and *S. chlorophenolica* by using previously described procedures (14). Plasmids were isolated from *E. coli* by using standard protocols (30). For DNA hybridization, genomic DNA of *S. paucimobilis* was digested with suitable restriction enzymes and subjected to gel electrophoresis. Separated DNA fragments were transferred from agarose gels to Hybond-N nylon membranes (Pharmacia Amersham Biotech) and probed with [α-^{32}P]dATP (BRIT, Hyderabad, India)-labeled DNA fragments. DNA probes were prepared from plasmid DNA either by restriction enzyme digestion followed by elution of the appropriate DNA fragments from an agarose gel or by PCR amplification. The primers used for amplification of different *lin* gene fragments, IS*6100*, and *tnpA* were based on DNA sequences of the corresponding genes in the GenBank database and can be supplied on request. PCR amplification was performed with a Neugen gene thermocycler (Techne Progeny, Cambridge, United Kingdom) or a Robocycler (Stratagene) by using standard protocols. Hybridization was performed at 68°C. After hybridization, the membranes were washed twice in a solution containing 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) plus 0.1% sodium dodecyl sulfate at 68°C and once in a solution containing 1× SSC plus 0.1% sodium dodecyl sulfate at room temperature. The membranes were then exposed to X-ray film (Kodak India, Mumbai, India).

Construction and screening of the genomic library of *S. paucimobilis* B90A. A genomic library of *S. paucimobilis* B90A was constructed as described previously for strain B90 (14) in cosmid vector pWE15 (Stratagene). This library was screened specifically for the presence of clones in which *lin* genes were associated with IS*6100*. Screening of the library was carried out by using [α-^{32}P]dATP-labeled *linA*, *linB*, *linC*, *linD*, *linE*, *linX*, and IS*6100* probes separately. The DNA probes for *lin* genes were prepared by PCR amplification by using primers designed for amplification from the open reading frames (ORFs) of *lin* genes. The IS*6100* probe was prepared by amplification of the internal fragment of IS*6100*. The cosmid library (around 2,000 colonies) of B90A was screened by using each probe under stringent conditions (68°C), followed by stringent washing as described above. The tentative clones selected after screening were further confirmed by Southern hybridization. After screening, 15 cosmid clones that gave positive signals with one or more *lin* genes and/or IS*6100* were selected (Table 2).

Determination of copy numbers of *lin* genes and IS*6100* in the *S. paucimobilis* strains. In order to determine the approximate copy numbers of IS*6100* and the *lin* genes, the genomic DNAs of *S. paucimobilis* B90A, B90, UT26, and Sp+ were

TABLE 2. Pattern of hybridization signals in *S. paucimobilis* B90A cosmid clones with *lin* genes and IS*6100* as probes

Cosmid	Hybridization signals with the following DNA probes:						
	linA	*linB*	*linC*	*linD*	*linE*	*linX*	IS*6100*
33	−[a]	−	−	+	+	−	+
35	−	+	−	−	−	−	+
36	+	−	+	−	−	+	+
39	+	−	−	−	−	+	−
42	−	−	−	+	+	−	+
43	+	−	−	−	−	+	−
45	−	+	−	+	−	−	+
49	−	+	−	−	−	−	+
50	+	−	−	−	−	+	+
52	−	+	−	−	−	−	+
53	+	−	−	−	−	+	−
55	−	−	+	−	−	−	+
56	−	+	−	−	−	−	+
57	+	−	+	−	−	+	+
59	−	−	+	−	−	−	+

[a] −, no hybridization signal; +, hybridization signal.

digested with restriction enzymes which do not cut within the *lin* genes or IS*6100* and were hybridized by using *lin* gene and IS*6100* fragments as the probes. The maximum numbers of chromosomal hybridizing bands obtained from experiments with different restriction enzymes were considered the copy numbers of the IS*6100* and *lin* genes. In order to detect any IS*6100* elements in *S. paucimobilis* ATCC 29837^T and *S. chorophenolica* DSM7098^T (which do not degrade HCH), BamHI-digested DNAs of these strains were hybridized with the [α-^{32}P]dATP-labeled IS*6100* probe. To determine the copy number of IS*6100* on pLIND33, the plasmid DNA was digested with PstI/EcoRI and hybridized with the internal fragment of IS*6100* as the probe. Determination of the copy number from the insert cloned in pLIND33 was necessary to predict the type of recombination leading to spontaneous deletion of a DNA fragment containing *linD*, *linE*, and *linR* from B90A.

DNA sequencing of *lin* genes and their flanking regions. DNA sequences were determined by using standard methodologies. Sequences of smaller stretches of DNA fragments cloned into pUC18/19 were determined with an automated DNA sequencer (ABI PRISM model 377, version 3; Applied Biosystems) at the Department of Biochemistry, South Campus, University of Delhi, Delhi, India. DNA sequences of *lin* genes from strain Sp+ were determined from amplified PCR products directly by using the primers that were used for PCR amplification. A nearly 8-kb insert from cosmid pLIND33 that gave positive hybridization signals with *linD*, *linE*, *linR*, and IS*6100* as probes (Table 2) was sequenced, both by making subclones and by primer walking by Microsynth GmbH (Balgach, Switzerland). In a similar manner a 2.2-kb insert that hybridized with *linB* from HindIII-digested pLINB35 was subcloned in pUC18 (Table 1). The construct pLINB23 was then sequenced by primer walking. The complete sequence of a ~41-kb insert from cosmid pLINA57 that hybridized with *linX*, *linA*, *linC*, and IS*6100* was also determined by primer walking by Microsynth GmbH. One copy of IS*6100* from UT26 and one copy of IS*6100* from Sp+ from the cloned DNA fragments in pUC18 (pLINUTIS and pLINSpIS, respectively [Table 1]) were sequenced by using M13 primers. The sequences were analyzed by using the DNASIS package (Pharmacia). In order to determine ORFs on the ~41- and ~8-kb inserts of cosmids pLINA57 and pLIND33, respectively, the ORF Finder program at the National Center for Biotechnology Information (followed by manual inspection of all potential coding regions and start and stop codons) was used. Potential ribosome sites in front of the start codons of each ORF were also identified manually. The BLAST program (3) was used for homology searches in the GenBank database, and ClustalW (8) was used for multiple alignment of sequences.

Generation of *lin* mutants and stability of *lin* genes. In order to generate mutants that do not degrade HCH and to study the stability of *lin* genes, cultures of B90A and Sp+ were grown at 28°C to the stationary phase on SM containing 1% glucose (14). A fresh culture was then raised from a 1:100-diluted inoculum from a culture grown overnight (10^8 cells/ml). Serial dilutions of each stationary-phase culture were plated on SM agar containing glucose (1%, wt/vol), and the number of single colonies was counted after 2 to 3 days. About 200 colonies of each strain were subsequently transferred to liquid SM medium containing 1% glucose and γ-HCH (or γ- and β-HCH in the case of B90A) at a concentration of 5 μg/ml. After the stationary phase was reached (3 to 4 days), γ- or β-HCH was extracted from the samples and analyzed with a gas chromatograph (GC 17A; Shimadzu, Kyoto, Japan). The extraction protocol and conditions used for the gas chromatography analysis have been described previously (14). After analysis for degradation of HCH, cultures that were not able to degrade HCH (mutants) were selected; genomic DNAs were isolated from these mutants and hybridized with [α-^{32}P]dATP-labeled *linA* or IS*6100* gene fragments as probes. In order to attribute the loss of *linA* of Sp+ to homologous recombination involving IS*6100* sequences, genomic DNAs of Sp+ and four mutants were digested with HindIII (a HindIII site is present in IS*6100* and not present in *linA* of Sp+) and BamHI (BamHI does not cut either IS*6100* or *linA*). The DNAs were then hybridized by using [α-^{32}P]dATP-labeled *linA* and IS*6100* probes separately. B90, a spontaneous mutant of B90A, which lacked *linD*, *linE*, and *linR*, was also tested to determine its ability to convert the two possible intermediates, chlorohydroquinone and hydroquinone, in γ-HCH degradation (Fig. 1) by using the method described by Nagata et al. (22).

Nucleotide sequence accession numbers. The nucleotide sequences of the cloned fragments containing the *lin* genes and their flanking regions in B90A have been deposited in the National Center for Biotechnology Information database under accession numbers AY331258, AY331259, and AY334273.

RESULTS

Hybridization of a genomic library of strain B90A with IS*6100* and *lin* gene probes. A genomic library was prepared from *S. paucimobilis* B90A DNA, an older laboratory stock culture of strain B90, which was described previously (14). This library was screened by hybridization with radioactively labeled probes for all *lin* genes and for IS*6100*. From around 2,000 cosmid clones, we selected 15 cosmids which positively hybridized to at least one of the probes (Table 2). Several patterns of cohybridization with different *lin* markers were observed. Cosmids that hybridized to *linC* invariably also hybridized with the IS*6100* probe. Two of these cosmids (cosmids 36 and 57) hybridized with *linA* and *linX* as well. One cosmid (cosmid 50) hybridized to *linA*, *linX*, and IS*6100* but not to *linC*. This suggested that this cosmid overlapped the two other cosmids described above. Interestingly, three cosmids that hybridized to *linA* and *linX* did not hybridize to IS*6100* (cosmids 39, 43, and 53). As argued below, these cosmids might contain the other copies of *linA* and *linX* present in strain B90A. All cosmids that hybridized to *linB* (cosmids 35, 45, 49, 52, and 56) also hybridized to IS*6100*, but in only one case (number 45) was hybridization detected with one other *lin* probe, namely, *linD*. This suggested that *linB* and *linD* are relatively close to each other (within 20 to 30 kb). Two cosmids (cosmids 33 and 42) hybridized with *linD* and *linE* and also with the IS*6100* probe.

To further analyze the patterns of hybridization with *linX* and *linA*, digested chromosomal DNAs of strain B90A (and strains B90, UT26, and Sp+) were hybridized with the same probes (Fig. 2). DNA that was digested with HindIII and hybridized with *linX* resulted in one hybridizing band at ~4.5-kb for Sp+ and UT26 DNAs (Fig. 2A, lanes 1 and 2). However, with strains B90 and B90A two bands hybridized to *linX* (Fig. 2A, lanes 3 and 4). This suggested that more than one copy of *linX* was present in strains B90A and B90. Similarly, when hybridized with a *linA* probe, DNAs from strains B90A and B90 produced two bands (Fig. 2B, lanes 2 to 5), whereas DNA from UT26 or Sp+ resulted in only one hybridizing band (Fig. 2B, lanes 6 to 9). The presence of two copies of *linA* in strain B90A was in agreement with previous results for strain B90 (14).

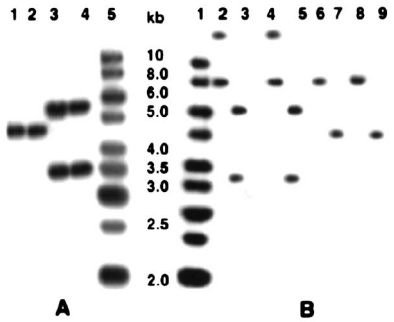

FIG. 2. (A) Southern blot of total DNAs of *S. paucimobilis* strains digested with HindIII and hybridized with [α-^{32}P]dATP-labeled *linX.* Lane 1, *S. paucimobilis* UT26; lane 2, *S. paucimobilis* Sp+; lane 3, *S. paucimobilis* B90; lane 4, *S. paucimobilis* B90A; lane 5, Gene Ruler DNA ladder mixture (MBI Fermentas). (B) Southern blot of total DNAs of *S. paucimobilis* strains digested with BamHI and HindIII and hybridized with [α-^{32}P]dATP-labeled *linA.* Lane 1, Gene Ruler DNA ladder mixture; lane 2, BamHI-digested DNA of *S. paucimobilis* B90A; lane 3, HindIII-digested DNA of *S. paucimobilis* B90A; lane 4, BamHI-digested DNA of *S. paucimobilis* B90; lane 5, HindIII-digested DNA of B90; lane 6, BamHI-digested DNA of *S. paucimobilis* UT26; lane 7, HindIII-digested DNA of UT26; lane 8, BamHI-digested DNA of *S. paucimobilis* Sp+; lane 9, HindIII-digested DNA of Sp+.

Sequence analysis of the *lin* genes and IS*6100* in strain B90A. Several cosmids were selected for further DNA sequencing. The insert of cosmid 57 (pLINA57) was sequenced completely. It contained an approximately 41-kb insert harboring 33 ORFs with clear database homologies (Fig. 3A and Table 3). As expected from the hybridization results, the insert of cosmid 57 contained a *linA* gene and a *linC* gene. According to the DNA sequence, the *linA* copy present in this region is the copy previously cloned from strain B90 and designated *linA1* to distinguish it from the slightly different second copy. The *linC* gene was identical to the gene previously reported for strain B90 (14). In contrast to *linA1* and *linC*, two copies of a *linX* gene were present; however, these copies were only 66% identical to each other. The *linX* copies were designated *linX1* and *linX2* (the *linX1* and *linX2* genes were represented by a ~3.5-kb fragment of HindIII-digested DNA that hybridized with the *linX* probe [Fig. 2A, lane 4]), and *linX1* exhibited 99% DNA sequence identity to *linX* of strain UT26 (22). Apart from the *lin* genes, three copies of a sequence (IS*6100*A, IS*6100*B, and IS*6100*C) identical to IS*6100* were present (Fig. 3A and Table 3), as were many other genes presumably not related to HCH degradation, since the potentially encoded polypeptides had relatively clear homologies to proteins with completely different functions (such as cytochrome *c* oxidase or Nif proteins). Interestingly, two copies of the IS*6100* element flanked the *linC* gene and the ORFM gene, possibly making a composite transposon. No target site duplication was observed when the boundary sequences of the different IS*6100* copies were compared (Fig. 3B).

A 2.2-kb region of cosmid 35 (pLINB35) containing the *linB* gene was also sequenced. This analysis revealed that *linB* in strain B90A and the immediate region upstream of *linB* were 99% similar to the corresponding regions in strain UT26 but that a copy of IS*6100* (IS*6100*D) was located downstream of *linB* (Fig. 3C).

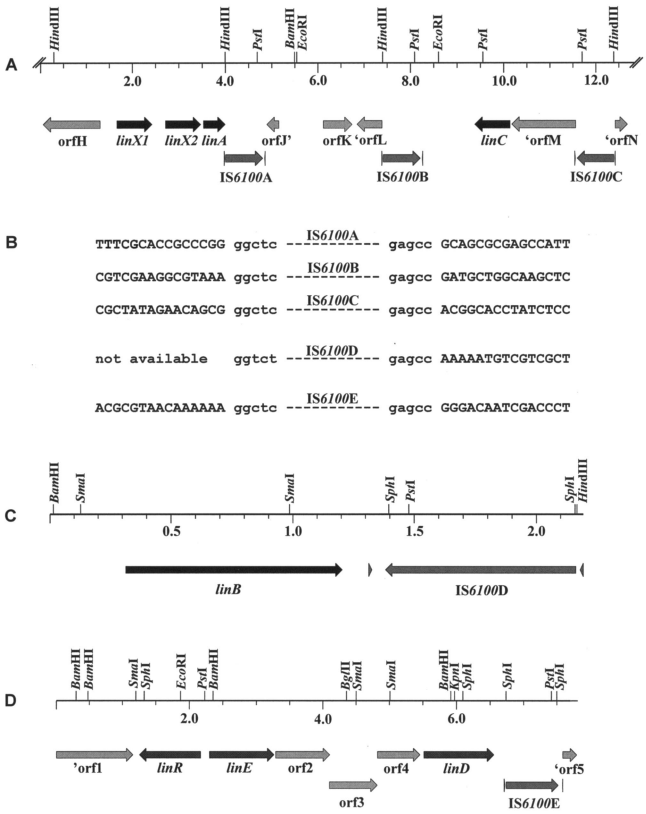

FIG. 3. (A) Partial physical and genetic map of pLINA57 containing the ~41-kb fragment from *S. paucimobilis* B90A. The ORFs deduced from the complete 13-kb nucleotide sequence are indicated by arrows showing the direction of transcription. Details of the coding regions are summarized in Table 3. (B) Comparison of the nucleotide sequences adjacent to five different IS*6100* copies, Nucleotide sequences in lowercase letters are common sequences at the terminal ends of each IS*6100*. IS*6100*A, IS*6100*B, and IS*6100*C are present in pLINA57, and IS*6100*D and IS*6100*E are present in pLINB23 and pLIND33, respectively. The sequence of one of the flanking regions of IS*6100*D could not be determined. (C) Physical and genetic map of the region of pLINB35 containing *linB* and IS*6100.* (D) Physical and genetic map of the region of pLIND33 containing *linD*, *linE*, *linR*, and IS*6100.*

A third separate 7,808-bp region with the *linD*, *linE*, and *linR* genes was sequenced from cosmid 33 (pLIND33) and several plasmid subclones. The relative position of these genes with respect to *linA1* is not known. The nucleotide sequences of *linD*, *linE*, and *linR* were around 99 to 100% identical to those of *linD*, *linE*, and *linR* of strain UT26 (Table 4). Even the ORFs between *linD* and *linE* were the same as those described for UT26 (17, 19). Interestingly, however, both the DNA sequences upstream of *linR* and the DNA sequences downstream of *linD* were different in strains B90A and UT26. Again, an intact copy of IS*6100* (IS*6100*E) was present downstream of *linD* (Fig. 3D). The hybridization data showed that yet another copy of IS*6100* was upstream of *orf1* (data not shown), but its exact location was not determined further.

Since the three cosmids that hybridized to *linA* and *linX* (cosmids 39, 43, and 53) did not hybridize to IS*6100* but sequencing of the *linA1* region of cosmid 57 showed that IS*6100* was located immediately downstream of *linA1*, we assumed

TABLE 3. Coding regions of the 41-kb insert of pLINA57 from *S. paucimobilis* B90A

Protein	Length (amino acids)	% Identity/% similarity	Putative function	Best hit bacterium (accession no.)
ORFA	306	39/49	Hypothetical protein	*Novosphingobium aromaticivorans* (ZP_00093610)
ORFB	323	47/66	TPP-dependent acetoin dehydrogenase alpha subunit	*Clostridium magnum* (AAA21744)
ORFC	327	47/64	Acetoin dehydrogenase (TPP dependent) beta subunit	*Clostridium magnum* (I40791)
ORFC1	79	45/67	Partial dihydrolipoamide acetyltransferase	*Klebsiella pneumoniae* (AAC13741)
ORFD	846	31/48	pfam00593, TonB_boxC, TonB-dependent receptor C-terminal region	*Novosphingobium aromaticivorans* (ZP_00095375)
ORFE	313	44/52	COG1975, xanthine and CO dehydrogenases, maturation factor	*Magnetospirillum magnetotacticum* (ZP_00054040)
ORFF	330	41/59	COG0303, MoeA, molybdopterin biosynthesis enzyme	*Brucella melitensis* (AAL53083)
ORFG	448	68/80	COG1249, pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component	*Novosphingobium aromaticivorans* (ZP_00094170)
ORFH	412	72/82	COG1960, acyl-coenzyme A dehydrogenases	*Novosphingobium aromaticivorans* (ZP_00095926)
LinX1	250	99/99	2,5-Dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase (pfam00106, short-chain dehydrogenase)	*Sphingomonas paucimobilis* UT26 (BAA04939)
LinX2	250	65/81	2,5-Dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase (pfam00106, short-chain dehydrogenase)	*Sphingomonas paucimobilis* UT26 (BAA04939)
LinA1	154	92/95	γ-Hexachlorocyclohexane dehydrochlorinase	*Sphingomonas paucimobilis* UT26 (BAA14369)
TnpA1	264	100/100	Transposase of IS*6100* (COG3316, transposase and inactivated derivatives)	*Aeromonas salmonicida* (CAD57187)
ORFJ	98	80/88	Putative glutathione *S*-transferase (COG0625, glutathione *S*-transferase)	*Sphingobium chlorophenolicum* (AAM96661)
ORFK	205	86/92	pfam02230, αβ-hydrolase_2, phospholipase/carboxylesterase	*Sphingobium chlorophenolicum* (AAM96663)
ORFL	175	94/96	Chloromaleylacetate reductase (COG1454, alcohol dehydrogenase, class IV)	*Sphingobium chlorophenolicum* (AAM96664)
TnpA2	264	100/100	Transposase of IS6100 (COG3316, transposase and inactivated derivatives)	*Aeromonas salmonicida* (CAD57187)
LinC	250	98/98	2,5-Dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase (pfam00106, short-chain dehydrogenase)	*Sphingomonas paucimobilis* UT26 (BAA04939)
′ORFM	(451)	30/49	pfam00593, TonB-dependent receptor C-terminal region	*Novosphingobium aromaticivorans* (ZP_00095375)
TnpA3	264	100/100	Transposase of IS*6100* (COG3316, transposase and inactivated derivatives)	*Aeromonas salmonicida* (CAD57187)
ORFN	(86)	37/59	Outer membrane protein (COG3047, outer membrane protein W)	*Pseudomonas putida* KT2440 (AAN66132)
ORFO	254	62/72	Hypothetical regulator (COG0664, cAMP-binding protein-catabolite gene activator)	*Novosphingobium aromaticivorans* (ZP_00096011)
ORFP	552	78/86	COG3278, cbb_3-type cytochrome oxidase, subunit 1	*Novosphingobium aromaticivorans* (ZP_00096008)
ORFQ	248	81/88	pfam02433, cytochrome *c* oxidase, monoheme subunit	*Novosphingobium aromaticivorans* (ZP_00096007)
ORFR	295	59/75	COG2010, cytochrome *c*, mono- and diheme variants	*Novosphingobium aromaticivorans* (ZP_00096005)
ORFS	476	69/80	COG0348, polyferredoxin	*Novosphingobium aromaticivorans* (ZP_00096004)
ORFT	158	46/62	COG5456, predicted integral membrane protein linked to a cation pump	*Novosphingobium aromaticivorans* (ZP_00096003)
ORFU	710	58/71	COG2217, cation transport ATPase	*Novosphingobium aromaticivorans* (ZP_00096002)
ORFV	1,200	70/79	Bifunctional proline dehydrogenase/δ-1-pyrroline-5-carboxylate dehydrogenase (COG4230, δ-1- pyrroline-5-carboxylate dehydrogenase; COG0506, proline dehydrogenase	*Sinorhizobium meliloti* (CAC41903)
ORFW	233	54/64	COG2755, lysophospholipase L1 and related esterases	*Novosphingobium aromaticivorans* (ZP_00094852)
ORFX	233	68/81	COG4181, predicted ABC-type transporter involved in lysophospholipase L1 biosynthesis, ATPase component	*Novosphingobium aromaticivorans* (ZP_00094853)
ORFY	840	50/64	COG3127, predicted ABC-type transporter involved in lysophospholipase L1 biosynthesis, permease component	*Novosphingobium aromaticivorans* (ZP_00094854)
ORFZ	(590)	43/62	Two-component hybrid sensor and regulator	*Pseudomonas fluorescens* (ZP_00084332)

TABLE 4. Coding regions of the insert of pLIND33 of *S. paucimobilis* B90A

Protein	Length (amino acids)	% Identity/% similarity	Putative function	Best hit Bacterium (accession no.)
ORF1	385	45/60	Hypothetical TonB-dependent receptor); pfam00593, TonB_dep_Rec, TonB-dependent receptor	*Novosphingobium aromaticivorans* (gi 23107064)
LinR	303	99/99	LysR-type transcriptional regulator for *linD*	*Sphingomonas paucimobilis* UT26 (gi 4092848)
LinE	321	100/100	Hydroquinone meta-cleavage dioxygenase	*Sphingomonas paucimobilis* UT26 (gi 4587228)
ORF2	269	35/51	β-Ketoadipate-enol-lactone hydrolase; pfam00561, abhydrolase, alpha/beta hydrolase fold	*Ralstonia solanacearum* (gi 17427307)
ORF3	238	26/42	Carboxylesterase; pfam02230, abhydrolase_2, phospholipase/carboxylesterase	*Burkholderia fungorum* (gi 22989674)
ORF4	210	33/47	Conserved hypothetical protein; COG2350, uncharacterized protein conserved in bacteria	*Ralstonia eutropha* (gi 11967267)
LinD	346	99/100	2,5-Dichlorohydroquinone reductive dechlorinase	*Sphingomonas paucimobilis* UT26 (gi 1731852)
TnpA5	264	100/100	Transposase of IS*6100* (COG3316, transposase and inactivated derivatives)	*Aeromonas salmonicida* (CAD57187)
ORF5	69	45/59	Multidrug resistance protein VceB (truncated, only C-terminal portion)	*Vibrio cholerae* (gi 2815578)

that the second *linA* copy and perhaps a third copy of *linX*, designated *linX3*, were present on cosmids 39, 43, and 53. A comparison of the restriction profiles of cosmids 39, 43, and 53 did not reveal any similarity with the profile of cosmid 57. Hybridization of the digests also confirmed that there was a second signal for the *linA* and *linX* probes (data not shown). When the PCR was conducted with conserved *linX* primers and a ~5.7-kb eluted fragment of HindIII-digested B90A genomic DNA (Fig. 2A, lane 4), a product was amplified which contained the third copy of *linX* (*linX*3), as determined by sequencing. This copy was 100% identical to *linX1*. Although the functionality of the *linX1*, *linX2*, and *linX3* genes was not established, the 99% DNA sequence similarity of *linX1* and *linX3* with *linX* of UT26 (20) and the intact *linX2* ORF (with only 66% similarity with *linX* of UT26) indicate that these genes (at least *linX1* and *linX3*) should be functional in B90A.

Association of IS*6100* copies with the *lin* genes in *S. paucimobilis* strains. Hybridization data for BamHI-digested genomic DNAs of B90A, Sp+, and UT26 revealed the presence of at least 11, 6, and 5 copies of IS*6100* in B90A, UT26, and Sp+, respectively (Fig. 4A). However, no signal could be detected with BamHI-digested genomic DNAs of *S. paucimobilis* ATCC 29837^T and *S. chlorophenolica* DSM 7098^T (non-HCH-degrading strains), indicating that IS*6100* was not present in these strains (data not shown). Further hybridization data for the cosmids from strain B90A and subsequent DNA sequencing (Fig. 3) demonstrated that several IS*6100* copies were associated with the *lin* genes. All five sequenced copies of IS*6100* from B90A, one copy of IS*6100* from Sp+, and one copy of IS*6100* from UT26 were identical over the complete 880 bp. To further investigate the copy number of IS*6100* in each strain and whether IS*6100* was actively transposed within *S. paucimobilis*, Southern hybridizations with digested chromosomal DNAs of strains B90A, B90, Sp+, and UT26 and mutants generated from Sp+ were performed (Fig. 4). *S. paucimobilis* B90A and B90 were originally derived from the same stock but had been separately maintained for different periods of time during cultivation in the laboratory. When genomic DNAs of strains B90A and B90 were digested with BamHI (an enzyme which does not cut within IS*6100*) and hybridized with the IS*6100* probe, at least 11 different bands were observed (Fig. 4A, lanes 2 and 3). The intensities of the bands were not the same in all cases, suggesting that for some fragments more than one copy of IS*6100* was present. Three different hybridizing bands were detected for strains B90A and B90 (Fig. 4A, lanes 2 and 3). For example, for the BamHI digests, the 2.5- and 8.5-kb bands of the B90A digest (Fig. 4A, lane 2) differed from the bands in the B90 digest (Fig. 4A, lane 3). One of these bands could be attributed to the loss of the *linD*, *linE*, and *linR* genes in strain B90. The differences in hybridization signals between B90A and B90 when IS*6100* was used as the probe perhaps can be attributed to the loss of one DNA fragment containing *linD*, *linE*, *linR*, and IS*6100* (Fig. 3D and 4B). The absence of *linD* and *linE* from B90 was also confirmed by Southern blot hybridization (Fig. 4B). This absence could have been due to recombination between two copies of IS*6100* flanking *linD*, *linE*, and *linR* in B90A. While both Sp+ and UT26 also contained multiple copies (six and five copies, respectively) (Fig. 4A, lanes 4 and 5) of the IS*6100* sequence, the copies were at different chromosomal positions (Fig. 4A). More fragments hybridizing to the IS*6100* probe with an apparently similar size were present in the genomic digests of Sp+, B90, and B90A than in the genomic digest of UT26. When the *linA* gene was amplified by PCR from strain Sp+ and sequenced, it was found that similar to *linA1* of strain B90A, the 3′ end of *linA* of Sp+ was replaced by 22 nucleotides of IS*6100*. However, the sequence of the region immediately downstream of *linA* in strain Sp+ did not show a continuation of the IS*6100* sequence like that reported for *linA1* of B90 (14), but hybridization data revealed the presence of yet another copy of IS*6100* further away from the 3′ end of *linA* in Sp+ (data not shown).

To investigate whether mutants could be obtained from *S. paucimobilis* in which HCH degradation activity had been lost, which perhaps could be attributed to the activity of IS*6100*, both strain B90A and strain Sp+ were repeatedly subcultured in SM medium with glucose but without γ-HCH. After about three passages on SM medium, about 200 colonies of both

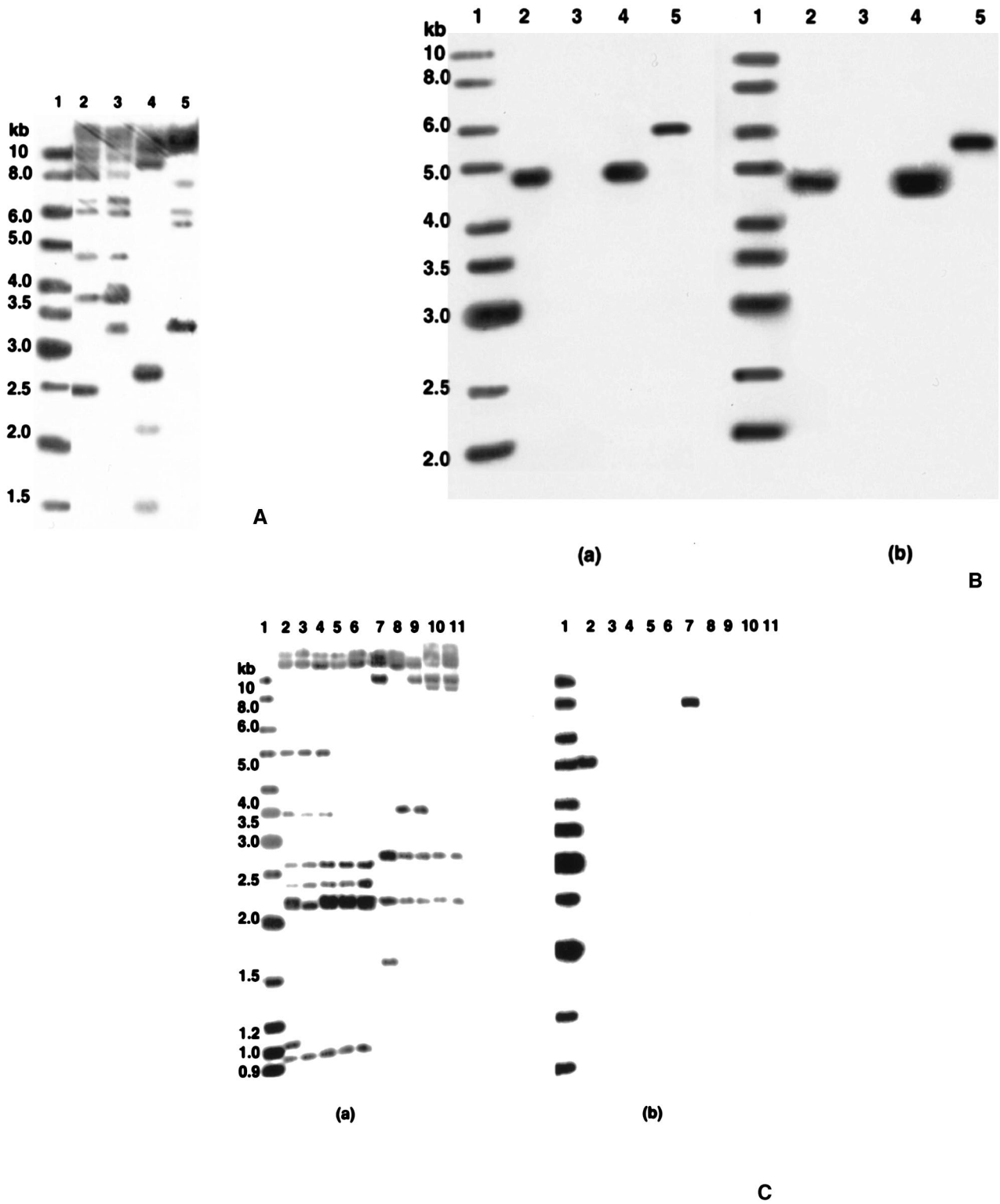

FIG. 4. (A) Southern blot hybridization of genomic DNAs of *S. paucimobilis* B90A, B90, Sp+, and UT26 digested with BamHI and hybridized with [α-^{32}P]dATP-labeled IS*6100.* Lane 1, Gene Ruler DNA ladder mixture; lane 2, B90A; lane 3, B90; lane 4, Sp+; lane 5, UT26. (B) Southern blot hybridization of PstI-digested genomic DNAs of *S. paucimobilis* B90A, B90, Sp+, and UT26 hybridized with [α-^{32}P]dATP-labeled *linD* (panel a) and *linE* (panel b) as probes. Lane 1, Gene Ruler DNA ladder mixture; lane 2, B90A; lane 3, B90; lane 4, Sp+; lane 5, UT26. (C) Southern blot hybridization of genomic DNAs of *S. paucimobilis* Sp+ mutants digested with HindIII and BamHI and hybridized with [α-^{32}P]dATP-labeled IS*6100* (panel a) and *linA* (panel b). Lane1, Gene Ruler DNA ladder mixture; lanes 2 to 6, HindIII-digested genomic DNAs of Sp+ and mutants 1 to 4; lanes 7 to 11, BamHI-digested genomic DNAs of Sp+ and mutants 1 to 4.

TABLE 5. Comparison of various HCH degradative genes and IS*6100* in *S. paucimobilis* B90A, UT26, and Sp+[a]

Gene	No. of nucleotides (no. of amino acids in):			G+C content (%) in:			Function[b]	Stability in:[c]		
	Strain B90A	Strain UT26	Strain Sp+	Strain B90A	Strain UT26	Strain Sp+		Strain B90A	Strain UT26	Strain Sp+
linA1	462 (154)	ND[d]	462 (154)	52.7	ND	52.7	Dehydrochlorinase	++	ND	−
linA2/*linA*	468 (156)	468 (156)	ND	53.9	53.9	ND	Dehydrochlorinase	++	+	ND
linB	888 (296)	888 (296)	888 (296)	62.5	62.5	62.5	Halidohydrolase	++	+	−
linC	750 (250)	750 (250)	750 (250)	64.5	64.5	64.5	Dehydrogenase	++	+	−
linD	1,038 (346)	1,038 (346)	1,038 (346)	61.8	61.8	61.8	Reductive dechlorinase	+	+	++
linE	963 (321)	963 (321)	963 (321)	60.1	60.1	60.1	Ring cleavage dioxygenase	+	+	++
linR	909 (303)	909 (303)	909 (303)	60.3	61.3	60.3	Transcriptional regulator	+	+	+
linX1	750 (250)	750 (250)	750 (250)	64.5	64.5	64.5	Dehydrogenase	++	+	+
linX2	750 (250)	ND	ND	64.5	ND	ND	Dehydrogenase	++	ND	ND
linX3	750 (250)	ND	ND	64.5	ND	ND	Dehydrogenase	++	ND	ND
tnpA	792 (264)	792 (264)	792 (264)	61	61	61	Transposase	+	nd[e]	−

[a] Summary of data presented in this paper and references 14 and 20.
[b] The functions of *lin* genes are based on the data from previous work (14) and on the data for corresponding *lin* genes of UT26 (20).
[c] ++, highly stable; +, stable; −, unstable.
[d] ND, not detected.
[e] nd, not determined.

strains were screened for degradation of HCH isomers by gas chromatography analysis. No mutant lacking the ability to degrade HCH isomers was detected with strain B90A, although the spontaneous mutant B90, which lacked *linD*, *linE*, and *linR*, had been obtained previously (unknowingly), probably due to repeated subculturing (14). We presume that the loss of the fragment (as described above) can be attributed to homologous recombination between two copies of IS*6100* flanking *linD*, *linE*, and *linR*. B90 was found to accumulate 2,5-dichlorohydroquinone (Holliger, unpublished data), and when resting cells of B90A and B90 were incubated with chlorohydroquinone and hydroquinone, these compounds accumulated in B90, but they were degraded further in B90A (Fig. 1). However, we detected four strain Sp+ mutants that lacked the ability to degrade α-, γ-, and δ-HCH. Genomic DNAs of these Sp+ mutants were isolated, digested with HindIII and BamHI, and hybridized with *linA* and IS*6100* probes (Fig. 4C). In all the mutants *linA* appeared to have been lost, but IS*6100* hybridization patterns different than the wild-type Sp+ pattern were found (Fig. 4C). When BamHI-digested genomic DNAs were used, all the Sp+ mutants lacked at least one copy of IS*6100* (1.7-kb BamHI fragment) (Fig. 4C, panel a, lanes 8 to 11), but this did not provide conclusive evidence that there is an association of IS*6100* with a deletion of *linA* through homologous recombination. However, a ~5.0-kb fragment in a HindIII digest that hybridized with both *linA* and IS*6100* in Sp+ (Fig. 4C, panel a, lane 2) was simultaneously lost in at least two mutants, designated mutants 3 and 4 (Fig. 4C, panel a, lanes 5 and 6). In conclusion, all Sp+ mutants that had lost *linA* produced different hybridization patterns than the wild type, and at least two of them (mutants 3 and 4) had lost one copy of IS*6100* along with *linA* (Fig. 4C, panel a, lanes 5 and 6). These results, which are consistent with homologous recombination of two direct IS*6100* copies and subsequent deletion of a *linA* fragment, also support the hypothesis that IS*6100* is directly associated with the stability of *linA* in Sp+. No differences were found among three Sp+ mutants (mutants 2, 3, and 4) in terms of the hybridization patterns when the other *lin* genes (*linB*, *linC*, *linD*, and *linE*) were used as probes (data not shown). All four mutants contained *linC*, *linD*, *linE*, and *linR*. However, one of them (mutant 1), in addition to lacking *linA*, lacked *linB* as well. This was confirmed by PCR amplification and DNA-DNA hybridization (data not shown). The instability of *lin* genes in UT26 has been described previously (20). The data on the stability of *lin* genes and their organization are summarized in Table 5.

DISCUSSION

The data presented in this paper show the extraordinary presence of multiple copies of IS*6100* in HCH-degrading strains of *S. paucimobilis* and the complete absence of IS*6100* in the non-HCH-degrading strains *S. paucimobilis* ATCC 29837^T and *S. chlorophenolica* DSM 7098^T. We have evidence that acquisition of IS*6100* by all HCH- degrading *S. paucimobilis* strains is important not only for establishment of the *linA* genes but also for shaping the genetic organization of the *lin* genes, and we also have evidence that the stability of the *lin* genotype is strongly influenced by IS*6100* activity. We reached this conclusion from the hybridization and sequencing data which showed that most *lin* genes are associated with IS*6100* in B90A, that *linD*, *linE*, and *linR* along with one copy of IS*6100* are deleted in the spontaneous mutant strain B90, and that *linA* and a copy of IS*6100* are deleted in at least two Sp+ mutants.

IS*6100* is a member of the IS*6* family and consequently forms a cointegrate as an end product of transposition (26). In *M. fortuitum* IS*6100* occurs as part of the composite transposon Tn*610* that confers resistance to sulfonamides. It seems to be an extremely promiscuous IS element. For instance, the sequenced copies of IS*6100* from strains B90A, Sp+, and UT26 (five, one, and one copies, respectively) were 100% identical to those of *M. fortuitum* (16). The DNA sequences of five IS elements from the nylon oligomer-degrading plasmid pOAD2 of *Arthrobacter* sp. were identical to the DNA sequence of IS*6100* (12). In the *Pseudomonas aeruginosa* plasmid R1033, IS*6100* is located downstream of Tn*1696*, at the 3′ end of the In*4* integron (7). IS*6100* is also present within the *tnpR* gene of

transposon Tn*5393b* from *Xanthomonas campestris* pv. vesicatoria, where it was shown to increase the expression of the streptomycin resistance genes *strA* and *strB* (35). Similarly, IS*6100* has been found in *Salmonella enterica* serovar Typhimurium (5), in plasmid pACM1 from *Klebsiella oxytoca* (27), in plasmid pTET3 in *Corynebacterium glutamicum* (36), and in plasmid pRASI in *Aeromonas salmonicida* (34). There has been no previous report of IS*6100* elements in any strain of *S. paucimobilis*, and using Southern blot hybridization, we were unable to detect IS*6100* elements in *S. paucimobilis* ATCC 29837^T and *S. chlorophenolica* DSM 7098^T. Thus, the previously published data on the presence of IS*6100* in different bacterial strains and the presence of identical copies of IS*6100* only in HCH-degrading *S. paucimobilis* strains indicate that IS*6100* plays a vital role in disseminating genes, including catabolic and antibiotic resistance genes, among different bacteria. These data also indicate that IS*6100* has been disseminated both widely and recently among different bacterial species without any change in nucleotide sequence. The presence of IS*6100* in distantly related bacterial species, ranging from *M. fortuitum* to *S. paucimobilis*, further suggests that IS*6100* elements have a very broad host range, and their presence on plasmids (even in strains in which the location has not been ascertained) cannot be ruled out.

The genetic structures of the *lin* genes in the different *S. paucimobilis* strains are a remarkable example of rearrangements and pathway evolution, although the implications of the rearrangements in the different strains are not fully understood. For example, data obtained in this work demonstrated that strains B90 and B90A contain two copies of a *linA* gene, whereas both Sp+ and UT26 carry only one copy. Although the exact specificity differences of *linA1* and *linA2* have not been unequivocally determined, the fact remains that strains B90A and B90 can (partially) degrade β-HCH, whereas Sp+ and UT26 cannot. In addition, three copies of a *linX* gene are present in strain B90A. One of the copies, *linX2*, differs considerably from the other two. Only one copy of *linX* has been found in strains UT26 and Sp+. While the enzymatic activity of the *linX* gene (*linX1*, *linX2*, and *linX3*) products for HCH degradation in B90A has not been studied yet, the presence of two copies of *linA* and three copies of *linX* might influence HCH degradation rates. In fact, degradation of α-, γ-, and δ-HCH proceeded at a higher rate in B90A than in UT26 and Sp+ (R. Lal, unpublished data).

Despite the different geographic locations at which they were first isolated, the HCH-degrading *S. paucimobilis* strains have identical *lin* genes (except *linA1* of B90A), even though the *lin* gene organization is not the same in all of the strains. This supports the hypothesis that the *lin* genes were distributed relatively recently and by and large have not accumulated strain-specific mutations yet and that they originated from a single source. We have very little information to determine whether the *lin* genes were assembled once in one microorganism and were subsequently disseminated or whether the *lin* genes were distributed on a self-transmissible DNA fragment to different suitable (*S. paucimobilis*) hosts. In two strains which we studied (B90A and Sp+) and in two other strains described previously (9, 37), the five *linA* genes had a mosaic organization, and at the same time their amino acid and nucleic acid sequences exhibited high levels of similarity. In B90A and B90, the C terminus of the *linA1* gene has been replaced by a copy of IS*6100*, and *linA* of Sp+ only contains the first 22 nucleotides of the IS*6100* sequence towards the C terminus and not a complete copy of IS*6100* (like *linA1* of B90A). A complete copy of IS*6100* appears to be present away from the 3′ end of the *linA* gene of Sp+ (the exact location of a complete copy of IS*6100* in the vicinity of the *linA* gene of Sp+ has not been determined yet), and the *linA* gene of UT26 and the *linA2* gene of B90A do not appear to be associated with IS*6100*. In addition, the G+C contents of all of the *linA* genes reported so far are lower than those of the *linB*, *linC*, *linD*, and *linE* genes, suggesting that *linA* might have been acquired by the strains through horizontal gene transfer from an external donor, as proposed previously (14, 20), by IS*6100* involvement. Eventually, the association of *linA1* with IS*6100* might have triggered a duplication process, thereby adding a second copy in B90A. This hypothesis is supported by the presence of IS*6100* near *linA1*. In fact, the current structure of the *linA1* and *linA2* regions in strain B90A suggests that the genetic organization of *linX* and *linA* in strain UT26 was the result of recombination between the 5′ ends of the *linA1* and *linA2* sequences (14). Additional gene reshuffling followed by further transposition or recombination between IS*6100* copies may then have led to activation of rest of the genes (*linB*, *linC*, *linD*, *linE*) that appear to be indigenous to the strain (20).

In spite of the fact that B90A contains 11 copies of IS*6100*, compared to the 6 and 5 copies in Sp+ and UT26, respectively, the *linA* gene was more stable in B90A than in Sp+ (this study) and UT26 (20), indicating that the copy number of IS*6100* may not be the only factor that contributes to the stability of *lin* genes. The stability of genes within a genome is controlled by several underlying mechanisms operating within the genome, including homologous recombination between elements at different genomic locations, duplications, deletions, inversions, translocations, and transductions (13). The loss of the *linA* gene in Sp+ was always associated with a change in the profile of the IS*6100* copies in all the mutants, and the loss in at least two mutants was accompanied by a loss of a copy of IS*6100* associated with *linA*. Likewise, the presence of IS*6100* flanking *linD* and *orf1* in B90A also suggests that there was a similar additional deletion event, facilitated by homologous recombination between these two copies, resulting in the formation of B90. This is a very recent step that took place in the laboratory.

In conclusion, the mosaic nature of the *linA* genes, the association of IS*6100* with the *lin* genes, and the deletions of IS*6100* associated with deletions of the *lin* genes indicate the underlying role that IS*6100* elements have played in establishing this pathway. Interestingly, the *lin* genes are not located in a single operon, like *linD*, *linE*, and *linR*, but are present in several different regions of the genome. This is in contrast to the genes for many catabolic pathways in pseudomonads, for example, which are more closely organized, but it is similar to the genes in other sphingomonads, such as the genes for polycyclic aromatic hydrocarbon degradation in *Sphingomonas aromaticivorans* (28) and mecocrop degradation in *Sphingomonas herbicidovorans* (van der Meer, unpublished data). The present study brought up several interesting questions, including questions related to finding the original host containing *linA*, the evolution of the β-HCH degradation pathway in B90A, and the mechanism of formation and the role of multiple copies of *lin*

genes in some strains, which will require a series of new experimental studies. On the applied side, the occurrence of stable HCH-degrading microorganisms at different locations on the globe suggests that microorganisms have the ability to adapt to this pollutant, which is a hopeful sign for remediation of HCH contamination.

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