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Complete Nucleotide Sequence of an Exogenously Isolated Plasmid, pLB1, Involved in γ-Hexachlorocyclohexane Degradation▽

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The α-proteobacterial strain *Sphingobium japonicum* UT26 utilizes a highly chlorinated pesticide, γ-hexachlorocyclohexane (γ-HCH), as a sole source of carbon and energy, and haloalkane dehalogenase LinB catalyzes the second step of γ-HCH degradation in UT26. Functional complementation of a *linB* mutant of UT26, UT26DB, was performed by the exogenous plasmid isolation technique using HCH-contaminated soil, leading to our successful identification of a plasmid, pLB1, carrying the *linB* gene. Complete sequencing analysis of pLB1, with a size of 65,998 bp, revealed that it carries (i) 50 totally annotated coding sequences, (ii) an IS*6100* composite transposon containing two copies of *linB*, and (iii) potential genes for replication, maintenance, and conjugative transfer with low levels of similarity to other homologues. A minireplicon assay demonstrated that a 2-kb region containing the predicted *repA* gene and its upstream region of pLB1 functions as an autonomously replicating unit in UT26. Furthermore, pLB1 was conjugally transferred from UT26DB to other α-proteobacterial strains but not to any of the β- or γ-proteobacterial strains examined to date. These results suggest that this exogenously isolated novel plasmid contributes to the dissemination of at least some genes for γ-HCH degradation in the natural environment. To the best of our knowledge, this is the first detailed report of a plasmid involved in γ-HCH degradation.

γ-Hexachlorocyclohexane (γ-HCH) (also called γ-BHC and lindane) is a halogenated organic insecticide which was once used worldwide but has since been prohibited in most countries due to its toxicity and long persistence in upland soil. Although γ-HCH is a representative man-made xenobiotic, some γ-HCH-degrading bacterial strains have been isolated and characterized (30, 32, 35, 42, 43, 47). Genes and enzymes for γ-HCH degradation were first well characterized for an α-proteobacterial strain, *Sphingobium japonicum* UT26, which converts γ-HCH to β-ketoadipate through the action of six enzymes: LinA (dehydrochlorinase), LinB (halidohydrolase), LinC (dehydrogenase), LinD (reductive dechlorinase), LinE (ring cleavage dioxygenase), and LinF (reductase) (14, 40). The *linA*-to-*linF* genes in UT26 are dispersed on the three large circular replicons: the *linA*, *linB*, and *linC* genes on the 3.6-Mb chromosome I; the *linF* gene on the 670-kb chromosome II; and the *linDE* operon with its regulatory gene (*linR*) on a 185-kb plasmid, pCHQ1 (39). Nearly identical *lin* genes have also been identified in other HCH-degrading bacterial strains, such as *Sphingobium indicum* B90 (31) and B90A (12) from India and *Sphingobium francense* Sp+ from France (7); most of the *lin* genes in these strains are closely associated with an insertion sequence, IS*6100* (7, 32). pCHQ1 is conjugally transferable from UT26 to another *Sphingomonas paucimobilis* strain (39), and a recent report showed that the *linA* and *linB* genes in other strains are also located on plasmids (7). These observations indicate that *lin* genes must be spread by mobile genetic elements (MGEs).

The recent determination of various bacterial genome sequences has revealed that the horizontal transfer of various phenotypic genes has played a significant role in the evolution of bacteria and in their adaptation to environmental changes (17, 45). There is no doubt that various MGEs have contributed greatly to horizontal gene transfer (44, 46, 48, 61), but the direct detection of such events in natural bacterial communities is rare. Exogenous isolation of MGEs, designated "exogenous plasmid isolation" when such MGEs are plasmids, has been developed to capture transferable plasmids directly from the natural microbial community by using bacterial conjugation systems (56). This technique involves conjugal mating of a suitable recipient with a natural microbial community and subsequent selection of transconjugants that have acquired a genetic marker. This approach has been used for the isolation of plasmids involved in resistance to mercury and in the degradation of 2,4-dichlorophenoxyacetic acid and naphthalene (3, 4, 54, 58, 60). Considering the fact that most bacterial cells in the environment are not easily culturable by conventional techniques (1), the exogenous plasmid isolation technique will provide novel insights into horizontal gene transfer in the natural environment.

In the present study, the exogenous plasmid isolation technique was applied to isolate a gene for LinB activity from HCH-contaminated soil. Considering the fact that LinB is a key enzyme in the degradation not only of γ-HCH but also of β-HCH (41), the *linB* gene is one of the most important genes in sites contaminated by a technical mixture of HCH (t-HCH) that consists of α, β, γ, and δ isomers. The present report provides a snapshot of the dynamism of the *linB* gene in the natural environment.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* cells were grown at 37°C in

TABLE 1. Bacterial strains and plasmids used in this study

Species and strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1*Δ(*lacZYA-argF*)ϕ80*lacZ* Δ*M15*	52
S17-1λ*pir*	*pro thi recA hsdR* λ*pir* lysogen; Tp^r Sm^r; chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7*	64
HB101	*hsdS20 recA13 ara-14 proA2 lacI1 galK2 rpsL20 xyl-5 mtl-1 supE44*	34
Pseudomonas putida		
KT2440	Wild-type strain	2
KT2440G	KT2440::Tn*Mod*-OGm; Gm^r	This study
Sphingobium japonicum		
UT26	HCH^{+a}; Nal^r	20
UT26DB	UT26 *linB*::Km^r	41
UT26DBT1	Transconjugant of UT26DB harboring pLB1	This study
Sphingomonas paucimobilis		
IAM12578	Wild-type strain	36
IAM12578G	IAM12578::Tn*Mod*-OGm; Gm^r	This study
Sinorhizobium meliloti 1021	Wild-type strain; Sm^r	37
Mesorhizobium loti MAFF303099	Wild-type strain; Pho^r	23
Burkholderia multivorans		
ATCC 17616	Wild-type strain	57
ATCC 17616G	ATCC17616::Tn*Mod*-OGm; Gm^r	This study
Acidovorax sp.		
KKS102	Wild-type strain	25
KKS102G	KKS102::Tn*Mod*-OGm; Gm^r	This study
Plasmids		
pUC18	pMB9 replicon; Ap^r	62
pTn*Mod*-OGm	pMB1 replicon, Tn*5* inverted repeat; Gm^r	11
pK18mob	pMB1 replicon; Mob^+ Km^r	53
pJP5608	R6K replicon; Mob^+ Tc^r	51
pNIT6012	pVS1 derivative, shuttle vector; Mob^+ Tc^r	19
pBBR1MCS-2	pBBR1 replicon, broad host range; Mob^+ Km^r	28
pRM11	pUC18 derivative carrying EcoRI-BamHI fragment containing *linB* of pLB1	This study
pRM14	pNIT6012 derivative carrying BamHI-SphI fragment containing *linB* of pRM11	This study
pRM15	pNIT6012 derivative carrying BamHI fragment containing *linB* of pLB1	This study
pRM26	pJP5608 derivative carrying PstI fragment of pRM11	This study
pK18OR	pK18mob derivative carrying HindIII-BamHI PCR fragment containing *oriV* and *repA*	This study
pK18BD3	pK18mob derivative carrying DraI-BamHI fragment of pLB1	This study
pLB1	Exogenously isolated plasmid carrying *linB*	This study
pLB1Tc	pLB1::pRM26	This study

[a] HCH^+, growth on γ-HCH.

Luria-Bertani (LB) broth (34), and *Sinorhizobium meliloti* and *Mesorhizobium loti* cells at 30°C in TY medium (6). Other strains were grown in 1/3LB broth (14) or W minimal medium (20) supplemented with γ-HCH as a carbon source at 30°C. The solid media were prepared by the addition of 1.5% agar. Antibiotics were used at final concentrations of 50 μg/ml for ampicillin and phosphomycin (Pho), 25 μg/ml for kanamycin (Km), 10 μg/ml for gentamicin (Gm), 15 μg/ml for tetracycline (Tc), 200 μg/ml for trimethoprim, and 1,000 μg/ml for streptomycin (Sm).

Methods of DNA manipulation. Established methods were used for the preparation of plasmid DNA, its digestion with restriction endonucleases, ligation, agarose gel electrophoresis, and the transformation of *E. coli* cells (34). Large plasmids from α-proteobacterial strains were extracted using the method described by Kado and Liu (22), and the transformation of bacterial cells by electroporation was performed as described previously (27). Nucleotide sequencing was performed with an ABI PRISM model 310 sequencer (Applied Biosystems, Foster City, CA). Southern blot analysis was carried out using the conventional protocols and a digoxigenin system (Roche Diagnostics, Mannheim, Germany). For the preparation of the *linB* probe, a *linB* gene fragment was amplified by PCR with UT26 total DNA as a template and the primers Bgl-linB-Xho.R (5′-GGGCTCGAGGATTATGCTGGGCGCAATC-3′) and linB.F (5′-TAAGGAGGAATATCGATGAGCCTC-3′). For the *repA* probe of pLB1, we used plasmid pLB1Tc (see below) as a template and the primers Hin_pLB1_65868.F (5′-CCCAAGCTTGTGCCACCGAAGTGAGC-3′) and pLB1_894_Bam.R (5′-CCCGGATCCGAACTTCTTCCGTCAACG-3′).

Microbiological methodology for a soil sample. The clay soil sample used in the present study was obtained from a field which had been contaminated with HCH isomers in Miyagi Prefecture, Japan. To determine the most probable number (MPN) of γ-HCH-degrading cells in the soil, 1-ml portions of serially diluted soil bacterial suspensions were inoculated into 9 ml of 1/10 W [containing the following per liter: KH_2PO_4, 170 mg; Na_2HPO_4, 980 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4$, 48.7 mg; $FeSO_4$, 0.52 mg; MgO, 10.75 mg; $CaCO_3$, 2.0 mg; $ZnSO_4$, 0.81 mg; $CuSO_4$, 0.16 mg; $CoSO_4$, 0.15 mg; and H_3BO_3, 0.06 mg) containing 10 ppm of γ-HCH and were incubated at 30°C for 2 weeks with shaking. An equal volume of ethyl acetate was mixed with each suspension to extract the solution that was used for the analysis of γ-HCH degradation with a Shimadzu GC-17A gas chromatograph equipped with an electron capture ^{63}Ni detector (Shimadzu, Kyoto, Japan) as described previously (41).

Exogenous plasmid isolation was performed using a modified version of the procedure described by Top et al. (60). The bacterial fraction in 10 g of soil

sample was suspended in phosphate-buffered saline (PBS) and filtered through 7-μm-pore-size filter paper (Advantec MFS, Inc., Tokyo, Japan) to remove nonbacterial materials. The filtered fraction was centrifuged at 10,000 × *g* for 20 min, and the cell pellet was resuspended in 0.5 ml of PBS buffer and mixed with 1 ml of an overnight culture of the recipient (UT26DB) cells. This mating mixture was concentrated by centrifugation at 10,000 × *g* for 2 min and spotted onto 1/3LB agar plates supplemented with cycloheximide (100 μg/ml). After overnight incubation at 30°C, cells were suspended in 1 ml of PBS, diluted, and spread on W agar plates supplemented with γ-HCH and Km.

Sequencing and computer analysis of pLB1. A 1.9-kb PstI fragment of pLB1 (bp 20285 to 22160) was cloned into the PstI site of an R6K-based Tc-resistant (Tc^r) plasmid, pJP5608. The resulting plasmid, pRM26, was conjugally transferred from *E. coli* S17-1λ*pir* to UT26DBT1 (see below), and selection was made for resistance to Tc. One such transconjugant carried the pLB1 derivative (pLB1Tc) in which pRM26 was integrated into pLB1 by single-crossover-mediated homologous recombination between the common PstI fragment shared by the two plasmids. To avoid contamination of endogenous plasmids in UT26DB, pLB1Tc was conjugally transferred from UT26DB to *E. coli* S17-1λ*pir* and purified by a standard alkaline lysis method (34). Shotgun sequencing of pLB1Tc was performed, the DNA fragments not covered by shotgun sequencing were directly amplified by PCR, and the nucleotide sequences of the resulting PCR products were determined.

Sequence assembly and calculation of G+C contents were performed using Genetyx 13 software (SDC, Tokyo, Japan). Annotation was performed using the GenBank CDS translations/PDB/SwissProt/PIR/PRF protein databases and the DDBJ/EMBL/GenBank DNA databases with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The conserved domains were searched using the InterProScan (http://www.ebi.ac.uk/InterProScan/) and the Pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml) programs. The putative amino acid sequences were used to generate a neighbor-joining tree by running the CLUSTALW program (http://www.ddbj.nig.ac.jp/search/clustalw-j.html).

Replication analysis of pLB1. The 2-kb DNA fragment containing the *repA* gene and its upstream region (bp 64908 to 894) was amplified by PCR using pLB1Tc as a template and the primers pLB1_64908.F (5′-TAAAAGCTTTCA TCGCTTTCTCC-3′) and pLB1_894_Bam.R (see above). The amplicon was cloned into the multiple cloning site of a narrow-host-range vector, pK18mob (Km^r), to obtain pK18OR as a minireplicon. Insertion of the 3.3-kb BamHI-DraI fragment of pLB1 (bp 36846 to 40144) (see Fig. 2 and Table 2) into pK18mob generated pK18BD3, which was used as a negative control. A broad-host-range vector, pBBR1MCS-2 (Km^r), was used as a positive control. pK18OR, pK18BD3, and pBBR1MCS-2 (150 ng each) were introduced into the UT26 cells by electroporation to obtain Km^r transformants.

Filter matings between pure cultures. The donor and recipient cells grown overnight were harvested by centrifugation, washed with TY broth, and resuspended in fresh TY broth. They were then mixed and subsequently spotted on a sterile 0.45-μm-pore-size cellulose acetate filter (Advantec) placed on a TY agar plate. After incubation at 30°C for 13 h, the cells on the filter were suspended in TY broth, diluted, and plated on selective agar plates. *S. meliloti* 1021, *M. loti* MAFF303099, and *E. coli* HB101 were selected with Sm, Pho, and Sm, respectively. The chromosomes of other recipient strains (*Pseudomonas putida* KT2440, *S. paucimobilis* IAM12578, *Burkholderia multivorans* ATCC 17616, and *Acidovorax* sp. strain KKS102) were marked with the Tn*Mod*-OGm-derived Gm^r gene. The introduction of this gene into the genomes of the parental strains was carried out by electroporation–mediated mutagenesis using pTn*Mod*-OGm (11).

Nucleotide sequence accession number. The complete sequence for the circular plasmid pLB1 has been deposited in DDBJ/EMBL/GenBank under accession number AB244976.

RESULTS

Exogenous isolation of a plasmid involved in γ-HCH degradation. In the present study, we used a clay soil sample from Miyagi Prefecture, Japan. The soil had been contaminated by t-HCH, and still contained low levels of HCH isomers (0.03 to 0.1 mg/liter, mainly α-HCH). The number of culturable bacterial cells on each 1/100 NB (30 mg of beef extract per liter and 50 mg of peptone per liter) agar plate was 1.4×10^7 CFU/g of soil. The number of indigenous γ-HCH-degrading bacterial cells was estimated to be at least 10^3 MPN/g of soil. The bacterial fraction collected from the soil was mated with a *linB* mutant of UT26 (UT26DB) (41), using the protocol described in Materials and Methods. We obtained one putative transconjugant, UT26DBT1, that was able to utilize γ-HCH as a sole carbon source on the W agar plate plus Km. The control experiments without UT26DB did not give rise to such transconjugants. We further confirmed that UT26DBT1 was indeed a transconjugant of UT26DB by the following three results: (i) the 16S rRNA gene of UT26DBT1 was completely identical to that of UT26; (ii) *linB*::Km-specific DNA fragments were amplified by PCR using the UT26DBT1 colony as a template; and (iii) three UT26-endogenous plasmids, pCHQ1 (185 kb), pUT1 (30 kb), and pUT2 (5 kb), were also observed in UT26DBT1 (Fig. 1). UT26DBT1 harbored one additional plasmid, designated pLB1. Southern blot analysis with the *linB* gene as a probe indicated that pLB1 carries the *linB* homologue(s) (Fig. 1).

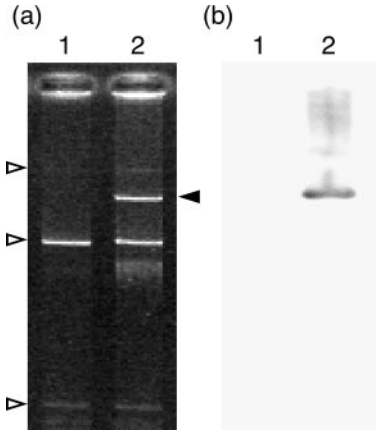

FIG. 1. Plasmids residing in UT26DB and its transconjugant UT26DBT1. Gel electrophoresis of plasmids (a) and Southern blot analysis with the *linB* gene as a probe (b) are shown. Lanes 1, UT26DB; lanes 2, UT26DBT1. The white arrowheads indicate endogenous plasmids pCHQ1 (185 kb), pUT1 (30 kb), and pUT2 (5 kb), respectively. The black arrowhead indicates pLB1.

Sequence analysis of pLB1. Our determination of the entire nucleotide sequence of pLB1Tc purified from *E. coli* S17-1λ*pir* cells revealed that its parental plasmid, pLB1, is 65,998 bp in size. The average G+C content of pLB1 is 60.2%, and this score approximately corresponds to those of plasmids from other α-proteobacterial strains, such as pNL1 (62%) from *Sphingomonas aromaticivorans* F199, pSymA (60%) from *S. meliloti* 1021, and pMLa (59%) from *M. loti* MAFF303099. pLB1 was found to carry 50 coding sequences (CDSs) (Fig. 2 and Table 2), which represent 72.1% of the total plasmid DNA. The remaining 18,385-bp regions consist of partial gene fragments and intergenic regions. The putative products of 24 of 50 CDSs showed low similarity (less than 48% identity) to those deposited in public databases.

(i) The IS*6100-linB* cluster. pLB1 carried two directly oriented copies of genes whose nucleotides were identical to *linB* from *S. indicum* B90A. To confirm the functionality of the two pLB1-specified *linB* genes, pRM14 and pRM15 were constructed such that each carried one of the two *linB* genes (bp 17148 to 18531 and 14965 to 16348, respectively) (Table 1). The introduction of these plasmids into UT26DB gave rise to transformants able to grow on a W agar plate containing

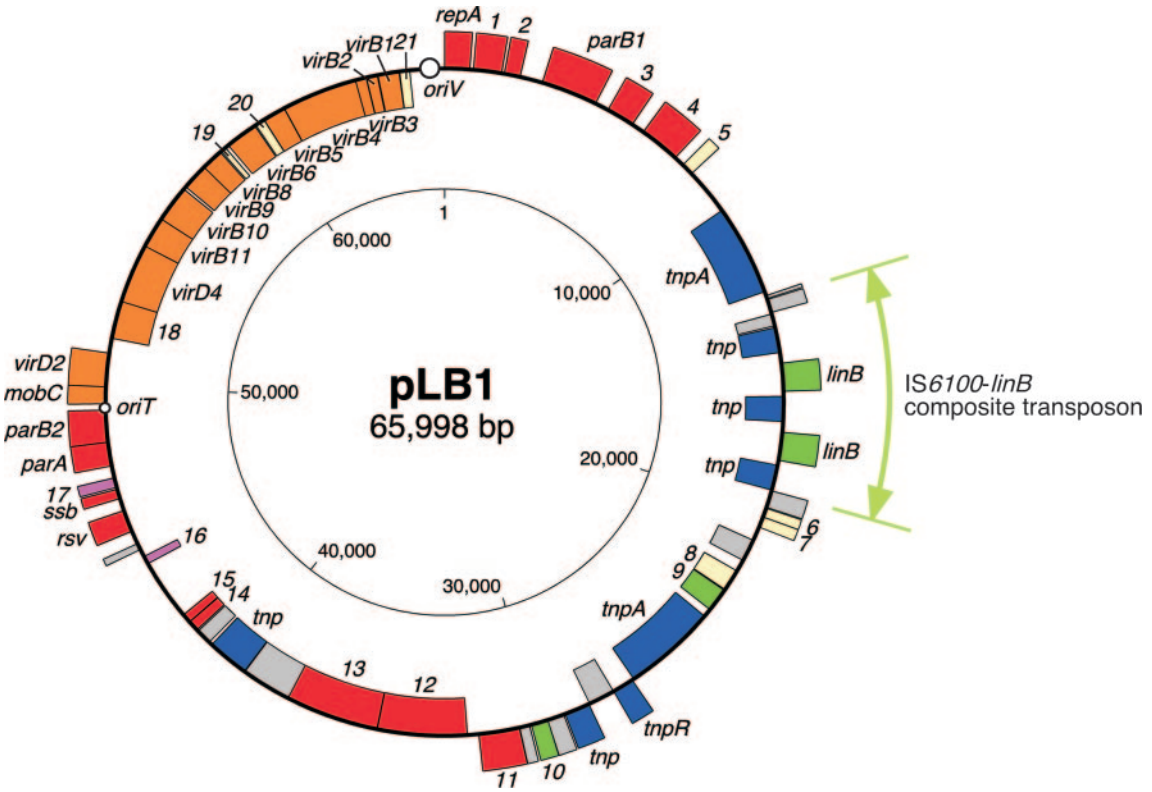

FIG. 2. Circular map of pLB1. CDSs or gene remnants outside the circle are transcribed in the clockwise direction and those inside in the counterclockwise direction. The putative functions of genes are shown by the following colors: red, replication and stable inheritance; orange, conjugative transfer; blue, transposase and resolvase; magenta, regulation; yellow, unknown; and green, others. Gene remnants are shown in gray. Large and small circles indicate the putative *oriV* and *oriT* regions, respectively. The region of the IS*6100-linB* cluster is shown by the light green arrow. The positions of *tnp* in the IS*6100-linB* cluster are almost equal to those of IS*6100* (Table 2).

γ-HCH as a carbon source, indicating that both *linB* genes on pLB1 are functional. The two *linB* genes and three complete and directly oriented copies of IS*6100* were clustered in the order IS*6100-linB*-IS*6100-linB*-IS*6100*, and this cluster was preceded by two truncated versions of this IS element. This 6-kb region including the IS*6100* remnants (bp 13187 to 19323) was designated the IS*6100-linB* cluster. Although the transposition of IS*6100* usually generates 8-bp direct repeats of its target site (8), no obvious duplication of such a target site was observed around this cluster.

(ii) Genes for replication. The predicted pLB1 replication initiator protein, RepA, is unique because only three homologues with homology at a low level were found in databases: RepA of *Agrobacterium radiobacter* K84 plasmid pAgK84 (33% identity), Rep of *Agrobacterium tumefaciens* 1D1422 plasmid pTAR (31% identity), and a hypothetical protein of a *Zymomonas mobilis* ZM4 plasmid (27% identity). An InterProScan search revealed that the pLB1 RepA protein contains in its middle part a putative winged-helix motif that would promote the binding to DNA. Although the upstream region of the *repA* gene (bp 64921 to 65998) showed no similarity to other well-characterized replication origin (*oriV*) sequences, this region was relatively A+T rich (55.8% of G+C content) and was found to contain seven 9-bp direct repeats (5′-kCwAwCwsd-3′) and one inverted repeat (Fig. 3). The direct repeats may serve as iterons (9), which are the interaction sites of iteron-type replication initiator proteins. Additionally, two putative DnaA boxes (38) were also located in this region (bp 64945 to 64953 and 65134 to 65142).

(iii) Genes for stable inheritance. The *parA-parB2* cluster (bp 47479 to 49253) is considered to be involved in the partitioning of pLB1. The putative ParA protein shows 28% identity to an IncC-like protein of *S. meliloti* MBA19 plasmid pMBA19a (Table 2) and belongs to the Walker-type ATPases (63). A phylogenetic tree of partitioning ATPases, in which each clade is thought to coincide with the genetic organization of the *par* loci (15, 16), demonstrated that ParA of pLB1 belongs to a novel clade (Fig. 4). The putative ParB2 protein has a functional domain (IPR003115) required for its binding to a specific DNA sequence (partition site). Another putative partitioning gene, *parB1* (bp 3079 to 4854), was found far away from the *parA-parB2* cluster. Although ParB1 also had the predicted functional domain, its C-terminal region, which was postulated to be involved in dimerization, showed a very low level of similarity to that of ParB2.

Two CDSs, *rsv* and *orf2*, encode a putative resolvase (site-specific serine recombinase) and nuclease, respectively. Their predicted products show homology with ParA and ParB of plasmid RP4, respectively (49), and they are considered to be involved in multimer resolution (13, 21). (Note that ParA and ParB of RP4 are different from ParA, ParB1, and ParB2 of pLB1.) Therefore, the Rsv and Orf2 proteins might contribute

TABLE 2. Genes, sites, and specific fragments on pLB1

Gene	Position[a]	Determined or estimated function	% Amino acid identity[b]	Source	Protein identification no. of closest relative
repA	1–783	Replication protein	33 (83/245)	*Agrobacterium tumefaciens* K84 plasmid pAgK84	YP_086770.1
orf1	895–1776	Putative primase	38 (111/291)	*Novosphingobium aromaticivorans* DSM 12444	ZP_00873452.1
orf2	1863–2360	Putative nuclease	29 (45/151)	*Nitrobacter hamburgensis* X14	ZP_00626575.1
parB1	3079–4854	Putative plasmid stabilization protein	29 (190/638)	*A. tumefaciens* K84 plasmid pAgK84	YP_086769.1
orf3	5293–6165	ArdC-like antirestriction protein	51 (152/294)	*Nitrobacter hamburgensis* X14	ZP_00627826.1
orf4	6577–7857	Fic-like filamentation protein	37 (70/187)	*Xyielia fastidiosa* Dixon	ZP_00653050.1
orf5	8186–8575	Conserved hypothetical protein	35 (47/132)	*Rhodobacter sphaeroides* 2.4.1	ABA81614.1
tnpA	10065–12956C	Tn*3* family transposase	92 (888/964)	*Acetobacter aceti* plasmid pAC5	AAF20014.1
—[c]	13082–13186	Resolvase N-terminal fragment	94 (33/35)	*Xanthomonas campestris* pv. Vesicatoria strain 85-10	YP_364058.1
—	13220–13639	IS*6100* transposase N-terminal fragment	100 (140/140)	*Salmonella enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
—	13694–14074C	IS*6100* transposase C-terminal fragment	100 (126/126)	*S. enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
tnp	14131–14925C	IS*6100* transposase	100 (264/264)	*S. enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
linB	15264–16154	1,4-TCDN[d] halidohydrolase	100 (296/296)	*Sphingobium indicum* B90A	AAR05978.1
tnp	16314–17108C	IS*6100* transposase	100 (264/264)	*S. enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
linB	17447–18337	1,4-TCDN halidohydrolase	100 (296/296)	*S. indicum* B90A	AAR05978.1
tnp	18497–19291C	IS*6100* transposase	100 (264/264)	*S. enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
—	19324–19866	Resolvase C-terminal fragment	75 (131/174)	*Zymomonas mobilis* subsp. *mobilis* ZM4	AAV90079.1
orf6	19898–20206	Putative inner membrane protein	56 (55/97)	*S. enterica* serovar Typhimurium LT2	NP_463387.1
orf7	20187–20519	Hypothetical protein	52 (45/86)	*Escherichia coli* B7A	ZP_00714508.1
—	20981–21688C	IS*4* transposase gene remnant	67 (161/238)	*N. hamburgensis* X14	ZP_00627545.1
orf8	22076–22762C	Hypothetical protein	34 (29/85)	*Synechococcus* sp. strain CC9605	YP_381146.1
orf9	22790–23551C	Short-chain dehydrogenase	53 (136/253)	*S. indicum* B90A	AAR05964.1
tnpA	23816–26773C	Tn*3* family transposase	69 (682/985)	*Gluconobacter oxydans* 621H plasmid pG0X1	YP_190365.1
tnpR	26832–27506	Resolvase	70 (141/199)	Uncultured bacterium plasmid pTB11	YP_112363.1
—	27519–28349C	Putative ring-hydroxylating dioxygenase gene remnant	41 (110/265)	*Burkholderia* sp. strain 383	YP_366455.1
tnp	28381–29175	IS*6100* transposase	100 (264/264)	*S. enterica* serovar Typhimurium G8430 plasmid pU302L	AAR05758.1
—	29230–29751	Resolvase gene remnant	89 (150/168)	*Pseudomonas syringae* pv. Syringae A2 plasmid pPSR1	NP_940689.1
orf10	29754–30278	Putative acetyltransferase	29 (47/158)	*P. fluorescens* Pf-5	YP_260527.1
—	30344–30655	LexA gene remnant	47 (47/100)	*Rhodoferax ferrireducens* DSM 15236	ZP_00695279.1
orf11	30666–31928	Putative UV protection protein	41 (176/422)	*Magnetococcus* sp. strain MC-1	ZP_00607746.1
orf12	32290–35097C	Hypothetical protein	68 (641/935)	*Aurantimonas* sp. strain SI85-9A1	ZP_01227946.1
orf13	35106–38039C	Putative Type III restriction-modification enzyme	45 (447/972)	*Thermoplasma volcanium* GSS1	NP_111994.1
—	38039–39613C	Adenine-specific DNA methylase C-terminal fragment	50 (270530)	*Azotobacter vinelandii* AvOP	ZP_00416345.1
tnp	39630–40952C	IS*5* family transposase	62 (260/418)	*Desulfovibrio desulfuricans* G20	YP_390215.1
—	41057–41551C	Adenine-specific DNA methylase N-terminal fragment	49 (90/183)	*A. vinelandii* AvOP	ZP_00416345.1
orf14	41607–41945C	Plasmid maintenance system antidote protein	85 (87/102)	*Rhodospirillum rubrum*	ZP_00270380.1
orf15	41949–42239C	Plasmid maintenance system killer protein	82 (79/96)	*N. hamburgensis* X14	ZP_00624977.1
orf16	44188–44445C	XRE family transcriptional regulator	41 (25/60)	*Rhizobium* sp. strain NGR234 plasmid pNGR234a	NP_443819.1
—	44697–44897	RNA polymerase sigma 70 factor fragment	44 (30/67)	*Thermosynechococcus elongatus* BP-1	NP_681289.1

Continued on following page

TABLE 2—*Continued*

Gene	Position[a]	Determined or estimated function	% Amino acid identity[b]	Source	Protein identification no. of closest relative
rsv	45325–46023	Resolvase	70 (153/217)	Plasmid RP4	AAA26414.1
ssb	46417–46731	Single-strand binding protein	50 (52/103)	*N. hamburgensis* X14	ZP_00627857.1
orf17	46789–47091	Putative transcriptional regulator	40 (34/85)	*Sphingopyxis alaskensis* RB2256	ZP_00579357.1
parA	47479–48237	ParA-like partition protein	28 (67/235)	*Sinorhizobium meliloti* MBA19 plasmid pMBA19a	AAX19280.1
parB2	48234–49253	ParB-like partition protein	30 (73/242)	*Bradyrhizobium* sp. strain BTAi1	ZP_00864437.1
oriT	49254–49443	Putative *oriT* region			
mobC	49444–49995	MobC-like protein	28 (47/163)	*Bradyrhizobium* sp. strain BTAi1	ZP_00864438.1
virD2	49982–51049	Relaxase	32 (104/320)	*Aeromonas punctata* HGB5 plasmid pFBAOT6	YP_067824.1
orf18	51526–52734C	Putative DNA primase	28 (58/202)	*X. fastidiosa* 9a5c plasmid pXF51	NP_061673.1
virD4	52724–54640C	VirD4 type IV secretion protein	45 (257/569)	*Bradyrhizobium* sp. strain BTAi1	ZP_00864242.1
virB11	54618–55610C	VirB11 type IV secretion protein	62 (201/324)	*Bradyrhizobium* sp. strain BTAi1	ZP_00864241.1
virB10	55607–56782C	VirB10 type IV secretion protein	38 (143/371)	*Mesorhizobium* sp. strain BNC1	ZP_00614245.1
virB9	56877–57719C	VirB9 type IV secretion protein	38 (108/279)	*R. etli* CFN42 plasmid p42d	NP_659886.1
virB8	57716–58399C	VirB8 type IV secretion protein	44 (97/220)	*S. meliloti* 1021 plasmid pSymA	NP_435958.1
orf19	58447–58620C	Hypothetical protein			
virB6	58740–59756C	VirB6 type IV secretion protein	28 (95/336)	*A. tumefaciens* strain C58 plasmid AT	NP_535541.1
orf20	59797–60102C	Hypothetical protein			
virB5	60105–60818C	VirB5 type IV secretion protein	36 (80/217)	*R. etli* CFN42 plasmid p42d	NP_659890.1
virB4	60832–63216C	VirB4 type IV secretion protein	51 (402/777)	*R. etli* CFN42 plasmid p42d	NP_659891.1
virB3	63203–63547C	VirB3 type IV secretion protein	43 (42/96)	*Bartonella henselae* strain Houston–1	YP_034052.1
virB2	63554–63895C	VirB2 type IV secretion protein	38 (38/99)	*Mesorhizobium* sp. strain BNC1	ZP_00614254.1
virB1	63917–64582C	VirB1 type IV secretion protein	47 (80/169)	*X. fastidiosa* 9a5c plasmid pXF51	NP_061658.1
orf21	64597–64920C	Hypothetical protein	48 (38/79)	*S. meliloti* 1021 plasmid pSymA	NP_435966.1
oriV	64921–65998	Putative *oriV* region			

[a] The letter C indicates that the gene is carried on the complementary strand.
[b] Values in parentheses refer to numbers of identical amino acids per the number examined.
[c] —, probable gene remnant.
[d] TCDN, 1,3,4,6-tetrachloro-1,4-cyclohexadiene.

to the stable maintenance of pLB1 in the host cells by the site-specific resolution and subsequent decatenation of the multimer form of pLB1. The putative products of *orf14* and *orf15* showed high similarity (82 to 85% identity) to antitoxin and toxin, respectively, of the classical proteic killer system for plasmid maintenance (26), and they may be involved in the genetic addiction system of pLB1.

(iv) Genes for conjugative transfer. Putative genes for conjugative DNA transfer, constituting the DNA transfer and replication (Dtr) system and mating pair formation (Mpf) system, were found in a 15-kb region of pLB1 (Fig. 2). The gene organization suggested that this region consists of two transcriptional units (*mobC-virD2* and *orf21-orf18*) (Fig. 5). The putative origin of transfer (*oriT*; bp 49254 to 49443) was deduced on the basis of typical features of other previously well-characterized *oriT* sequence (65). This region, which had a relatively low G+C content (52.6%), contained inverted repeats (bp 49302 to 49327 and 49386 to 49425) and the putative *nic* site (5′-TATCCCGC-3′), which showed significant similarity to the RP4-type site (49).

We believe that the four putative gene products (MobC, VirD2, Orf18, and VirD4) play a role in the Dtr system. MobC contained at its C terminus the MobC domain (PF05713) that is believed to act as a molecular wedge for the relaxosome-induced melting of *oriT* DNA (66), and thus MobC may facilitate relaxosome function. VirD2 has been predicted to serve as a relaxase capable of site-specific nicking of plasmid DNA at *oriT*, because it contains each of the three defined motifs that are conserved in the relaxases of IncP-1 and Ti plasmids (50). As is the case with TraC of IncP-1 plasmids (49), the *orf18* product is assumed to be transported to the recipient cells during the conjugative transfer of single-stranded plasmid DNA and is thought to subsequently initiate the synthesis of the complementary strand. The presence of the nucleotide-binding Walker motifs A and B in VirD4 allowed us to categorize this protein as being isofunctional with TrwB from an

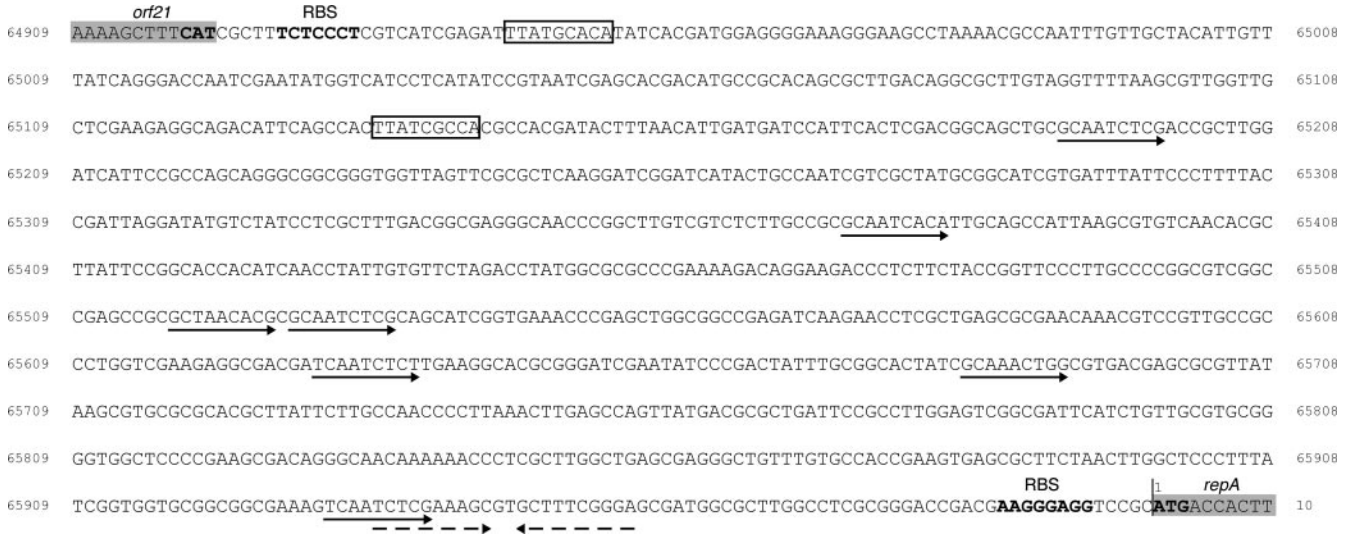

FIG. 3. Structure of the putative *oriV* region of pLB1. The 9-bp direct repeats (5′-kCwAwCwsd-3′), which may serve as iterons, are shown by solid arrows, and one inverted repeat is indicated by dashed arrows. Putative DnaA-binding sequences are boxed, and parts of *repA* and *orf21* are shaded. Putative ribosome-binding sites and start codons of *orf21* and *repA* are shown in boldface.

IncW plasmid, TraG from IncP-1 plasmids, and VirD4 from the Ti plasmid, all of which are necessary for conjugal DNA transfer.

The products of the 10 pLB1-specified genes (*virB1* to *virB6* and *virB8* to *virB11*) putatively involved in the Mpf system showed 28% (VirB6) to 62% (VirB11) identity with the products of the *virB* gene clusters of symbiotic plasmids from rhizobial strains (Table 2). The level of identity was relatively low, but the functional domains of the putative products were conserved, suggesting that they are functional as the Mpf system. The gene order of the *virB* cluster on pLB1 was fundamentally the same as that in the Mpf gene clusters in various conjugative plasmids (Fig. 5) (55). The position of the Dtr-related gene cluster relative to that of the Mpf-related cluster on pLB1 was

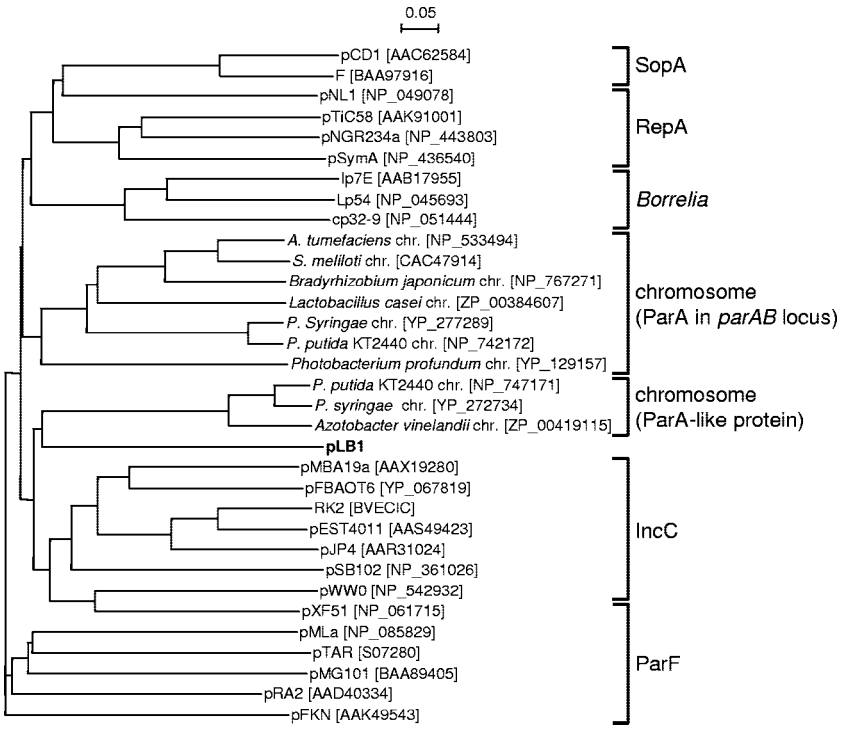

FIG. 4. Phylogenetic tree of putative Walker-type partition ATPases. Lengths of horizontal lines reflect relative evolutionary distances among the 33 Walker-type partition ATPases encoded by various plasmids or chromosomes. The GenBank accession numbers of respective proteins are shown in brackets. Groups of similar sequences are labeled. pLB1 is shown in boldface. The scale bar indicates 0.05 substitution per site.

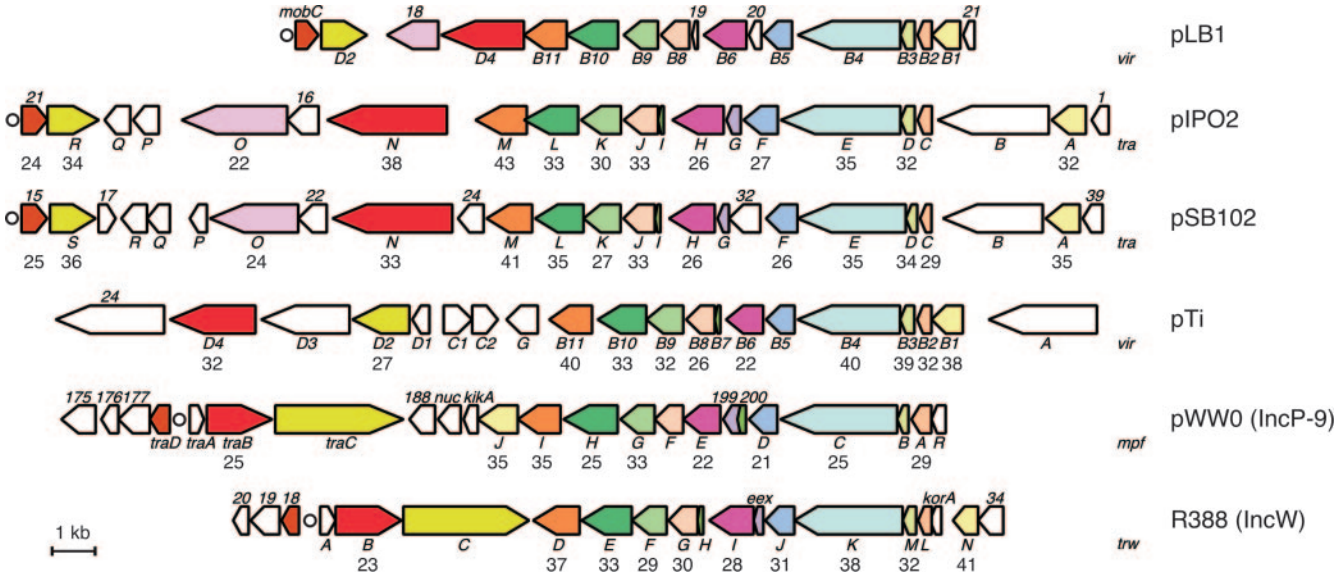

FIG. 5. Genetic organization of the putative transfer region of pLB1 and comparison with related systems. Genes encoding similar functions are displayed in the same color. Circles indicate the putative *oriT* regions of the respective plasmids. The numbers below the genes indicate the percent amino acid identity (>20%) to the corresponding gene product from pLB1. The lengths of TraO homologues are various, because only their C-terminal regions are conserved, which contain a putative functional domain for primase. The GenBank accession numbers of the respective nucleotide sequences are as follows: broad-host-range cryptic plasmid pIPO2, NC_003213; mercury resistance plasmid pSB102, NC_003122; *A. tumefaciens* strain C58 plasmid pTi, NC_003065; catabolic plasmid pWW0, NC_003350; and antibiotic resistance plasmid R388, BR000038.

furthermore conserved in pIPO2 and pSB102, although several CDSs (such as *traB*, *traP*, and *traQ*) are located within the Mpf-related gene clusters on the latter two plasmids. This indicates that the putative transfer region of pLB1 is more compact than those of other plasmids. The VirB7 protein of Ti plasmid and its homologues in other Mpf systems of various plasmids encode outer-membrane-anchored lipoproteins to stabilize VirB9 and its homologues (18, 29). The *virB7* homologues are flanked by *virB6* and *virB8* homologues in various plasmids. Although *orf19* of pLB1 had a size similar to that of other *virB7*-related genes, the *orf19* product showed no similarity with other VirB7-related proteins. The functions of the two genes, *orf20* and *orf21*, in the pLB1 *virB* cluster remain unknown.

Identification of the region essential for replication. The most probable region essential for the replication of pLB1 is the *repA* gene and its upstream region (bp 64908 to 894). To confirm this possibility, this 2-kb region of pLB1 was cloned into a narrow-host-range vector, pK18mob (Km^r), to obtain a minireplicon, pK18OR. Electroporation of UT26 cells with pK18OR gave rise to the Km^r transformants. Southern blot analysis of such transformants with the *repA* gene as a probe confirmed that pK18OR exists as an autonomously replicating unit in UT26 (data not shown). Control experiments using pK18BD3, a pK18mob variant into which another part of pLB1 was inserted, gave no Km^r transformant at all. These results demonstrate that the *repA* gene and its upstream region of pLB1 are functional as a replication unit in UT26.

Conjugal transferability of pLB1. To investigate the self-transmissibility of pLB1, the donor strain UT26DB harboring pLB1Tc, a Tc^r variant of pLB1 with a replication origin of plasmid R6K (R6Kγ*ori*), was separately mated with seven recipient strains: *S. paucimobilis* IAM12578G, *S. meliloti* 1021, *M. loti* MAFF303099, *B. multivorans* ATCC 17616G, *Acidovorax* sp. strain KKS102G, *P. putida* KT2440G, and *E. coli* HB101. The Tc^r transconjugants of IAM12578G, 1021, and MAFF303099 were obtained at frequencies ranging from 10^{-7} to 10^{-5} per donor (Table 3). Gel electrophoresis and Southern blot analysis of plasmids residing in the transconjugants led to the confirmation of the successful transfer of pLB1Tc without any obvious structural changes in the three α-proteobacterial strains (data not shown). Because transconjugants are thought to have no *pir* gene, which is necessary for the replication of plasmids carrying the R6Kγ*ori* sequence, we concluded that pLB1Tc replicated by using the pLB1-derived replication machinery in the transconjugants. On the other hand, no Tc^r transconjugants were obtained when ATCC

TABLE 3. Conjugal transfer of pLB1Tc from UT26DB[a]

Recipient[b]	Conjugation frequency[c]
Sphingomonas paucimobilis IAM12578G	$(4.1 \pm 1.3) \times 10^{-5}$
Sinorhizobium meliloti 1021	$(4.4 \pm 5.1) \times 10^{-7}$
Mesorhizobium loti MAFF303099	$(5.6 \pm 0.6) \times 10^{-6}$
Burkholderia multivorans ATCC 17616G	$<3.3 \times 10^{-9}$
Acidovorax sp. strain KKS102G	$<6.0 \times 10^{-9}$
Pseudomonas putida KT2440G	$<9.3 \times 10^{-9}$
Escherichia coli HB101	$<1.8 \times 10^{-9}$

[a] The donor cells were grown overnight at 30°C in 1/3LB medium containing Tc.

[b] The optical density of the recipient cells at 660 nm was 0.9. Counterselection was made for resistance to Pho in the case of *Mesorhizobium*, to Sm in the cases of *Sinorhizobium* and *Escherichia coli*, and to Gm in the cases of the other recipient strains.

[c] Conjugation frequency is expressed as the number of transconjugants per donor. The data represent the range from three independent experiments.

17616G, KKS102G, KT2440G, and HB101 were used as recipients ($<10^{-9}$ per donor).

DISCUSSION

Our previous observation that the six structural *lin* genes are dispersed on the UT26 genome suggested that each *lin* gene is separately distributed in other environmental bacteria (39). Based on this suggestion, the exogenous plasmid isolation technique was successfully employed in the present study to capture pLB1 that carried only *linB* genes. Since other *lin* genes have been reported to be located on plasmids (7, 39), there may be other indigenous plasmids carrying other *lin* genes, such as pCHQ1, whose self-transmissibility has been demonstrated (39), and it should be possible to isolate them by techniques similar to that used here. However, we have not yet succeeded in isolating plasmids carrying other *lin* genes by using *linA* and *linRED* mutants of UT26 as recipients (data not shown). Endogenous plasmids in UT26 might have inhibited the capturing of exogenous plasmids. Plasmid-free recipient strains are desirable for exogenous plasmid isolation, and the use of such strains will allow us to conduct a more comprehensive analysis of the dynamism of *lin* genes in the natural environment.

The *linB* genes on pLB1 were found to be organized as the IS*6100-linB* cluster. It is known that IS*6100* is closely associated with *lin* genes (32). IS*6100* belongs to the IS*6* family and has been found in various bacterial chromosomes and plasmids (8), such as pZWL0 from *Pseudomonas* sp. strainWBC-3 (33), pOAD2 from *Arthrobacter* sp. strain KI72 (24), and pTET3 from *Corynebacterium glutamicum* LP-6 (59). On these plasmids, two copies of IS*6100* form a composite transposon carrying catabolic or antibiotic resistance genes (24, 33, 59). The organization of the IS*6100-linB* cluster on pLB1 suggests that this cluster also behaves as a composite transposon, giving rise to the potential to exhibit highly efficient dissemination. Considering the fact that the IS*6100-linB* cluster on pLB1 is flanked by divided gene remnants (bp 13082 to 13186 and 19324 to 19866), which might have formerly encoded a resolvase, we believe that the cluster has recently been disseminated into archetypal pLB1.

Almost all putative genes of pLB1 involved in its replication, stable inheritance, and conjugative transfer functions showed low levels of identity to the homologues so far reported, indicating that the basic plasmid mechanisms of pLB1 (replication, stable inheritance, and conjugation) are novel. pLB1 seems to belong to a new incompatibility group of plasmids, at least on the basis of RepA sequence similarity. Our present results provide a novel insight into the evolution of bacterial catabolic plasmids, because most well-characterized catabolic plasmids are categorized into the IncP-1, IncP-2, IncP-7, and IncP-9 groups of *Pseudomonas* origin (10). Interestingly, ParA of pLB1 was found to be related to the chromosomal ParA-like proteins from *P. putida*, *Pseudomonas syringae*, and *Azotobacter vinelandii*, whose genes are solely separated from their canonical *parAB* loci (Fig. 4). The ParA homologues in the *parAB* loci of bacterial genomes are believed to be ATPases essential for the partitioning of replicons and thus have been investigated in recent studies (16), while the ParA homologues separately encoded alone at the other loci have not been studied. The present findings suggest that the latter group of *parA*-like genes have some unknown functions for the partitioning of chromosomes and/or endogenous plasmids.

In the present study, pLB1 was found to be able to be conjugally transferred to α-proteobacterial strains but not to β- or γ-proteobacterial strains. The frequency of conjugation of pLB1Tc to IAM12578G was higher than that to the other two α-proteobacterial strains, indicating that pLB1 is optimally transferable to *Sphingomonadaceae* family strains. To the best of our knowledge, there has been no previous indication of the transfer of catabolic plasmids from sphingomonads to bacterial strains not belonging to the *Sphingomonadaceae* family (5). Therefore, our present results are the first demonstration that a catabolic plasmid from sphingomonads is transferable to strains outside the order.

In conclusion, the features of pLB1 indicate that it is a novel class of plasmids. In the present study we demonstrated that genes for the degradation of recently released xenobiotics can be distributed by a novel plasmid in the natural environment. Further modification of this work will enable us to access novel genetic sinks in the environment and to make significant observations related to bacterial evolution via MGEs.

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