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Efficient Plasmid Mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*

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pIP501 is a streptococcal conjugative plasmid which can be transmitted among numerous gram-positive strains. To identify a minimal mobilization (*mob*) locus of pIP501, DNA fragments of pIP501 were cloned into nonconjugative target plasmids and tested for mobilization by pIP501. We show that nonmobilizable plasmids containing a specific fragment of pIP501 are transmitted at high frequencies between *Lactococcus lactis* subsp. *lactis* strains if transfer (*tra*) functions are provided in *trans* by a pIP501 derivative. Independent transfer of the mobilized plasmid was observed in up to 44% of transconjugants. A 2.2-kb segment containing *mob* was sequenced. This DNA segment is characterized by three palindromes (*palI*, *palII*, and *palIII*) and a 202-amino-acid open reading frame (ORFX) of unknown function. The smallest DNA fragment conferring high frequency mobilization was localized to a 1.0-kb region (extending from pIP501 coordinates 3.60 to 4.60 on the 30.2-kb map) which contains *palI* ($\Delta G = -27$ kcal/mol [ca. −110,000 J/mol]). A 26-bp sequence identical to *palI* is present on pIP501, upstream of the plasmid copy control region. Further homologies with the *palI* sequence are also found with the related *Enterococcus faecalis* conjugative plasmid pAMβ1. The region containing *mob* maps outside the previously described segment mediating pIP501 conjugation. Our results with *recA* strains indicate that the *mob* site is a hot spot for cointegrate formation.

Broad-host-range conjugative (*tra*$^+$) plasmids encoding macrolides-lincosamides-streptogramin B have been found in streptococci of groups A to D. They range from 26 to 33 kb in size and are considered related, since they cross-hybridize and display similar restriction enzyme patterns (see references 12 and 23 for reviews). The 30.2-kb plasmid pIP501 isolated from *Streptococcus agalactiae* (24) confers resistance to macrolides-lincosamides-streptogramin B and to chloramphenicol and has already been used to develop several useful gene-cloning systems (1, 15). It was recently shown that pIP501 replicates by a unidirectional theta-type mechanism (29) like the closely related *tra*$^+$ *Enterococcus faecalis* plasmid pAMβ1 (8, 11).

pIP501 encodes transfer functions which allow its transmission into a wide variety of streptococci (4, 11, 23, 24) and other gram-positive bacteria such as lactococci (27), staphylococci (14, 36), lactobacilli (18, 27, 42), *Listeria* spp. (9), pediococci (20), and *Leuconostoc* spp. (33). The genes required for conjugation (*tra* genes) have been localized to two separate regions (A and B) on pIP501 spanning 16 kb; a separate 4.5-kb region (region C) was reported to influence host range and stability of pIP501 in the recipient strain (25, 26) (see Fig. 1).

The development of a recombinant plasmid transfer system based on pIP501 is potentially useful, particularly since many gram-positive bacteria are poorly transformable (31). A system based on pIP501 has already been developed to mobilize two nonconjugative derivatives of pIP501 (pVA838 and pSA3) by cointegrate formation (34, 38). Cointegrates between homologous incompatible conjugative and target plasmids are first selected in the donor and then transferred as a single species. Resolution occurs in the recipient, and the conjugative plasmid segregates by virtue of its incompatibility.

In this article, we describe the cloning and sequencing of a region (called *mob*) of the conjugative plasmid pIP501 which mediates high-efficiency mobilization when present on a second target plasmid. A genetic analysis of *mob* and flanking regions is presented. Our results show that this region does not contain an origin of transfer (*oriT*) of pIP501, as *mob* plasmids are mobilized via a *recA*-dependent mechanism. Rather, it appears that *mob* is a recombinational hot spot for cointegrate formation between pIP501 and the mobilized plasmids.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains are described in Table 1. *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains were grown on M17 medium (42) in which lactose was replaced by glucose and incubated at 30°C. *Escherichia coli* was grown on Luria-Bertani medium (35) and incubated at 37°C. When present, antibiotics were added at the following concentrations (in micrograms per milliliter) for *L. lactis* subsp. *lactis*: erythromycin, 10; chloramphenicol, 10; streptomycin, 2,000; rifampin, 100; and fusidic acid, 250. For *E. coli*, 150 μg of erythromycin and 100 μg of ampicillin per ml were added.

Plasmid constructions. The plasmids used are described in Table 2. DNA manipulations and gel electrophoresis were carried out as recommended by suppliers or by standard methods (35). Helper plasmid pIL205 (Fig. 1) was used in most crosses. The two target vectors used for *mob* cloning were theta-replicating pIL253 (pIL) (37) and rolling-circle-replicating pG$^+$host5 (pGH) (3, 30). To subclone *mob* (Table 2), a 4.9-kb fragment of the *mob*$^+$ *tra* plasmid pIL::4.9 was excised by using *Sac*I sites and first cloned into the *E. coli* pBluescript (pBS) KS+ plasmid (Stratagene, La Jolla, Calif.), resulting in pBS::4.9. *Sal*I-linearized pIL was then inserted at the unique *Sal*I site of pBS::4.9, resulting in pIL-BS::4.9. A hybrid plasmid consisting only of pIL and

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TABLE 1. Bacterial strains

Strain	Description[a]	Reference
L. lactis subsp. *lactis*		
IL1403	WT, plasmidless, R^-M^-	10
IL1441	Sm^r mutant of IL1403	10
IL1419	Fus^r Rif^r mutant of IL1403	27
L. lactis subsp. *cremoris*		
MG1363	WT, plasmidless, R^-M^-	16
MG1614	Sm^r Rif^r mutant of MG1363	16
VE1122	*rec* derivative of MG1363	13
E. coli TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F'(*traD36 proAB*$^+$ *lacI lacZ*ΔM15)	19

[a] WT, wild type; Sm^r, Fus^r, and Rif^r; resistance to streptomycin, fusidic acid, and rifampin, respectively.

pBS KS+ (pIL-BS) served as a negative control for mobilization tests. Nested deletions were generated by the Stratagene ExoIII-mung bean nuclease protocol. One series was performed on pBS::4.9 digested by *Pst*I and *Not*I (ExoIII-sensitive end), resulting in five deletion derivatives. Each subclone was linearized by *Sal*I and joined to *Sal*I-linearized pIL; the resulting hybrid plasmid was transferred into an *L. lactis* subsp. *lactis* strain containing pIL205 for mobilization tests. Deletion derivatives of pBS::2.2 were obtained with ExoIII-mung bean nuclease after *Sac*I and *Sma*I (ExoIII-sensitive end) or *Kpn*I and *Xho*I (ExoIII-sensitive end) treatment.

Bacterial transformation. Competent-cell transformation of *E. coli* cells was performed as described elsewhere (35). *L. lactis* subsp. *lactis* strains were electrotransformed as described previously (27) and modified according to the method described by Holo and Nes (22): M17-glucose broth was supplemented with 0.2 M sucrose and 2% glycine instead of DL-threonine. Electroporation was carried out with a Gene Pulser and a Pulse Controller apparatus (Bio-Rad Laboratories, Richmond, Calif.). As *L. lactis* subsp. *lactis* IL1403 is readily transformed by electroporation (27), plasmids to be tested for *mob* activity were constructed (with either pIL-BS or pGH) in *E. coli* and then transferred to IL1403 to test for mobilization.

Determination of conjugation and mobilization frequencies. Matings were performed on solid surfaces as described previously (27). All matings were performed on agar containing 100 μg of DNase I (Sigma) per ml. To assay *mob*

TABLE 2. Plasmids

Plasmid	Description[a]	Source or reference(s)
Vectors		
pIL	Em^r, 5 kb, high	37
pBS	Ap^r, 2.9 kb, high	Stratagene
pGH	Em^r, 5.2 kb, high	3, 30
Helpers		
pIP501	Em^r Cm^r *tra*$^+$, 30.2 kb, low	24
pIL205[b]	Cm^r *tra*$^+$ 27.3 kb, low	This study
pVA1700	Cm^r *tra*$^+$, 26.5 kb, low	25
pVA1701	Km^r *tra*$^+$, 29.7 kb, low	25
pVA1702	Km^r *tra*$^+$, 25.2 kb, low	25
Constructions[c]		
pIL::7.6	Em^r *mob*$^+$, 12.6 kb, high; pIL carrying a 7.6-kb fragment resulting from partial *Sau*3AI digestion of pIL205	This study
pIL::4.9	Em^r *mob*$^+$, 9.9 kb, high; pIL carrying a 4.9-kb *Hin*dIII fragment of pIL205	This study
pBS::4.9	Em^r Ap^r; *Sac*I-linearized pBS joined to a 4.9-kb *Sac*I fragment of pIL::4.9	This study
pIL-BS	$Em^r Ap^r$; *Sal*I-linearized pBS joined to *Sal*I-linearized pIL	This study
pIL-BS::4.9	Em^r Ap^r; *Sal*I-linearized pBS::4.9 joined to *Sal*I-linearized pIL	This study
pBS::3.0	Ap^r; *Hin*dIII-linearized pBS joined to a *Hin*dIII fragment of pBS::4.9	This study
pIL-BS::3.0	Em^r; Ap^r; *Sal*I-linearized pBS::3.2 joined to *Sal*I-linearized pIL	This study
pBS::2.7	Ap^r; *Spe*I-*Hin*dII-linearized pBS joined to a 2.7-kb *Spe*I-*Hin*dII fragment of pBS::4.9	This study
pIL-BS::2.7	Em^r Ap^r; *Sal*I-linearized pBS::2.7 joined to *Sal*I-linearized pIL	This study
pBS::2.2	Ap^r; ExoIII deletant of *Pst*I-*Not*I of pBS::4.9 carrying a 2.2-kb fragment	This study
pIL-BS::2.2[d]	Em^r Ap^r; *Sal*I-linearized pBS::2.2 joined to *Sal*I-linearized pIL	This study
pBS::1.5	Ap^r; pBS::2.2 lacking a 750-bp *Spe*I fragment	This study
pIL-BS::1.5	Em^r Ap^r; *Sal*I-linearized pBS::1.5 joined to *Sal*I linearized pIL	This study
pGH::2.2[d]	Em^r; *Eco*RI-linearized pGH joined to a 2.2-kb *Eco*RI fragment of pBS::2.2	This study
pGH::1.5	Em^r; pGH::2.2_2 lacking a 750-bp *Spe*I-*Eco*RI fragment	This study
pGH::1.0	Em^r; pGH::2.2_2 lacking a 1,181-bp *Sna*BI-*Eco*RI fragment	This study

[a] High and low, plasmid copy numbers of 50 to 100 and 1 to 10, respectively; Em^r, Ap^r, Cm^r, and Km^r, resistance to erythromycin, ampicillin, chloramphenicol, and kanamycin, respectively.
[b] See Fig. 1.
[c] *mob* activities of subclones are given in Fig. 2.
[d] Cloned in both orientations.

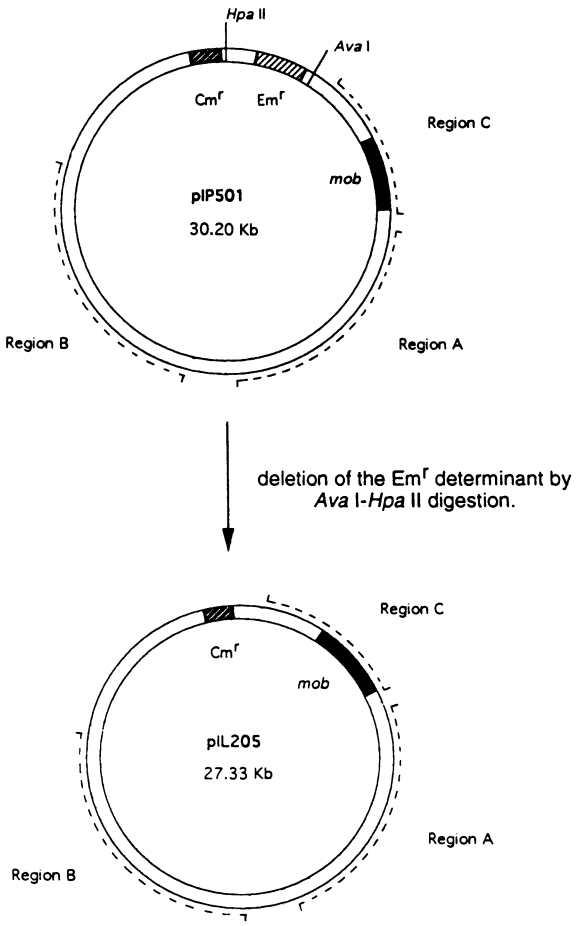

FIG. 1. Construction of the *tra*$^+$ *mob*$^+$ helper plasmid pIL205. Regions A, B, and C, required for conjugative transfer, as defined by Krah and Macrina (25, 26) (see Discussion) are stippled. The 2.2-kb sequenced *mob* fragment (solid bar) maps in region C. The Em^r determinant of pIP501 was deleted by *Ava*I-*Hpa*II digestion to construct the helper plasmid pIL205.

activity, the donor strain contained both the *tra*$^+$ helper plasmid (pIL205) and a test plasmid carrying the putative *mob* fragment. The conjugation frequency of transfer of pIL205 (f_{tra}) was expressed as the number of chloramphenicol-resistant (Cm^r) transconjugants per recipient strain, and the mobilization frequency of transfer of the *mob* plasmid (f_{mob}) was expressed as the number of erythromycin-resistant (Em^r) transconjugants per recipient. Cotransfer frequencies of helper and target plasmids were determined by counting Cm^r clones among at least 100 Em^r transconjugants picked on erythromycin and chloramphenicol plates.

Examination of plasmid content. Plasmid preparations (2) or whole-cell lysates (32) were separated by agarose gel electrophoresis. Gels were analyzed by Southern hybridization (39) using [α-^{32}P]dCTP nick-translated (Amersham Corp., Arlington Heights, Ill.) plasmid DNA probes.

DNA sequence analysis. Double-stranded DNA was prepared with Qiagen columns (Diagen, Düsseldorf, Germany). Dideoxynucleotide chain termination DNA-sequencing reactions were carried out on double-stranded DNA with the *Taq* Dye Primer Cycle Sequencing kit (Applied Biosystems, San Jose, Calif.) using a Perkin Elmer polymerase chain reaction apparatus. Sequencing reactions were primed with fluorescent oligonucleotides and analyzed on the 370A DNA sequencer (Applied Biosystems). The reported sequences were determined on both strands.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number is L07895.

TABLE 3. Transfer frequencies[a] of *mob*-containing plasmids between *L. lactis* subsp. *lactis* strains

Cross	Plasmid content of donor strain[b]	f_{tra}[c]	f_{mob}[c,d]	% of cotransfer[e]
1	pIP501 (2)	1.0×10^{-2}		NT
2	pIL205 (17)	7.0×10^{-3}		NT
3	pIL (1)	$<1.0 \times 10^{-9}$		NT
4	pIL::7.6 (1)	$<2.1 \times 10^{-9}$		NT
5	pVA1700 (2)	2×10^{-1}		NT
6	pIL205, pIL (2)		3.3×10^{-7} (−)	NT
7	pIL205, pIL-BS (1)		1.1×10^{-6} (−)	NT
8	pIL205, pIL::7.6 (2)		4.4×10^{-3} (+)	85
9	pIL205, pIL::4.9 (2)		4.9×10^{-3} (+)	80
10	pIL205, pIL-BS::4.9 (1)		7.8×10^{-3} (+)	NT
11	pIL205, pIL-BS::2.7 (2)		6.1×10^{-7} (−)	NT
12	pIL205, pIL-BS::3.0 (1)		2×10^{-6} (−)	NT
13	pIL205, pIL-BS::2.2[f] (8)		5.2×10^{-3} (+)	100
14	pIL205, pIL-BS::1.5[g] (2)		2.8×10^{-4} (+)	NT
15	pIL205, pIL-BS::1.5[h] (1)		5.8×10^{-7} (−)	NT
16	pIL205, pGH (2)		1.8×10^{-8} (−)	NT
17	pIL205, pGH::2.2 (4)		2.6×10^{-5} (+)	99
18	pIL205, pGH::1.5 (4)		4.2×10^{-5} (+)	56
19	pIL205, pGH::1.0 (2)		9.0×10^{-5} (+)	99
20	pVA1700, pGH::2.2 (2)		1.2×10^{-9} (−)	NT

[a] f_{tra} is used for donor strains containing only one plasmid; f_{mob} is used for donor strains containing the helper plasmid pIL205 (or pVA1700) and a second plasmid for which mobilization was tested.
[b] The number of determinations is given in parentheses.
[c] Number of transconjugants per input recipient cell.
[d] *mob* phenotype conferred by the *mob* fragment is given in parentheses. Note that pIL-BS derivatives showed somewhat higher overall mobilization (+) and background (−) activities than pGH derivatives.
[e] Of helper and target plasmid markers; determined by replica plating 100 Em^r transconjugants on chloramphenicol-containing plates. NT, not tested.
[f] The 2.2-kb insert was tested in both orientations, giving comparable results.
[g] The putative ORFX transcript is in the same direction as the pIL replication fork.
[h] The putative ORFX transcript converges with the pIL replication fork.

RESULTS

General strategy for cloning the *mob* region of pIP501. DNA fragments of pIP501 were subcloned onto a nonmobilizable vector and tested for efficient mobilization in the presence of a conjugative helper plasmid. The helper plasmid pIL205, a Cm^r and erythromycin-sensitive (Em^s) derivative of plasmid pIP501 (Fig. 1), exhibits the same high f_{tra} as pIP501 (27) in lactococcal crosses (Table 3, crosses 1 and 2). pIL (37) and pGH (3) are Em^r lactococcal vectors which are nonconjugative and nonmobilizable (Table 3, crosses 3 and 16) and were used as the target plasmids for tests of *mob* activity.

A 2.2-kb DNA fragment of pIP501 is sufficient to promote high-frequency mobilization of a target plasmid. *Sau*3A partial fragments (ranging from 2 to 10 kb) of conjugative plasmid pIL205 were joined to the unique *Bam*HI site of pIL and transformed into *L. lactis* subsp. *lactis* IL1403 containing pIL205. The transformants were used as donors in matings with the plasmidless Sm^r recipient strain IL1441 (10), selecting for Em^r and Cm^r. Among 100 transconjugants, one was chloramphenicol sensitive (Cm^s), indicating that it

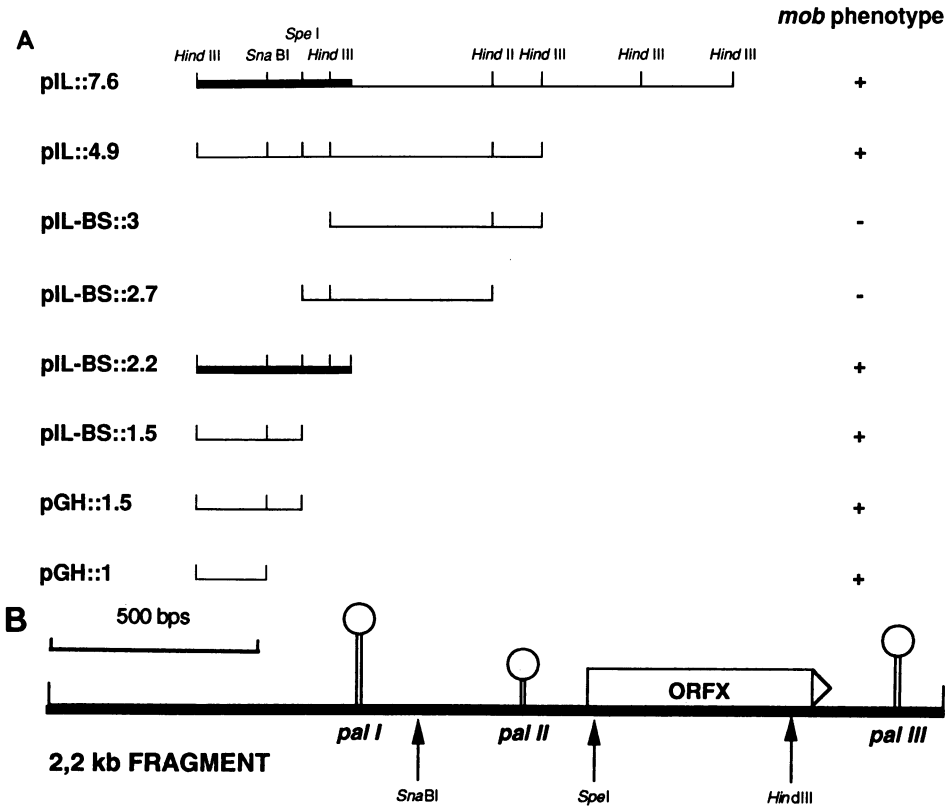

FIG. 2. Deletion analysis and organization of the *mob* region of pIP501. (A) The pIP501 DNA fragments carried by pIL and pGH vectors are shown below the restriction map of the 7.6-kb *mob* fragment. *mob* activity of deletion derivatives of the *tra* plasmid pIL::7.6 was determined during matings between *L. lactis* subsp. *lactis* strains. Solid bar, the sequenced region. (B) An expanded diagram of the 2.2-kb *mob*$^+$ fragment of pIP501. Palindromic sequences *palI*, *palII*, and *palIII*, which have stem lengths of 21, 8, and 15 bp, respectively, and a potential open reading frame (ORFX) of 202 amino acids are indicated.

did not contain the helper plasmid. It contained a single plasmid consisting of pIL with a 7.6-kb insert (pIL::7.6). pIL::7.6 was nonconjugative (Table 3, cross 4). However, in the presence of pIL205, it was transferred at frequencies close to those found for pIL205 (Table 3, crosses 2 and 8). Furthermore, 20% of Em^r and streptomycin-resistant (Sm^r) transconjugants contained only pIL::7.6, suggesting that pIL::7.6 is mobilized as a discrete species or that transiently formed cointegrates can be immediately resolved and segregated in the recipient. We consider that pIL::7.6 contains a *mob* site. Restriction sites identified by mapping of pIL::7.6 were used to further subclone *mob* (Fig. 2A). One subclone, plasmid pIL::4.9 (Fig. 2A), is mobilized by pIL205 as efficiently as pIL::7.6 (Table 3, cross 9).

Since transformation with ligated DNA in *L. lactis* subsp. *lactis* is inefficient, further *mob* subclonings were performed in *E. coli* by using a pIL-BS hybrid as an *E. coli-L. lactis* shuttle vector. The presence of pBS sequences did not significantly affect f_{mob} (Table 3, cross 7). *mob* was subsequently localized to a 2.2-kb DNA fragment (Fig. 2A). Transfer frequencies of plasmids pIL-BS containing the 2.2-kb *mob* fragment in either orientation were comparable (Table 3, cross 13). However, in contrast to results with larger *mob* cloned segments, all transconjugants of pIL-BS::2.2 contained the helper plasmid.

DNA sequence and structural analysis of the 2.2-kb *mob* fragment. The nucleotide sequence of the 2.2-kb fragment was determined for both strands (Fig. 3). The GC content of the fragment is 34%, similar to the 34.5% GC content of *S. agalactiae*, the native plasmid host. Both strands of the *mob* sequence were submitted to a computer search for open reading frames greater than 100 amino acids. The search revealed a single open reading frame (ORFX) of 202 amino acids, unrelated to any protein sequences in GenBank (Fig. 2B and 3). No ribosome-binding site preceding the potential TTG or ATG start site was found. Gram-positive consensus RNA polymerase-binding sites upstream of ORFX were also not found.

Three perfect palindromic sequences, designated *palI*, *palII*, and *palIII* (having 21-, 8-, and 15-bp stems, respectively) are present on the *mob* segment (Fig. 2B and 3). *palII* and *palIII* flank ORFX and could modulate its expression. The largest palindrome, *palI*, lies 689 bp upstream of the ORFX coding region and has a free energy of −27 kcal/mol [ca. −110,000 J/mol]) (43). A 26-bp sequence identical to part of *palI* is present upstream of the copy control region of pIP501 (5) (Fig. 4). Furthermore, the closely related *E. faecalis* conjugative plasmid pAMβ1 (40) also contains a sequence (*palIII'*) highly homologous to *palI* (Fig. 4). Additional sequence comparison revealed that the sequence between *palI* and *palII* is also present on pAMβ1, in a separate region encoding the plasmid topoisomerase (1a).

Orientation-dependent mobilization by subfragments. Plasmids containing the 2.2-kb fragment in either orientation were *mob*$^+$ (Table 3). *mob* was finally localized to 1.5- and 1.0-kb fragments (see below). However, transfer of pIL

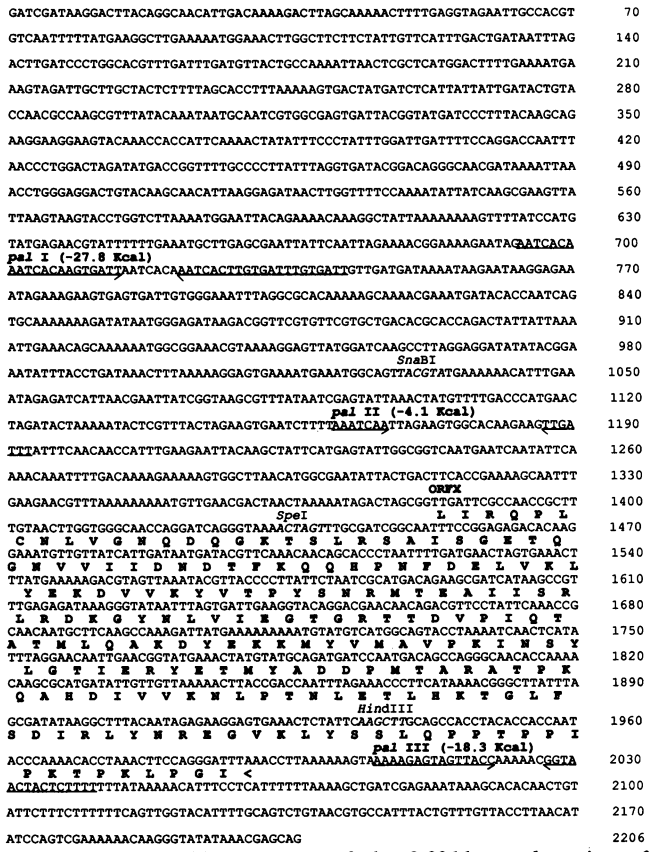

FIG. 3. Nucleotide sequence of the 2,206-bp *mob* region of pIP501. The predicted amino acid sequence of ORFX is indicated below the corresponding DNA sequence. *palI*, *palII*, and *palIII* are indicated (half-barbed arrows). Their estimated free energies are indicated in parentheses (1 cal = 4.184 J).

plasmids containing these smaller fragments was efficient for just one orientation of the *mob* fragment (Table 3, compare crosses 14 and 15). Analysis of the nonmobilizable plasmids revealed that the *mob* fragments carry a truncated ORFX and might, therefore, produce a nonterminated transcript. This putative transcript is convergent with the pIL253 replication fork (8) and might affect *mob* activity.

mob can mobilize rolling-circle plasmid. The first target vector used to clone *mob* is a derivative of the *tra*$^+$ *E. faecalis* plasmid pAMβ1 (10). It was recently reported that pAMβ1 and pIP501 are closely related replicons (6, 29). We, therefore, continued analysis of the *mob* region on pGH (3, 30), a rolling-circle plasmid totally unrelated to pIP501. The 2.2-kb *mob* fragment cloned into pGH was active (Table 3, cross 17). The 1,457-bp *Eco*RI-*Spe*I fragment was inserted into pGH and also gave a *mob*$^+$ phenotype (Table 3, cross 18), confirming that only *palI* and *palII* are required for transfer. pGH::1.5 showed about 40% independent transfer (Table 3, cross 18).

The 1,042-bp *Eco*RI-*Sna*BI fragment also mediated mobilization, indicating that *palII* is not necessary for transfer (Table 3, cross 19). However, 99% of cotransfer was observed with these crosses.

Plasmid profiles in donor and recipient strains. Plasmid contents of donor strains containing pIL205 and pGH::2.2 and of recipients after conjugational transfer were examined. Whole-cell lysates separated on agarose gels were analyzed by Southern hybridization using a segment of pGH::2.2 as a probe (Fig. 5). The plasmid profile of pGH::2.2 was typical in all strains except one Cm^r Em^r recipient, expected to contain both plasmids, for which only a faint, slowly migrating band, corresponding to a DNA species larger than the helper, was observed. Overexposure of the gel revealed that monomeric pGH::2.2 was present. Donors and recipients in which pGH contained smaller *mob* fragments also gave profiles similar to those obtained with pGH::2.2 (data not shown). These results indicate that interaction between the helper and *mob* plasmids probably occurs and that the product, possibly a cointegrate, may not always be resolved.

Localization of the *mob* fragment on the pIP501 map. Two regions (A and B) of pIP501 were reported to be sufficient for conjugal transfer in *E. faecalis* recipient strains (25). A stability locus was mapped in a third region, region C; this region was proposed to be required for establishment of transconjugants in non-*E. faecalis* strains such as *Streptococcus sanguis* without being involved in actual transfer (Fig. 1) (26). Comparison of restriction maps of *mob*$^+$ plasmids and the parental plasmid pIP501 showed that the 2.2-kb *mob* fragment maps between coordinates 3.6 and 5.8 kb of the published pIP501 map (15), within region C (Fig. 1). This localization was confirmed by Southern hybridizations (39) of two conjugative derivatives of pIP501, pVA1701 (containing regions A, B, and C) and pVA1702 (containing regions A and B) (25, 26), using the 2.2-kb *Eco*RI fragment of pBS::2.2 as a DNA probe. Hybridization with pIP501 and pVA1701, but not with pVA1702, confirmed that the 2.2-kb fragment maps within the C region of pIP501 (not shown).

Effect of *recA* on transfer of *mob* plasmids. To determine the mechanism of transfer of the *mob* plasmids, two kinds of experiments were performed. First, we tried to mobilize pGH::2.2 by the helper plasmid pVA1700, lacking the *mob* region. Plasmid pGH::2.2 was not mobilized (Table 3, cross 20), suggesting that homology between target and helper plasmids is necessary to mediate mobilization. It is notable that pVA1700 conjugates at the same high frequencies as pIL205, suggesting that the C region is not necessary for conjugal transfer between lactococci (Table 3, cross 5).

Second, pGH::2.2 and pIL205 were introduced into *L. lactis* subsp. *cremoris* VE1122, a *recA* derivative of *L. lactis* subsp. *cremoris* MG1363 (13), and into the isogenic *rec*$^+$ strain MG1363 (16). Mobilization frequencies were deter-

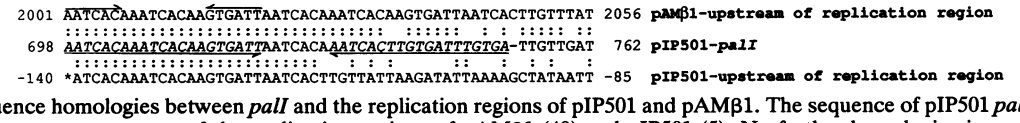

FIG. 4. Sequence homologies between *palI* and the replication regions of pIP501 and pAMβ1. The sequence of pIP501 *palI* is aligned with DNA sequences present upstream of the replication regions of pAMβ1 (40) and pIP501 (5). No further homologies in sequenced flanking regions were found. pIP501 *palI*, pAMβ1 *palIII'*, and a secondary palindrome present in all three sequences are indicated (half-barbed arrows). A break in the alignment made to maximize similarity is indicated by a dash. The DNA sequence data are not available for the region beyond the point marked by the asterisk. Pairwise identities of bases are indicated (:).

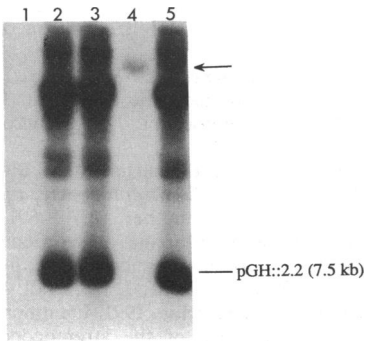

FIG. 5. Detection of *mob* plasmids in conjugational crosses. Whole-cell lysates prepared from *L. lactis* overnight cultures were separated on a 0.7% agarose gel. Southern hybridizations were performed by using as a probe a pBR322 DNA fragment present on *mob* plasmid pGH::2.2. This probe reveals only pGH::2.2 and not the helper plasmid pIL205. Lane 1, IL1403; lane 2, plasmid pGH::2.2 in IL1403; lane 3, pGH::2.2 and helper plasmid pIL205 in the IL1403 donor strain; lane 4, Em^r Cm^r transconjugants of the IL1441 recipient strain; lane 5, Em^r Cm^s transconjugant of the IL1441 recipient strain. A slowly migrating species hybridizing to pGH::2.2 in lane 4 is indicated (arrow). The strong hybridization signals just below the level of the arrow in lanes 2, 3, and 5 correspond to high-molecular-weight linear multimers of pGH::2.2 (21).

mined by using the above strains as donors and the isogenic *L. lactis* subsp. *cremoris* MG1614 as the recipient strain (16). The conjugative helper plasmid pIL205 transferred efficiently from both rec^+ and *recA* donor strains ($f_{tra} = 2 \times 10^{-4}$ and 2×10^{-3}, respectively). In contrast, while pGH::2.2 was efficiently mobilized from the rec^+ donor strain ($f_{mob} = 4 \times 10^{-5}$), no efficient mobilization of pGH::2.2 from the *recA* donor strain was observed ($f_{mob} = 6.4 \times 10^{-8}$).

Surprisingly, a larger *mob* fragment, present on pIL::4.9 (Fig. 2A), was efficiently mobilized from the *recA* donor strain (the additional 2.7 kb did not promote mobilization). The transfer frequency (2×10^{-5}) was comparable to that obtained with the wild-type donor strain (3×10^{-5}). Taken together, these results suggest that efficient recombination must occur in the donor strain to get high-frequency *mob*-mediated transfer. This recombination step may be aided by functions encoded on the 2.7-kb DNA segment adjacent to *mob*.

DISCUSSION

***mob* region of pIP501.** We localized a DNA segment of pIP501 (mob^+) which, when cloned onto a nontransmissible plasmid, confers high-frequency plasmid mobilization from a donor strain containing a tra^+ derivative of pIP501. *mob* activity was demonstrated on both theta and rolling-circle plasmids. The sequence of a 2.2-kb *mob*-containing fragment reveals a 202-amino-acid open reading frame (ORFX) flanked by two small palindromes plus a third highly palindromic region (*palI*). Fragments smaller than 2.2 kb could also promote mobilization, with the smallest fragment being 1 kb long and containing only *palI*.

A 26-bp sequence identical to part of *palI* is present elsewhere on pIP501 (5). Interestingly, these two sequences appear to flank the entire transfer region. Homologies with *palI* are also present upstream of the replication region of the closely related conjugative plasmid pAMβ1 (40). Further sequence similarities between *mob* and pAMβ1 are found in a region near the topoisomerase gene.

The mechanism of mobilization. Our data point strongly to the hypothesis that *mob* acts by cointegrate formation and is not an *oriT* sequence as usually defined (45). First, recipient strains receiving both plasmid markers contained the plasmids in cointegrate form (Fig. 5) (monomeric *mob* plasmids were also present but in low amounts). Second, no transfer was observed if the helper and the target were totally nonhomologous. Third, no transfer of pGH::2.2 from *recA* donor strains was observed.

Although these results strongly suggest that homology-mediated cointegrate formation must occur in the donor, our data also indicate that this is a site-specific process, since many pIP501 fragments tested did not confer a *mob* phenotype (Table 3). Possibly, the *mob* fragment is a recombinational hot spot, a hypothesis which would explain the elevated transfer frequencies. Palindromic regions have been previously observed to stimulate illegitimate recombination (7) and have also been identified as sites of specific recombination (17). If recombination and resolution are elevated at the *mob* site, this could explain the relatively high frequency of independently transferred target plasmids (up to 44% with certain *mob* fragments); a cointegrate would be formed in the donor and resolved upon entering the recipient, with concomitant loss of the mobilizing plasmid. It is notable that the presence of the 4.9-kb fragment containing *mob* (Fig. 2A) on the target plasmid conferred mobilization from the *recA* donor strain. Since the additional 2.7 kb of DNA did not mediate transfer alone, we suppose that it contains a recombination function active in *cis* which helps cointegrates to form. This type of phenomenon was described for pT181 and pE194, in which a site-specific recombination site, RS_A, is the target of an adjacent protein, Pre (17).

The transfer functions of pIP501 were previously mapped to three distinct regions, A, B, and C (25, 26). A and B were sufficient to mediate plasmid conjugation between *E. faecalis* strains, while C, in which *mob* is mapped, was proposed to be involved in stable establishment of pIP501 in a broad spectrum of non-*E. faecalis* recipient strains (26). How can these data be reconciled with our present results? One explanation for the stability phenotype of the C region is that *mob*, or *palI*, is a site for multimer formation and resolution (in addition to the *res* site at which multimers are depolymerized by resolvase [41]). In our model, the stability phenotype could be due to the resolution of multimers in the C region.

***mob*-containing plasmids as new tools for plasmid transfer.** The ability of *mob* to mobilize plasmids can be exploited to introduce genetic information into nontransformable hosts. Since previous experiments show that pIP501 can be transferred to nontransformable *Lactobacillus* strains (28), it is likely that *mob*-containing plasmids can be mobilized in these strains. Our test system for mobilization employed two broad-host-range cloning vectors. Such tools should facilitate genetic analyses of a wide variety of strains.

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