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Characterization of Multiple Regions Involved in Replication and Mobilization of Plasmid pNZ4000 Coding for Exopolysaccharide Production in *Lactococcus lactis*

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We characterized the regions involved in replication and mobilization of the 40-kb plasmid pNZ4000, encoding exopolysaccharide (EPS) production in *Lactococcus lactis* NIZO B40. The plasmid contains four highly conserved replication regions with homologous *rep* genes (*repB1*, *repB2*, *repB3*, and *repB4*) that belong to the lactococcal theta replicon family. Subcloning of each replicon individually showed that all are functional and compatible in *L. lactis*. Plasmid pNZ4000 and genetically labeled derivatives could be transferred to different *L. lactis* strains by conjugation, and pNZ4000 was shown to be a mobilization plasmid. Two regions involved in mobilization were identified near two of the replicons; both included an *oriT* sequence rich in inverted repeats. Conjugative mobilization of the nonmobilizable plasmid pNZ124 was promoted by either one of these *oriT* sequences, demonstrating their functionality. One *oriT* sequence was followed by a *mobA* gene, coding for a *trans*-acting protein, which increased the frequency of conjugative transfer 100-fold. The predicted MobA protein and the *oriT* sequences show protein and nucleotide similarity, respectively, with the relaxase and with the inverted repeat and *nic* site of the *oriT* from the *Escherichia coli* plasmid R64. The presence on pNZ4000 of four functional replicons, two *oriT* sequences, and several insertion sequence-like elements strongly suggests that this EPS plasmid is a naturally occurring cointegrate.

Lactococci are known to harbor conjugative plasmids that are used for industrial strain improvement since they encode important metabolic traits such as lactose fermentation, protease activity, bacteriophage resistance, or production of exopolysaccharide (EPS). Therefore, these plasmids are studied for their functional properties as well as for their mode of replication and transfer capacities. Two different mechanisms of replication are known to operate in *Lactococcus lactis*: rolling circle and theta replication. Rolling circle replication seems to be restricted to relatively small lactococcal plasmids with cryptic functions (19). Two of these, the related promiscuous plasmids pWV01 and pSH71, have been developed into widely used cloning and expression vectors (6). The replication regions of several theta replicating lactococcal plasmids that encode metabolic functions have been analyzed, and all are members of a family of highly related, compatible theta replicons as first identified for plasmid pCI305 (17, 35). They all contain a homologous *repB* gene encoding the replication protein. The conserved region upstream of *repB* is likely to include the origin of replication and also contains 22-bp repeats which have a replicon-specific regulatory role in plasmid replication and an inverted repeat overlapping the *repB* promoter which is a RepB binding site (7).

The capacity for conjugal transfer is an important characteristic of some lactococcal plasmids. Self-transmissible conjugative plasmids have the ability to form effective cell-to-cell contact, while mobilization plasmids are able only to prepare their DNA for transfer (36). The conjugation process in gram-negative bacteria is initiated at the origin of transfer (*oriT*) by the formation of a relaxosome, usually containing a relaxase and accessory DNA binding proteins. The relaxase catalyzes the cleavage of a specific phosphodiester bond at the *nic* site in the *oriT*, after which it is covalently linked to the 5′ end of the cleaved strand through a tyrosyl residue. Single-stranded DNA is transferred to the recipient cell and subsequently ligated through the cleaving-joining activity of the relaxase, resembling the process of leading-strand replication by rolling circle replication (20). To date, very little is known about genes required for conjugation in lactococci and other gram-positive bacteria (12). The chromosomally encoded sex factor and the homologous conjugative element pRS01 of *L. lactis* 712 and ML3, respectively, can mediate a high-frequency transfer of nonconjugative lactose plasmids and confer a cell aggregation (Clu) phenotype (1, 10). The sex factor *cluA* gene encodes a protein that is involved in cell aggregation during conjugation (14). On the bacteriophage resistance plasmid pCI528, a 2-kb region involved in conjugative mobilization has been identified. It contains a putative *oriT* and a *mobA* gene which is predicted to encode a protein involved in mobilization (24).

While EPS production by lactococci has long been known to be a plasmid-encoded trait, it was only recently established that structural genes involved in EPS biosynthesis are located on these plasmids (37, 38). The best-characterized EPS plasmid to date is the 40-kb pNZ4000 from *L. lactis* NIZO B40, which contains a 12-kb gene cluster encoding EPS biosynthesis (37). Furthermore, it contains multiple replicons, since we were able to separate pNZ4000 in two *Xho*I-*Sph*I fragments that upon labeling with an erythromycin resistance (Ery^r) marker could each replicate in *L. lactis* (37). In this study, we report the identification and characterization of the regions involved in plasmid replication and mobilization of this EPS plasmid. Plasmid pNZ4000 contains four functional replicons and two regions involved in mobilization; one codes for an active *trans*-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
E. coli DH5α		16
L. lactis NIZO B40	Lac$^+$ Eps$^+$ multiplasmid strain harboring pNZ4000	37
L. lactis MG1363	Plasmid free	11
L. lactis MG1614	Rif^r Str^r, plasmid free	11
L. lactis IL1403	Plasmid free	3
L. lactis NZ4010	Rif^r Str^r Eps$^+$, MG1614 harboring pNZ4000	37
Plasmids		
pCI182	Tet^r, 8.0-kb pBR322 derivative carrying the Tn*919 tetM* gene	18
pUC19Ery	Ery^r, 3.8-kb pUC19 carrying the Ery^r gene of pIL253	37
pUC18Ery	Ery^r, 3.8-kb pUC18 carrying the Ery^r gene of pIL253	37
pNZ4000	40-kb plasmid encoding EPS production	37
pNZ4001	Ery^r, 7.1-kb derivative of pUC19Ery carrying *repB1*	This study
pNZ4002	Ery^r, 7.2-kb derivative of pUC19Ery carrying *repB2*	This study
pNZ4003	Ery^r, 7.5-kb derivative of pUC18Ery carrying *repB3*	This study
pNZ4004	Ery^r, 6.7-kb derivative of pUC19Ery carrying *repB4*	This study
pNZ4006	Ery^r, 5.0-kb derivative of pUC19Ery carrying a 1.2-kb *Eco*RI-*Xba*I fragment of pNZ4000 with *orfD1*	This study
pNZ4007	Ery^r, 7.9-kb derivative of pUC19Ery carrying a 4.1-kb *Eco*RI-*Xba*I fragment of pNZ4000 with *orfD2*	This study
pNZ4010	Ery^r, 45-kb, pNZ4000 containing an integrated copy of pNZ4006	This study
pNZ4017	Ery^r, 48-kb, pNZ4000 containing an integrated copy of pNZ4007	This study
pNZ124	Cm^r, 2.8-kb pSH71 replicon	28
pNZ4021	Cm^r, 3.6-kb derivative of pNZ124 carrying *oriT1*	This study
pNZ4022	Cm^r, 3.4-kb derivative of pNZ124 carrying *oriT2*	This study
pNZ4023	Cm^r, 5.8-kb derivative of pNZ124 carrying *oriT1* and *mobA*	This study
pNZ4025	Tet^r, 10.2-kb derivative of pUC18 carrying *repB1* and *tetM*	This study
pNZ4026	Tet^r, 11.4-kb derivative of pCI182 carrying *repB2*	This study
pNZ4027	Tet^r, 10.9-kb derivative of pCI182 carrying *repB4*	This study

[a] Lac$^+$, lactose fermenting; Eps$^+$, EPS producing; Rif^r, rifampin resistant; Str^r, streptomycin resistant; Tet^r, tetracycline resistant; Ery^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

acting mobilization protein, and both contain a *cis*-acting *oriT* region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in L-broth-based medium at 37°C (33). *L. lactis* was grown at 30°C in M17 broth (Difco Laboratories) supplemented with 0.5% glucose (GM17). If appropriate, the media contained chloramphenicol (10 μg/ml), erythromycin (10 μg/ml for *L. lactis* and 150 μg/ml for *E. coli*), rifampin (50 μg/ml), streptomycin (100 μg/ml), tetracycline (25 μg/ml), or ampicillin (100 μg/ml).

DNA isolation, manipulation, and transfer. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook et al. (33). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with Qiagen columns as instructed by the manufacturer. Isolation and transformation of *L. lactis* plasmid DNA were performed as previously described (5). For whole-cell lysates of *L. lactis*, 1.5 ml of a late-log-phase culture was harvested and suspended in 100 μl of a buffer containing 30 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 25% sucrose, 10 μg of lysozyme ml^{-1}, and 0.1 mg of RNase ml^{-1}. This suspension was incubated at 37°C for 30 min. Lysis was achieved by addition of 100 μl of 2% sodium dodecyl sulfate and vortexing at top speed for 1 min, after which the lysate was treated with 20 μg of proteinase K ml^{-1} at 37°C for 30 min. Conjugation was performed by filter matings as described before (37). The ratio of donor and recipient was 2:1.

Nucleotide sequence analysis. Automatic double-stranded DNA sequence analysis was performed on both strands with an ALF DNA sequencer (Pharmacia Biotech). Sequencing reactions, performed with an AutoRead sequencing kit, were initiated by using fluorescein-labeled universal and reverse primers and continued with synthetic primers in combination with fluorescein-15-dATP, following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed using the PC/GENE program (version 6.70; IntelliGenetics). The GenBank database (February 1998 release) was screened for homologies by using TFASTA.

Construction of plasmids. For replicon screening and plasmid integration, the *E. coli* plasmid pUC19Ery or pUC18Ery, carrying the Ery^r gene, or pCI182, carrying the tetracycline resistance (Tet^r) gene, was used. For plasmids pNZ4001, pNZ4002, and pNZ4004, a 3.3-kb *Eco*RI-*Xba*I fragment, a 3.4-kb *Eco*RI fragment, and a 2.9-kb *Sau*3AI fragment of pNZ4000 were cloned into pUC19Ery digested with *Eco*RI-*Xba*I, *Eco*RI, and *Bam*HI, respectively. For plasmid pNZ4003, a 3.7-kb *Xho*I-*Hin*cII fragment of pNZ4000 was cloned into *Sal*I-*Sma*I-digested pUC18Ery. To construct plasmid pNZ4025, a 3.3-kb *Eco*RI-*Xba*I fragment of pNZ4000 was cloned in pUC18 (41) digested with *Eco*RI-*Xba*I, and subsequently the pCI182 *tetM* gene was cloned on a 4.2-kb *Hin*cII fragment in the pUC18 *Hin*cII site. For plasmids pNZ4026 and pNZ4027, a 3.4-kb *Eco*RI and a 2.9-kb *Sau*3AI fragment of pNZ4000 were cloned in pCI182 digested with *Eco*RI or *Bgl*II, respectively.

To obtain Ery^r derivatives of pNZ4000 (pNZ4010 and pNZ4017), plasmids pNZ4006 and pNZ4007 were constructed. Plasmids pNZ4006 and pNZ4007 are pUC19Ery derivatives carrying 1.2- and 4.1-kb *Eco*RI-*Xba*I fragments of pNZ4000, respectively. These plasmids were used for plasmid integration by a single crossover to form pNZ4010 and pNZ4017, respectively.

For functional analysis of the putative *oriT* regions, fragments containing the *oriT1* or *oriT2* sequence were cloned in plasmid pNZ124. Plasmid pNZ4021, carrying *oriT1*, was constructed by cloning a 1.8-kb *Bgl*II-*Xho*I fragment of pNZ4000 in *Bgl*II-*Xho*I-digested pNZ124. Plasmid pNZ4022, carrying *oriT2*, was constructed by cloning a Klenow enzyme-treated 0.64-kb *Nsp*V-*Nco*I fragment of pNZ4000 in pNZ124 linearized with *Sca*I. To study the functionality of *mobA*, plasmid pNZ4023, carrying both *oriT1* and *mobA*, was constructed by cloning a 3.0-kb *Bgl*II-*Acc*I fragment of pNZ4000 with a Klenow enzyme-treated *Acc*I site in *Bgl*II-*Sca*I-digested pNZ124. All plasmids were constructed in *E. coli*.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the replication and mobilization regions are available under GenBank accession no. AF03685, AF03686, and AF03687.

RESULTS AND DISCUSSION

The EPS plasmid pNZ4000 contains four functional replicons. The 40-kb plasmid pNZ4000 is essential for EPS production in strain NIZO B40 and includes the 12-kb *eps* gene cluster involved in EPS biosynthesis (37). The nucleotide sequence of the EPS plasmid was determined, and analysis of the data revealed the unusual presence of four highly homologous replication regions that belong to a family of lactococcal theta replicons (35) which are located outside the *eps* gene cluster (Fig. 1). DNA fragments carrying these putative replicons were cloned into pUC19Ery or pUC18Ery, which can be used as replicon screening vectors in *L. lactis*. The resulting plasmids

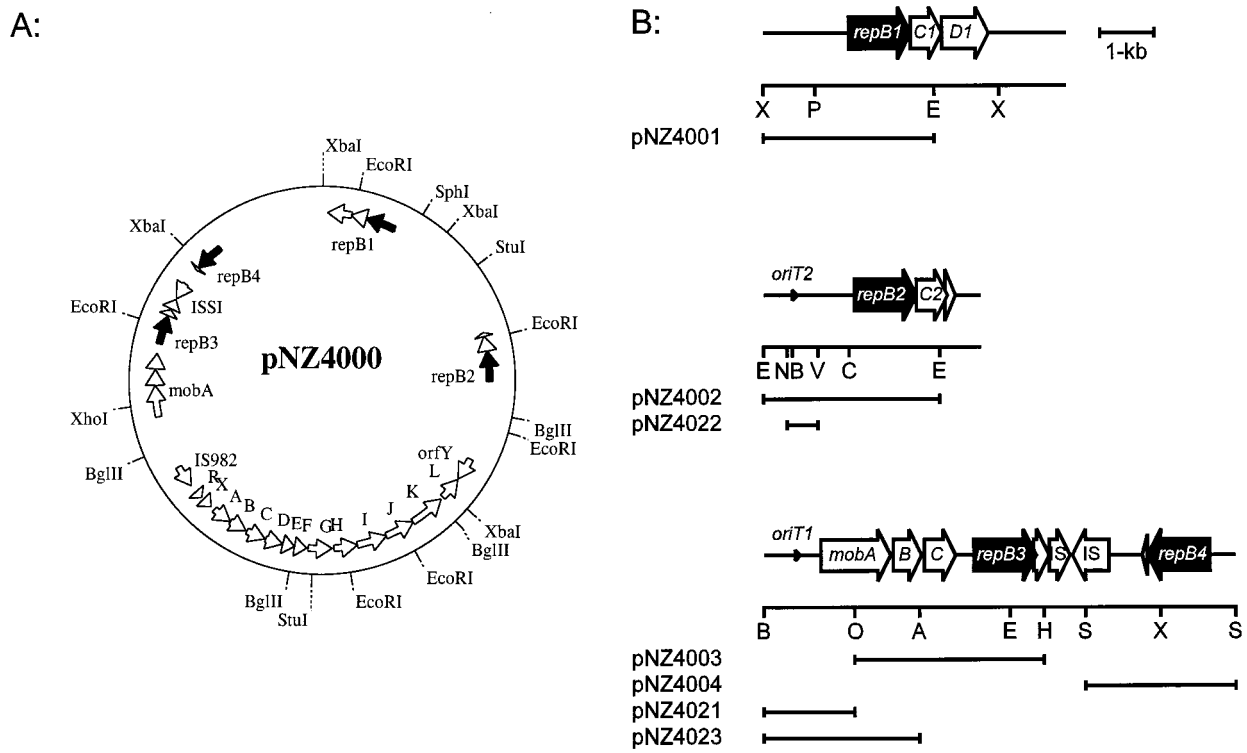

FIG. 1. (A) Physical and genetic map of plasmid pNZ4000. The *eps* gene cluster is located between IS*982* and *orfY*. (B) Physical and genetic maps of the replication and mobilization regions of pNZ4000. The fragments used for functional analysis are depicted below. A, *Acc*I; C, *Cla*I; E, *Eco*RI; H, *Hin*cII; N, *Nco*I; O, *Xho*I; P, *Sph*I; S, *Sau*3AI; V, *Nsp*V; X, *Xba*I. For *Acc*I, *Cla*I, *Hin*cII, *Nco*I, *Nsp*V, and *Sau*3AI, only sites relevant for subcloning are included. Sequences are available under GenBank accession no. AF036485, AF03686, and AF03687.

(pNZ4001, pNZ4002, pNZ4003, and pNZ4004) were transformed to *L. lactis* MG1363, and in all cases Ery^r transformants that harbored plasmids with the expected configuration were obtained (results not shown). These results indicate that all four replicons are functional in *L. lactis*. Since plasmid replication requires only one of these replicons, pNZ4000 must have derived fragments of several plasmids, which might have formed cointegrates during conjugation processes. This conclusion is corroborated by the presence of complete and truncated copies of ISS*I*-like elements (Fig. 1), since it is known that ISS*I* mediates cointegration of the *L. lactis* ML3 lactose plasmid pSK08 with the conjugal plasmid pRS01 (29). In addition, a complete copy of an IS*982*-like element is present on pNZ4000 (Fig. 1).

GenBank analysis of the proteins encoded by the *repB* genes of the four replicons on pNZ4000 showed them to be highly homologous to putative replication proteins of several other lactococcal plasmids which all carry a single replicon and belong to a family of lactococcal theta replicons (34), including pVS40 (86.0% identity with RepB1) (39), pWV04 (98.5% identity with RepB2) (34), pCI528 (99.8% identity with RepB3) (23), and pFV1201 (99.2% identity with RepB4) (GenBank accession no. X96949). The upstream regions of the *repB* genes of pNZ4000 were highly conserved and corresponded to those found in the other lactococcal replicons as first identified for pCI305 (17). They all contain an A/T-rich region that could be the recognition site for host-encoded functions involved in replication (35), a 22-bp sequence repeated 3.5 times which was shown to have a replicon-specific regulatory role in plasmid replication (7), and two inverted repeats, one of which overlapped the −35 region of the *repB* promoter (inverted repeat 1 [IR1]) and was found to be a RepB binding site (7). The upstream region of *repB1* showed a slightly different architecture and contained only a 2.5-times-repeated 22-bp direct repeat.

All four replicons are compatible but show differences in organization. The minimal replicons for *repB1*, *repB2*, and *repB4* were labeled with the *tetM* gene to generate pNZ4025, pNZ4026, and pNZ4027, respectively. These plasmids were combined with either pNZ4001, pNZ4002, pNZ4003, or pNZ4004 and transformed to MG1363 to make six strains including all combinations of different replicons carrying a set of Ery^r and Tet^r genes (Table 2). All plasmids had comparable copy numbers, as judged from the intensity of ethidium bromide-stained plasmid DNA separated by agarose gel electrophoresis (results not shown). Stable transformants were ob-

TABLE 2. Segregational stability of the four replicons of pNZ4000

Replicon		Fraction of plasmid-containing cells[a]		
Tet^r	Ery^r	Tet^r	Ery^r	Tet^r + Ery^r
repB1	*repB2*	0.8	0.9	0.9
repB1	*repB3*	0.9	0.01	0.01
repB4	*repB1*	1.0	1.0	0.9
repB2	*repB3*	0.7	0.05	0.06
repB2	*repB4*	0.8	0.9	0.7
repB4	*repB3*	0.9	0.08	0.07

[a] After 35 generations without selection pressure, cultures containing the Tet^r and Ery^r replicons were plated on medium containing tetracycline, erythromycin, both tetracycline and erythromycin, or no antibiotics, and the fraction of antibiotic-resistant colonies was determined.

tained for all heteroplasmid combinations following selection for Ery^r and Tet^r, indicating that these replicons are compatible. The compatibility of the plasmids carrying different replicons was confirmed by determining the segregational stability after growth for 35 generations in medium containing no antibiotics (Table 2). Plasmids carrying the replicons with *repB1*, *repB2*, and *repB4* formed highly stable heteroplasmid combinations. In contrast, the segregational stability of the *repB3*-containing replicon was significantly lower than that of the others. This was also observed when this replicon was present as a single replicon in MG1363. After 20 generations without selection pressure, 61% of the population was plasmid containing; after 40 generations 16%, and after 60 generations only 3%, of the population contained plasmids. The reason for the difference in stability between the *repB3*-containing replicon and the other three highly homologous replicons is unclear. It seems that there is interference with the maintenance functions of the *repB3*-containing replicon which are not directly involved in replication. The *orfC* genes located downstream of and partly overlapping the *repB* genes (Fig. 1) are not likely to be involved in this process. The predicted OrfC proteins are homologous to RepB287 (45 and 43% identity for OrfC1 and OrfC2, respectively) encoded by the *Tetragenococcus halophilus* theta-replicating plasmid pUCL287. RepB287 is not essential for replication, as is OrfC, but its presence reduces the copy number and the segregational stability (2). The N-terminal parts of the OrfC proteins are highly conserved and contain a helix-turn-helix motif which is probably involved in DNA binding. While *repB1* and *repB2* are followed by almost complete *orfC* genes, *orfC3* and *orfC4* encode only the N-terminal parts of OrfC-like proteins. If the role of the lactococcal OrfC was similar to that of RepB287, we would expect the stability of the replicons containing *repB1* and *repB2* to be lower than that of the replicons containing *repB3* and *repB4*, which is not as we observed.

Downstream of *orfC1* and *orfC2*, we found a partly overlapping third ORF (*orfD1* and *orfD2*, respectively [Fig. 1]). The predicted gene product OrfD1 shows considerable homology (47% identity) to the product of an *hsdS*-like gene from the lactococcal plasmid pIL2614, which encodes the specificity subunit of a type IC restriction-modification system (34). The *hsdS*-like gene is the last of a putative operon of five genes, the first two of which are replication genes homologous to *repB* and *orfC*. These are followed by three genes coding for the endonuclease, methylase, and specificity subunits, respectively, of a type I replication-modification system (34). This finding indicates that pNZ4000 and pIL2614 contain similarly organized and homologous operons, the one in pNZ4000 lacking the genes encoding the endonuclease and methylase subunits.

The EPS plasmid pNZ4000 is a mobilization plasmid. We have previously shown that plasmid pNZ4000 can be conjugally transferred together with the lactose plasmid from the *L. lactis* NIZO B40 to the recipient strain MG1614 (37). To study the intraspecific conjugative transfer of pNZ4000 in more detail, the Ery^r derivatives pNZ4010 and pNZ4017 were used. These plasmids were transformed to the plasmid-free strain MG1363, and the resulting strains were used as donors in filter matings with strain MG1614. Conjugative transfer of either of these plasmids between these isogenic *L. lactis* subsp. *cremoris* strains occurred at a frequency of 10^{-6} per donor. Plasmid pNZ4017 was also transformed to the plasmid-free *L. lactis* subsp. *lactis* strain IL1403, from which it could be transferred to *L. lactis* subsp. *cremoris* MG1614 at a frequency of 10^{-8} per donor. These results demonstrate that pNZ4000 can be mobilized from strains MG1363 and IL1403. It is likely that differences in chromosomal conjugation functions account for the differences in transfer efficiency of the pNZ4000 derivatives from both *L. lactis* subspecies, which are known to share approximately 70 to 80% sequence identity in characterized genes and differ by the presence of a large chromosomal inversion of about half of the genome (13, 21). Furthermore, MG1363 harbors the sex factor that encodes conjugative functions (12), which may play a role in mobilization of pNZ4000.

TABLE 3. Transfer frequencies of pNZ124 derivatives from MG1363 to MG1614

Plasmid(s)	Genotype				Transfer frequency[a]
	oriT1	*oriT2*	*mobA* in *cis*	*mobA* in *trans*	
pNZ124	−	−	−	−	$<10^{-10}$
pNZ4021	+	−	−	−	10^{-7}
pNZ4021, pNZ4017	+	−	−	+	10^{-5}
pNZ4022	−	+	−	−	10^{-7}
pNZ4022, pNZ4017	−	+	−	+	10^{-5}
pNZ4023	+	−	+	−	10^{-5}

[a] Number of transconjugants per donor (average of two independent experiments).

pNZ4000 contains two functional *oriT* sites. Mobilization involves a *cis*-acting *oriT* region and a *trans*-acting gene encoding a relaxase (20). Nucleotide sequence analysis of pNZ4000 revealed the presence of a region upstream of *repB3* (Fig. 1), which is almost identical (98.3% identity) to a 2.0-kb fragment involved in mobilization of the lactococcal plasmid pCI528 (24). It contains a *mobA* gene encoding a putative mobilization protein. The upstream region of the *mobA* gene contains three inverted repeats and a direct repeat and has been postulated to be the *oriT* region (24). We tested the functionality of the putative *oriT* sequence (*oriT1*) by cloning it in the nonconjugative plasmid pNZ124 and transforming the resulting plasmid pNZ4021 to strain MG1363. This strain was mated with MG1614 and chloramphenicol-resistant transconjugants were selected (Table 3). The 1.8-kb region containing the *oriT1* sequence was sufficient to achieve conjugal transfer of the nonconjugative plasmid pNZ124, showing that the cloned fragment contains a functional *oriT*.

A second *oriT* region sharing 96.6% identity in 417 nucleotides with *oriT1* was found upstream of *repB2*. It was cloned as a 0.64-kb fragment in pNZ124, and the resulting plasmid, pNZ4022, had the same transfer frequency as pNZ4021 (Table 3), indicating the presence of two functional *oriT* sequences on pNZ4000, one upstream of *mobA* (*oriT1*) and one upstream of *repB2* (*oriT2*) (Fig. 1), oriented in opposite directions.

The *oriT* site of the streptococcal plasmid pMV158 is homologous to sequences of several plasmids from gram-positive hosts (15). However, no significant homology between the *oriT* regions of pNZ4000 and these sequences could be detected. In contrast, the pNZ4000 *oriT* sequences contain an inverted repeat (IR3) which is highly homologous to that of the *oriT* from IncI1 plasmid R64 (Fig. 2). This includes the R64 mobilization protein NikA binding site (9). Moreover, the homology between the pNZ4000 *oriT* sequences and that of R64 also includes the sequence next to the repeat containing the *nic* site (Fig. 2). In the absence of experimental evidence, we therefore postulate that these sequences may contain the pNZ4000 *nic* sites. The streptococcal plasmid pIP501 and the staphylococcal plasmid pGO1 *oriT* regions are homologous to *oriT* sequences of several gram-negative plasmids. They all contain a conserved sequence with the *nic* site next to a nonconserved inverted repeat centered around the nucleotide sequence 5′-

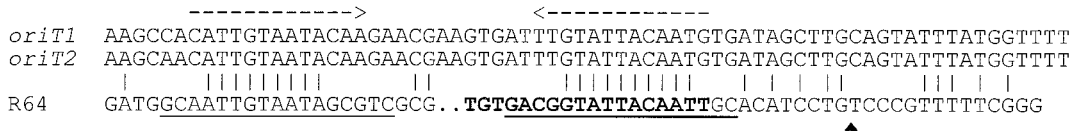

FIG. 2. DNA sequence alignment of the IR3 segments of the *oriT* regions found on pNZ4000 (*oriT1* and *oriT2*) and the sequence of the IncI1 plasmid R64 *oriT*. For R64 *oriT*, the inverted repeat is underlined, the NikA binding site is indicated in boldface, and the *nic* site is indicated with an arrowhead (9).

GAA-3′ (4, 40). Although no significant homology between these *oriT* regions and those of pNZ4000 could be detected, the IR3 sequence of each of the pNZ4000 *oriT* regions is also situated around a 5′-GAA-3′ nucleotide sequence.

***mobA* encodes a product *trans*-acting on *oriT*-carrying plasmids.** The involvement of *mobA* in mobilization was studied by comparing the transfer frequencies of plasmids carrying only *oriT* sequences or carrying *oriT* and *mobA* either in *cis* or in *trans* (Table 3). When *mobA* was provided in *trans* on pNZ4017, the transfer frequencies of pNZ4021 and pNZ4022 increased significantly. The same effect was achieved by when *mobA* was present in *cis* as on plasmid pNZ4023, containing *oriT1* and *mobA*. These results indicate that *mobA* encodes a *trans*-acting element involved in mobilization.

To verify the relaxation activity of the *mobA* gene product (25), whole-cell lysates of MG1363 harboring *oriT1*- or *oriT2*-carrying plasmids with or without *mobA* (in *cis* or in *trans*) were separated by agarose gel electrophoresis. The plasmid profiles of pNZ4021 and pNZ4022 showed a significant increase in open circular plasmid DNA only when pNZ4017 was present (approximately half of the *oriT*-carrying plasmids were in the open circular form). Moreover, pNZ4023 carrying *oriT1* and *mobA* showed a similar high degree of open circular DNA (data not shown). These results indicate that the plasmids carrying *oriT* fragments are relaxed by the *trans*-acting *mobA* gene product.

The predicted MobA protein reveals significant homologies (approximately 30% identity) with three mobilization proteins found on antibiotic resistance plasmids of *Staphylococcus aureus* (30–32) and moderate homology (23% identity in 388 amino acids) with the N-terminal part of TraI from the *E. coli* IncPα plasmid RP4. TraI is a relaxase and forms together with TraJ the relaxosome at *oriT* (26). TraI contains three conserved regions found in several relaxases (27). Motifs I and III are involved in catalyzing the cleaving-joining reaction. Motif I contains a conserved tyrosine residue which after nicking is covalently attached to the 5′ end of the cleaved DNA. Motif III contains a conserved histidine residue that is likely to activate the tyrosine of motif I by proton extraction. Motif II contains a conserved serine and is thought to be involved in DNA recognition (27). Multiple sequence alignment of MobA, the four homologous proteins, and the *E. coli* plasmid R64 relaxase NikB, which is homologous to TraI (8), showed that the three conserved domains and the tyrosine, serine, and histidine residues needed for relaxase activity are present in MobA (Fig. 3). This conservation strongly suggests that the lactococcal MobA is a relaxase which is involved in nicking the *nic* sites of the *oriT* sequences (Fig. 2), which is corroborated by the formation of open circular DNA of plasmids carrying an *oriT* sequence when *mobA* is present (see above).

On pNZ4000, a second ORF, here designated *mobB*, was found downstream of *mobA*, the putative start codon of which overlaps the stop codon of *mobA*. This configuration resembles that of the *S. aureus* plasmid pC223, which contains two overlapping mobilization genes *orf1* and *orf2* (30, 32). In addition to the homologous Orf1 and MobA proteins, the predicted MobB protein shares moderate homology (24% identity) with Orf2 of pC223. A third ORF, designated *mobC*, was detected 16 bp downstream of the stop codon of *mobB*. Its gene product showed no homology to any protein in the GenBank database, and the involvement of *mobC* in the conjugation process remains to be established.

The region on pNZ4000 containing the mobilization genes and the third replicon has a high degree of homology with the same regions on pCI528. Plasmid pCI528 is a 46-kb plasmid encoding the production of a hydrophilic polymer containing glucose and rhamnose that reduces phage adsorption to its lactococcal host (22). Although pCI528 does not encode EPS production whereas pNZ4000 does, there may be a close relationship between the two plasmids or their ancestors.

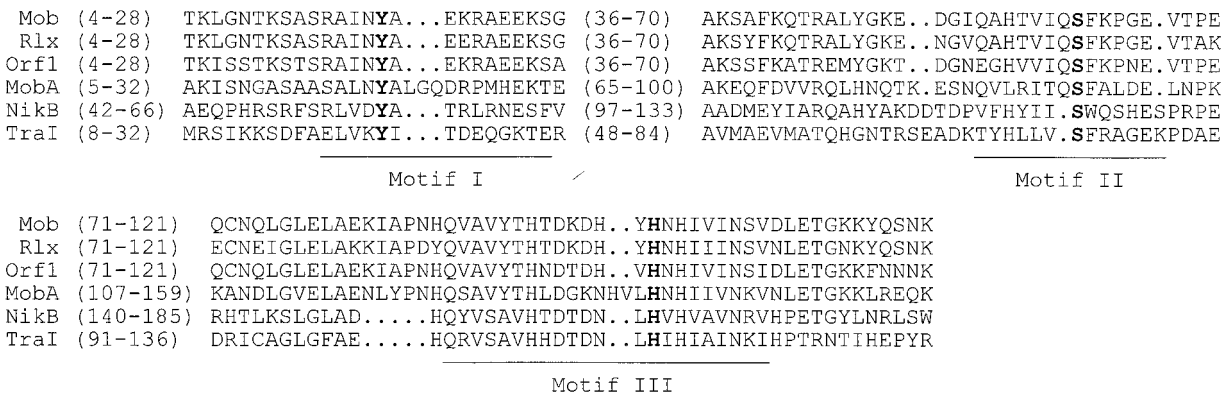

FIG. 3. Amino acid sequence comparison of the three conserved regions involved in relaxase activity as determined for TraI (27) for the relaxases MobA (Mob), Rlx, and Orf1 from *S. aureus* plasmids pC221, pS194, and pC223, respectively, MobA (MobA) from pNZ4000, NikB from *E. coli* plasmid R64 (8), and TraI from the *E. coli* plasmid RP4. The tyrosine (motif I) and histidine (motif III) residues involved in cleaving-joining reaction, and the serine residue (motif II) involved in DNA binding, are indicated in boldface.

In summary, we demonstrated that plasmid pNZ4000 contains four homologous and active replicons that are compatible with each other. It contains two functional *oriT* sequences. One *oriT* is followed by the *mobA* gene coding for a *trans*-acting protein. The predicted MobA protein and the *oriT* sequences are homologous to the R64 relaxase and the *oriT*. The R64 relaxase is known to nick a site which is also conserved in the *oriT* sequences of pNZ4000.

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