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Genome Plasticity among Related *Lactococcus* Strains: Identification of Genetic Events Associated with Macrorestriction Polymorphisms†

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The genomic diversity of nine strains of the *Lactococcus lactis* subsp. *cremoris* (NCDO712, NCDO505, NCDO2031, NCDO763, MMS36, C2, LM0230, LM2301, and MG1363) was studied by macrorestriction enzyme analysis using pulsed-field gel electrophoresis. These strains were considered adequate for the investigation of genomic plasticity because they have been described as belonging to the same genetic lineage. Comparison of *Apa*I and *Sma*I genome fingerprints of each strain revealed the presence of several macrorestriction fragment length polymorphisms (RFLPs), despite a high degree of similarity of the generated restriction patterns. The physical map of the MG1363 chromosome was used to establish a genome map of the other strains and allocate the RFLPs to five regions. Southern hybridization analysis correlated the polymorphic regions with genetic events such as chromosomal inversion, integration of prophage DNA, and location of the transposon-like structures carrying conjugative factor or oligopeptide transport system.

Until recently, analysis of the general structure and the gene order of the chromosomes of prokaryotic cells was held back by the lack of genetic tools for mapping bacterial genomes. The introduction of physical methods for the construction of chromosome maps, such as the "top-down" approach using pulsed-field gel electrophoresis (PFGE), has had a large impact on the genome characterization of a wide range of bacteria (10, 16).

Comparative studies of genome maps at the interspecies level allow one to define a conserved global architecture for circular bacterial chromosomes: almost all bacterial chromosomes contain several ribosomal operons (*rrn*) that are transcribed divergently from the origin of replication (*oriC*), which appears to be located opposite the terminus site (*terC*). Comparisons of the genomes of bacteria that belong to the same or related genera provide useful data about the structural maintenance of the chromosome itself as well as about the maintenance and disruption of gene order during evolution. With the exception of *Bacillus* (8, 51) and, to a lesser extent, *Leptospira* (79), the genome organization is highly conserved for most bacteria, as observed for *Escherichia coli* and *Salmonella* (30, 40), *Borrelia* (9, 50), *Haloferax* (42), *Mycobacterium* (53, 54), *Mycoplasma* (25), *Streptomyces* (33), and *Neisseria* (15), although some rearrangements are present.

At the intraspecies or strain level, comparative analysis yields information on the macrodiversity (defined as the variability of gene arrangement or macrorestriction polymorphisms) of a particular organism. Studies have shown that the extent of chromosome rearrangement depends largely on the species studied. Genome rearrangement among strains of *E. coli* (2, 52), *S. enterica* serovar Typhimurium (39), *Clostridium perfringens* (6), *Streptococcus thermophilus* (61), *Mycoplasma galliseptum* (70), *Halobacterium salinarium* (23), and *Thermococcus thermophilus* (67) is seen as minimal, despite the identification of inversions, insertions, deletions, and translocations of some regions. In contrast, a greater genomic diversity or a significant mosaic structure has been observed between strains of *S. enterica* serovar Typhi (41), *Rhodobacter capsulatus* (49), *Bacillus cereus* (7), *Leptospira interrogans* (79), and *Pseudomonas aeruginosa* (59).

The next step in the analysis of genomic macrodiversity is the identification of the genetic events (homologous or site-specific recombination, insertion-excision, transposition, etc.) responsible for such macrodiversity and the DNA sequences (IS elements or other repetitive sequences, prophages, duplicated genes, etc.) involved in genome rearrangement. For this purpose, it is essential to compare the genomes of strains belonging to the same genetic lineage (isogenic strains). However, experimental data that associate PFGE-generated restriction polymorphisms with identified genetic events are available only for three gram-negative bacteria (*E. coli* [52], *P. aeruginosa* [60], and *Neisseria gonorrhoeae* [19]) and seven gram-positive bacteria. In lysogenized strains of *Staphylococcus aureus* (4, 65) and *C. perfringens* (5), restriction polymorphism was correlated with prophage integration, whereas in *S. thermophilus* (62), *Bacillus subtilis* (71), and *B. cereus* (27), small deletions mediated by homologous recombination between tandemly repeated *rrn* operons were observed. In *Streptomyces lividans* (58) and *S. ambofaciens* (32), large DNA amplifications and deletions involved homologous recombination between specific loci (AUD).

Lactococcus lactis is a gram-positive mesophilic bacterium that is extensively used as a starter culture in the manufacture of dairy products. The PFGE technique has been used for several studies on the genome of *L. lactis* for estimation of the genome size and genome fingerprinting (37, 43, 68), analysis of plasmid stability (76), and study of integration sites of transposon Tn*917* derivatives (26). The chromosome maps of four independent lactococcal strains have been published to date: *L. lactis* subsp. *lactis* DL11 (73) and IL1403 (34) and *L. lactis* subsp. *cremoris* MG1363 (35) and FG2 (12). The genome of strain IL1403 has been completely sequenced recently (3). Hybridization data (14) showed that these two subspecies have a nucleotide divergence of 20 to 30%, the same order of magnitude as that observed between *E. coli* and *S. enterica* serovar

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† This paper is dedicated to Orian Le Bourgeois.

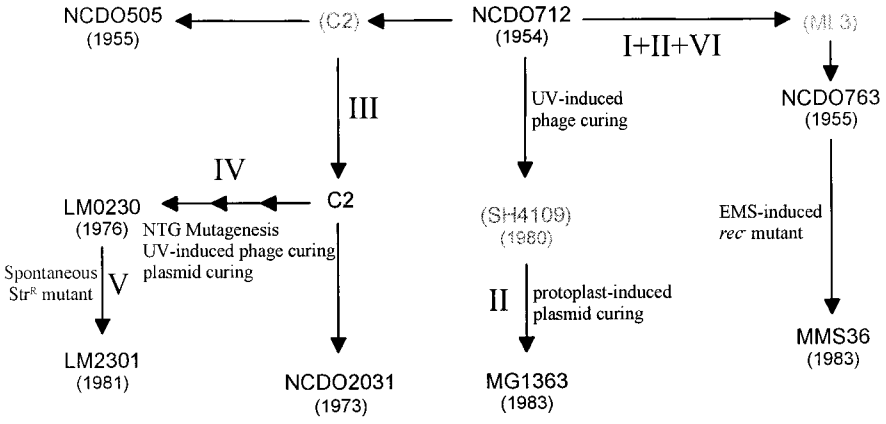

FIG. 1. Biological and genomic relationship of strains derived from *L. lactis* subsp. *cremoris* strain NCDO712. Parentheses indicate strains that were not included in this study. I, Inversion of half of the chromosome in regions 1 and 3; II, excision of the uncharacterized prophage in region 2; III, excision of the complete conjugative sex factor in region 5; IV, excision of the ϕT712 prophage in region 4; V, chromosomal deletion of a fragment containing the *opp-pepO* operon in region 3; VI, partial deletion of the conjugative sex factor in region 5. EMS, ethyl methanesulfonate; NTG, nitrosoguanidine; Str, streptomycin.

Typhimurium. Comparative genome analysis of the four strains at a physical level revealed a tight correlation in the position of restriction sites for the two *L. lactis* subsp. *lactis* strains but not for the *L. lactis* subsp. *cremoris* strains. In addition, no similarity was found between the two subspecies. At the genetic level, i.e., gene order, different rearrangements were observed. Intersubspecies comparison revealed a large inversion that covers nearly half of the chromosome (35), whereas there was translocation/inversion of four discrete regions between the two *L. lactis* subsp. *cremoris* strains (12). However, the order of genes within the rearranged segments is largely conserved. These studies were performed on genetically unrelated strains, and so it was not possible to identify the mechanisms which brought about these events.

To investigate the plasticity of the lactococcal genome, we initiated a comparative analysis of the genomes of nine strains belonging to the same genetic lineage. The *L. lactis* subsp. *cremoris* strain MG1363 (17) is a plasmid-free derivative of strain NCDO712, the ancestor of lactococcal strains NCDO763, NCDO505, NCDO2031, and C2 (13). Three other strains were also analyzed: MMS36, a recombination-deficient mutant of strain NCDO763 (1); strain LM0230, a plasmid-free derivative of strain NCDO2031 (46); and strain LM2301, a streptomycin-resistant mutant of strain LM0230 (75). These different strains constitute the most extensively studied group of lactococcal strains in genetic analysis and molecular biology. We recently demonstrated that one restriction polymorphism observed between the chromosomes of strains MG1363 and NCDO763 was associated with a single inversion of half of the chromosome (11).

In this study, we used combined PFGE and Southern hybridization to determine restriction fragment length polymorphisms (RFLPs) among the nine lactococcal strains, to assign these RFLPs to particular regions of their genome, and to identify the genetic events that could be correlated with these genome rearrangements.

MATERIALS AND METHODS

Bacterial strains. The genetic relationship between the *L. lactis* subsp. *cremoris* strains used in this study is presented in Fig. 1. Wild-type lactococcal strains were grown at 30°C in M17 broth (69). For the Lac$^-$ derivative strains LM0230, LM2301, and MG1363, the M17 broth was supplemented with 0.5% glucose (GM17 broth). When required, erythromycin was used at 5 μg/ml. Particles of temperate bacteriophages were obtained after UV induction of the lysogenic strain by the method of McKay and Baldwin (45).

DNA manipulation. Plasmid isolation, restriction digestion, ligation, and transformation in *E. coli* were performed as described by Sambrook et al. (63). Restriction enzymes and T4 DNA ligase were purchased from either Boehringer Mannheim or New England Biolabs and used as recommended by the suppliers. DNA restriction fragments were purified from agarose gel using the Prep-A-Gene DNA purification kit (Bio-Rad). *Lactococcus* strains were transformed by electroporation (56), except that the cells were grown in GM17 supplemented with 2% glycine. Lactococcal genomic DNA used for PFGE analysis was purified and digested in agarose plugs as described previously (35). Bacteriophage DNA was extracted from phage particles by the method of Trautwetter et al. (72).

PFGE, determination of fragment sizes, and Southern hybridization. PFGE were performed using a contour-clamped homogeneous electric field system (Pulsaphor Plus; LKB-Pharmacia) in 0.05 M TBE (1 M TBE is 1 M Tris base, 1 M boric acid, and 20 mM EDTA), as described previously (36). Restriction fragments were resolved under the following typical PFGE conditions: 2.5-s pulse time for 11 h for fragments of <100 kb, 7.5-s pulse time for 13 h for fragments of 100 to 300 kb, 15-s pulse time for fragments of 250 to 500 kb, and 30-s pulse time for fragments of 450 to 700 kb. Fragment sizes were determined manually by measurement of photographed gels. λ DNA concatemers, obtained using the procedure of Waterbury and Lane (77), were used for fragments larger than 48.5 kb. Smaller fragments were measured by comparison with a 1-kb DNA ladder (Gibco-BRL). Southern hybridizations were done on dried agarose gels as described previously (35).

Estimation of the degree of genomic relatedness. Similarity of restriction patterns was expressed using the Dice coefficient (S_D) by the method of Grothues and Tümmler (22). The relatedness between two restriction patterns, A and B, is given by the ratio of twice the number of bands found in both patterns (n_{AB}) to the sum of all bands in the two patterns ($N_A + N_B$): $S_D = 2n_{AB}/(N_A + N_B)$. In our case, n_{AB}, N_A, and N_B were obtained by summing the numbers of *Apa*I and *Sma*I restriction fragments. Strains were clustered by the unweighted pair group method with arithmetic mean (UPGMA) (66) using the Neighbor program (version 3.5c) of the PHYLIP package.

RESULTS

Restriction analysis of the *L. lactis* subsp. *cremoris* strains. The physical map of the MG1363 chromosome (35) was constructed using the restriction endonucleases *Apa*I (GGGCCC), *Sma*I (CCCGGG), and *Not*I (GCGGCCGC) and the intron-encoded endonuclease I-*Ceu*I (44). *Apa*I and *Sma*I generated a suitable number of restriction fragments and thus were used for the comparative study of the restriction patterns of the nine lactococcal strains. Visual inspection of ethidium bromide-stained PFGE gels showed that the chromosomal *Apa*I and *Sma*I fingerprints were very similar for these strains (Fig. 2A and B). For each strain, the size of every restriction fragment was estimated by direct comparison with restriction fragments of the MG1363 chromosome. In addition, the sizes of polymorphic restriction fragments were measured by comparison with DNA ladders. Depending on the strain, *Apa*I and *Sma*I

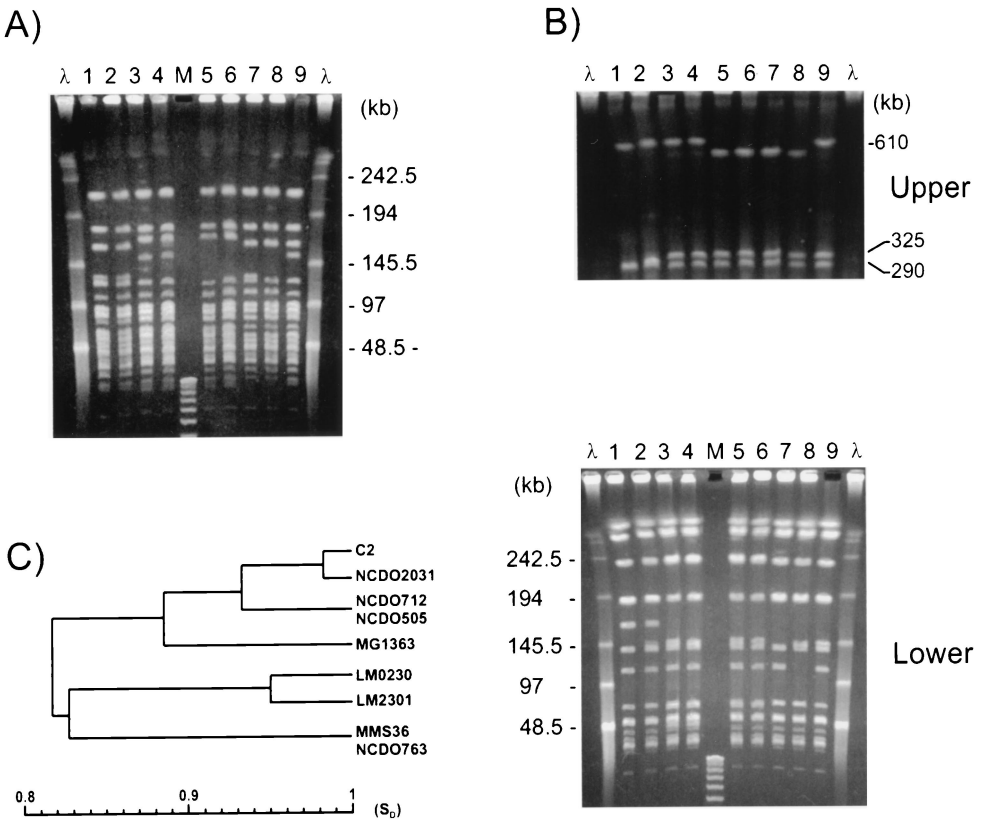

FIG. 2. Macrorestriction patterns of *L. lactis* chromosomes. Lanes: 1, NCDO763; 2, MMS36; 3, NCDO712; 4, NCDO505; 5, NCDO2031; 6, C2; 7, LM2301; 8, LM0230; 9, MG1363; λ, lambda DNA concatemers. (A) *Apa*I digestion. Electrophoresis conditions were 7.5 s for 13.5 h. (B) *Sma*I digestion. In the upper panel, electrophoresis conditions for separation of the larger *Sma*I fragments were 15 s for 13.5 h. In the lower panels, electrophoresis conditions were 8 s for 13.5 h. (C) Dendogram of similarity values.

endonucleases yielded a different number of restriction fragments (ranging from 39 to 45 and from 24 to 27, respectively), and the corresponding genome size was calculated by adding the sizes of every *Apa*I or *Sma*I restriction fragment (Table 1). The largest genome size difference was observed between strains NCDO712-NCDO505 and strain LM2301.

Strains were ordered according to the similarity of the *Apa*I and *Sma*I macrorestriction fragment patterns by cluster analysis (Fig. 2C). The high values of the Dice coefficient (S_D), 0.81 to 1, reflect the close genomic relatedness between the nine strains studied. Two pairs of strains (NCDO712-NCDO505 and NCDO763-MMS36) gave indistinguishable fingerprints ($S_D = 1$), suggesting that they have identical genome maps. In addition, the fingerprints of the NCDO2031 and C2 chromosomes were identical ($S_D = 0.986$) except for a 3-kb size increase for one of the Sm11A fragments. On the basis of these results, the name NCDO712 is used in this report for both strains NCDO712 and NCDO505, the name NCDO763 is used for both strains NCDO763 and MMS36, and the name NCDO2031 is used for strains both NCDO2031 and C2 when referring to genome organization.

Identification of the polymorphic regions on the MG1363 chromosome. Six *Sma*I fragments (Sm1, Sm2, Sm5A, Sm6, Sm8, and one of the Sm11 fragments) and nine *Apa*I fragments (Ap3, Ap4, one of the Ap10 fragments, Ap11, one of the Ap12 fragments, one of the Ap14 fragments, Ap26, one of the Ap27 fragments, and Ap28) present on the MG1363 chromosome were subjected to a size polymorphism in at least one fingerprint of the other strains (Table 1). Correlation of the location of these *Apa*I and *Sma*I fragments on the physical map of MG1363 chromosome allowed us to define five regions where genome rearrangements could account for the observed macrorestriction polymorphism (Fig. 3). Region 1 corresponds to the absence of fragments Sm2 and Ap12B on the NCDO763 chromosome. Region 2 corresponds to the absence of fragments Sm5A and Ap3 on the NCDO712 and NCDO2031 chromosomes. Region 3 corresponds to the absence of restriction fragments Sm6 and Ap11 on the NCDO763 chromosome and of Sm6, Ap10C, and Ap11 on the LM2301 chromosome. Region 4 corresponds to the absence of restriction fragments Sm8 and Ap14, Ap27, and Ap28 on the LM0230 and LM2301 chromosomes. Region 5 corresponds to the loss of fragments Sm1 and Ap4 on the NCDO763 chromosome and to the loss of Sm1, Ap4, and Ap26 fragments in strains NCDO2031, LM0230, and LM2301.

Correlation of genome rearrangements with singular genetic events. The nine strains analyzed in this study have various genetic differences involving the presence of plasmid DNA and temperate bacteriophage (13, 46) or the location of conjugative sex factor (18, 47) and oligopeptide transport systems (74, 78). We made the assumption that these different genetic events would produce genome rearrangements large enough to be correlated with most of the RFLPs observed above. To characterize which genetic event(s) could be associated with the macrorestriction polymorphisms, each of the five regions was analyzed in more detail. We recently demonstrated that the macrorestriction polymorphism in regions 1 and 3 between the MG1363 and NCDO763 chromosomes was

TABLE 1. Sizes of polymorphic *Sma*I and *Apa*I restriction fragments[a]

Fragment size[b] (kb)	Presence of fragment in strain[c]:								
	MG1363[d]	NCDO712	NCDO505	NCDO763	MMS36	NCDO2031	C2	LM0230	LM2301
*Sma*I fragments									
610	+ (Sm1)	+	+	−	−	−	−	−	−
580	−	−	−	+	+	−	−	−	−
550	−	−	−	−	−	+	+	+	+
325	+ (Sm2)	+	+	−	−	+	+	+	+
290	−	−	−	+	+	−	−	−	−
220	−	+	+	−	−	+	+	−	−
180	+ (Sm5A)	−	−	+	+	−	−	+	+
160	−	−	−	+	+	−	−	−	−
130	+ (Sm6)	+	+	−	−	+	+	+	−
105	+ (Sm8)	+	+	+	+	+	+	−	+
58	−	−	−	−	−	−	−	+	+
55*	−	+	+	−	−	+	+	−	−
45	−	−	−	−	−	−	+	−	−
42	+ (Sm11A)	+	+	+	+	+	−	+	+
36*	−	+	+	−	−	−	−	−	−
30*	−	+	+	−	−	−	−	−	−
24*	−	−	−	+	+	−	−	−	−
*Apa*I fragments									
165	−	+	+	−	−	+	+	−	−
160	+ (Ap3)	−	−	+	+	−	−	+	+
145	+ (Ap4)	+	+	−	−	−	−	−	−
120	−	−	−	+	+	−	−	−	+
100	−	−	−	−	−	+	+	+	+
75	+ (Ap10C)	+	+	+	+	+	+	+	−
69	+ (Ap11)	+	+	−	−	+	+	−	−
67	−	−	−	−	−	−	−	+	−
65	+ (Ap12B)	+	+	−	−	+	+	+	+
55*	−	+	+	+	+	+	+	−	−
49	+ (Ap14A)	+	+	+	+	+	+	−	−
29	−	−	−	−	−	−	−	+	+
19	−	−	−	+	+	−	−	−	−
16	+ (Ap26)	+	+	+	+	−	−	−	−
13	+ (Ap27B)	+	+	+	+	+	+	−	−
12	+ (Ap28)	+	+	+	+	+	+	−	−
11*	−	+	+	−	−	+	+	−	−

[a] A detailed table containing the number and size of the *Apa*I and *Sma*I restriction fragments of each strain is available upon request to the authors.
[b] Asterisks indicate fragments assigned to plasmid DNA (see text).
[c] The mean genome size for a given strain was calculated assuming an error of the order of 5%: MG1363, 2.5 ± 0.1 Mb; NCDO712 and NCDO505, 2.6 ± 0.1 Mb; NCDO763 and MMS36, 2.6 ± 0.1 Mb; NCDO2031, 2.6 ± 0.1 Mb; C2, 2.6 ± 0.1 Mb; LM0230, 2.4 ± 0.1 M; LM2301, 2.4 ± 0.1 Mb.
[d] Restriction fragments of strain MG1363 were labeled according to reference 35.

caused by the inversion of half of the chromosome in strain NCDO763 and was mediated by homologous recombination between two copies of an IS element (11). This inversion modified the size of two *Apa*I (Ap11 and Ap12B) and two *Sma*I (Sm2 and Sm6) fragments of the MG1363 chromosome, which were replaced, respectively, by 19- and 120-kb *Apa*I and 290- and 160-kb *Sma*I fragments on the NCDO763 chromosome.

(i) Identification of restriction fragments linked to plasmid DNA. Strains NCDO712, NCDO505, NCDO763, MMS36, NCDO2031, and C2 are known to contain plasmid DNA (13). All these strains contain a 55-kb lactose-protease (Lac-Prt) plasmid and some other cryptic plasmids. To assign particular restriction fragments to plasmid DNA, the following assumption was made: any additional fragment visualized only for this group of strains and not involved in genome rearrangements described in this study was considered to be plasmid DNA. An asterisk in Table 1 indicates the restriction fragments associated with plasmid DNA. Note that circular DNAs such as plasmids have a different electrophoretic mobility in PFGE from that of linear DNA molecules (24, 64). Plasmids in the open circular form (OC) do not migrate in PFGE whatever their size, whereas small covalently closed circular forms (CCC) migrate with anomalous mobility depending on the electrophoresis conditions. This indicates that a plasmid DNA will be visualized in PFGE only if be cut by restriction endonucleases.

Some restriction fragments were confirmed to be plasmid linked by hybridization using IS*S1* as probe (Table 2). IS*S1* was known to be present as one copy in the MG1363 chromosome, located on the Sm6 (130 kb) and Ap10C (75 kb) fragments (Fig. 3). This IS element was also found at two copies on the Lac-Prt plasmid of strain NCDO763 (55). Hybridization results showed that in addition to the Sm6 and Ap10C fragments revealed in all nine strains, one 55-kb *Apa*I fragment and one 55-kb *Sma*I fragment appeared on the NCDO712 and NCDO2031 fingerprints whereas only one 55-kb *Apa*I fragment appeared on the NCDO763 fingerprint. Furthermore, the 55-kb *Apa*I and *Sma*I fragments were unambiguously associated with the Lac-Prt plasmid by hybridization with the *lacG* gene (encoding the phospho-β-galactosidase of the Lac-Prt plasmid) as a probe (data not shown). The lack of the 55-kb *Sma*I hybridizing fragment in the NCDO763 fingerprint could be explained by the absence of the *Sma*I site from the Lac-Prt

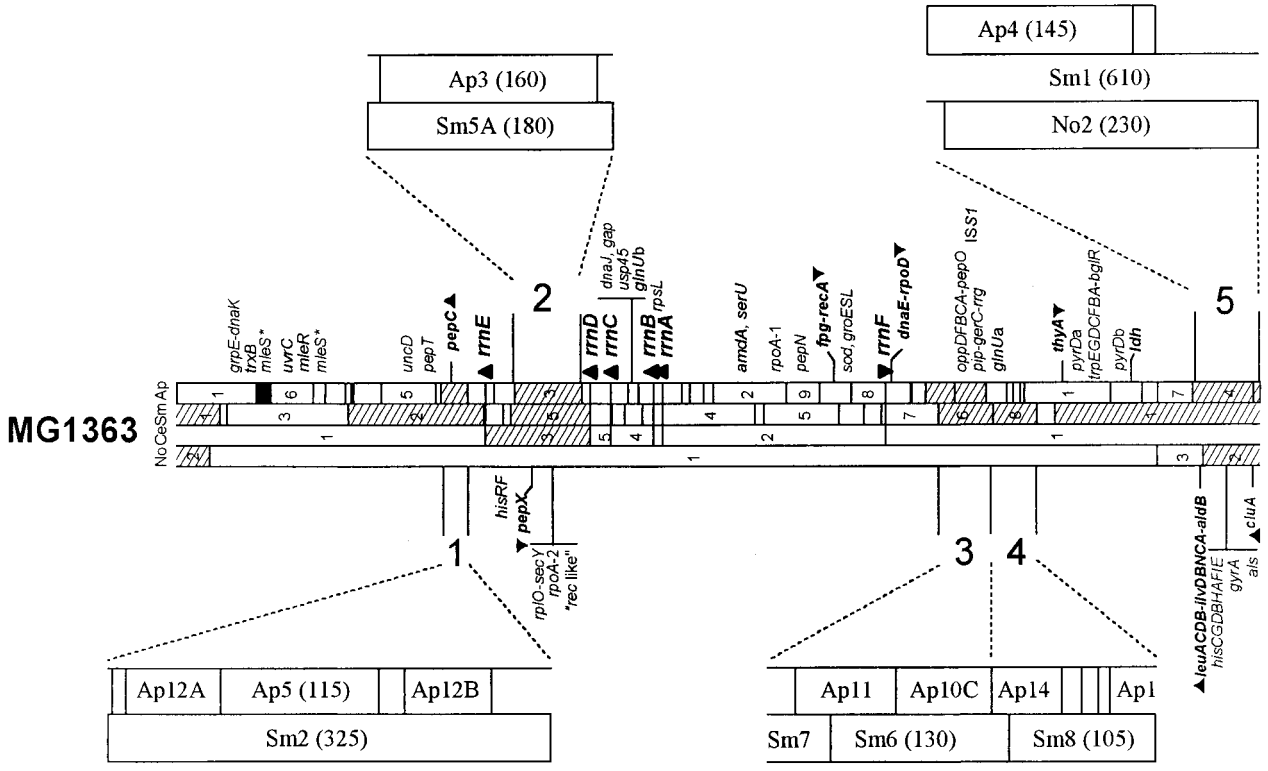

FIG. 3. Locations of the five polymorphic regions of the MG1363 chromosome. Parentheses indicate the sizes (in kilobases) of the restriction fragments. The genome map of strain MG1363 is drawn according to Le Bourgeois et al. (35). Abbreviations; Sm, *Sma*I; Ap, *Apa*I; No, *Not*I; Ce, I-*Ceu*I.

plasmid due to point mutation or a deletion too small to be seen by PFGE.

(ii) Effect of the lysogenic status of strains on the macrorestriction polymorphism. Strains NCDO712, NCDO505, NCDO2031, and C2 are known to be lysogenic for a temperate bacteriophage (respectively named φT712, φT505, φT2031, and φTC2), but strain NCDO763 is not (13). In addition, strain MG1363 was obtained from a UV-induced prophage-free derivative of NCDO712 (17) and strain LM0230 is a spontaneous prophage-cured mutant obtained by nitrosoguanidine treatment of strain C2 (46). *Hin*dIII and *Pst*I restriction enzyme analysis of the φT712, φT505, φT2031, and φTC2 genomes revealed an identical restriction pattern (data not shown), strongly suggesting that the four phages were very closely related. In addition, their genome contains two *Apa*I sites but no *Sma*I site. The genome size of φT712 was estimated to be 45 kb. When hybridized to the genomes of the nine strains, the entire phage DNA gave multiple signals of variable intensities (Table 2). By using different *Pst*I restriction fragments of φT712 as more specific probes, we found that one *Sma*I fragment (Sm6) and three *Apa*I fragments (Ap14A, Ap27, and Ap28) of strains NCDO712, NCDO763, NCDO2031, and MG1363 contained the prophage DNA. In contrast, the genomes of strains LM0230 and LM2301 lacked the entire φT712 prophage DNA. The remaining hybridizing fragments (i.e., 165- or 160-kb and 33-kb *Apa*I fragments and 220-, 180-, and 22-kb *Sma*I fragments) were presumed to contain sequences that have weak homology to the φT712 DNA. The size of the deletion generated by the excision of φT712 prophage DNA was confirmed by performing Southern hybridizations using the Sm8 (105-kb) fragment from MG1363 chromosome as a probe (Table 2). This fragment strongly hybridized with the 105-kb *Sma*I fragment in all strains except for strains LM0230 and LM2301, where a 58-kb fragment was revealed. For *Apa*I fingerprints, the probe strongly hybridized with five fragments in all strains except LM0230 and LM2301, where only three fragments of 205, 29, and 8 kb were observed.

Another macrorestriction polymorphism that correlates better with the lysogenic status of the strain is the presence of two 220- and one 180-kb *Sma*I fragments on the genome of lysogenic strains NCDO712 and NCDO2031. In the nonlysogenic strains MG1363, NCDO763, LM0230, and LM2301, macrorestriction patterns revealed only one 220- and two 180-kb *Sma*I fragments. A 40-kb shift in the size of *Sma*I fragments would be in good agreement with the presence of a lactococcal temperate bacteriophage, and because no such 40-kb shift was observed in *Apa*I fingerprints, the regions that included the Sm5A or Sm5B fragments were analyzed in more detail. Southern hybridization was performed using the Ap3 fragment (160 kb) of MG1363 as a probe. This *Apa*I fragment is located in the Sm5A fragment on the physical map of the MG1363 chromosome (Fig. 3). The Ap3 probe hybridized with one 180-kb *Sma*I fragment in strains NCDO763, MG1363, LM0230, and LM2301 and with one 220-kb *Sma*I fragment in strains NCDO712 and NCDO2031-C2 (Table 2). For the *Apa*I fingerprints, the Ap3 probe hybridized with the 160-kb *Apa*I fragment in strains NCDO763, MG1363, LM0230, and LM2301 and also with a unique 165-kb *Apa*I fragment in strains NCDO712 and NCDO2031 (Table 2). From these results, we concluded that an uncharacterized excisable element of 40 kb, which contains one *Apa*I site on its genome, was accountable for the macrorestriction polymorphism observed in this region.

TABLE 2. Sizes of hybridizing restriction fragments

Probe used	Enzyme tested	Fragment[a] (kb)	Presence of fragment in strain[b]:					
			MG1363	NCDO712	NCDO763	NCDO2031	LM0230	LM2301
IS*S1*	*Sma*I	130 [Sm6]	+	+	+ (160)	+	+	+ (105)
		55	−	+	−	+	−	−
	*Apa*I	75 [Ap10C]	+	+	+	+	+	+ (120)
		55	−	+	+	+	−	−
ϕT712	*Sma*I	220* [Sm4]	+	+	+	+	+	+
		180* [Sm5]	+	−	+	−	+	+
		105 [Sm8]	+	+	+	+	−	−
		22 [Sm14]	+	+	+	+	+	+
	*Apa*I	165*	−	+	−	+	−	−
		160* [Ap3]	+	−	+	−	+	+
		49 [Ap14]	+	+	+	+	−	−
		13 [Ap27]	+	+	+	+	−	−
		12 [Ap28]	+	+	+	+	−	−
		33 [Ap19]	+	+	+	+	+	+
Sm8	*Sma*I	105 [Sm8]	+	+	+	+	+ (58)	+ (58)
	*Apa*I	205 [Ap1]	+	+	+	+	+	+
		49 [Ap14A]	+	+	+	+	+ (29)	+ (29)
		13 [Ap27B]	+	+	+	+	−	−
		12 [Ap28]	+	+	+	+	−	−
		8 [Ap29]	+	+	+	+	+	+
Ap3	*Sma*I	180 [Sm5A]	+	+ (220)	+	+ (220)	+	+
	*Apa*I	160 [Ap3]	+	+ (165)	+	+ (165)	+	+
No2	*Not*I	230 [No2]	+	+	+ (200)	+ (175)	+ (175)	+ (175)
	*Sma*I	610 [Sm1]	+	+	+ (580)	+ (550)	+ (550)	+ (550)
	*Apa*I	205 [Ap1]	+	+	+	+	+	+
		145 [Ap4]	+	+	+ (120)	+ (100)	+ (100)	+ (100)
		16 [Ap26]	+	+	+	−	−	−
5′-*cluA*	*Sma*I	610 [Sm1]	+	+	+ (580)	−	−	−
	*Apa*I	50*	+	+	+ (25)	−	−	−
		16 [Ap26]	+	+	+	−	−	−
5′-*oppD*	*Sma*I	130 [Sm6]	+	+	+ (160)	+	+	−
		55	−	+	−	+	+	−
	*Apa*I	75 [Ap10C]	+	+	+	+	+	−
		69* [Ap11]	+	+	+ (19)	+	+ (67)	−
		55	−	+	−	+	+ (65)	−
oppFB[c]	*Sma*I	130 [Sm6]	+	+	+ (160)	+	+	−
		55	−	+	−	+	+ (58)	−
	*Apa*I	75 [Ap10C]	+	+	+	+	+	−
		55	−	+	−	+	+ (65)	−

[a] Fragment names are given in brackets. Asterisks indicate a weak hybridization signal. Sizes different from the size given in this column are shown in parentheses.
[b] NCDO712 refers to NCDO712 and NCDO505 genomes; NCDO763 refers to NCDO763 and MMS36 genomes; and NCDO2031 refers to NCDO2031 and C2 genomes.
[c] Probes *oppCA* and *pepO* gave the same hybridization pattern as *oppFB*.

(iii) Genome rearrangement involving the conjugative sex factor. Lactose plasmid conjugation in strains NCDO712 and NCDO763 frequently involves plasmid cointegration with a 50-kb cryptic conjugative element (sex factor), an event that causes a cell aggregation phenotype and provides high-frequency transfer ability (75). The sex factor is located on a low-copy-number plasmid, pRS01, in strain ML3 (47). In contrast, it has been demonstrated that the sex factor was integrated into the chromosomes of strains NCDO712, NCDO505, and NCDO763 but not of strain NCDO2031 and was located on the largest *Sma*I fragment in strain MG1363 (18). In addition, these authors observed that the sex factor could be present as an occasionally labile extrachromosomal band in strain MG1363. Identification of the genome polymorphism that involved the sex factor was attempted by hybridization experiments using different probes. We have previously shown that the size variation of the Sm1 fragment (610 kb) is associated with the same size variation of the No2 fragment (230 kb) in strains NCDO763, NCDO2031, and LM2301 (37). Thus, fragment No2 of the MG12363 chromosome was used as probe for Southern hybridization with the genome of the nine strains. To demonstrate that this chromosomal rearrangement was strictly linked to the presence of the sex factor, we performed a similar experiment using the 5′ end of the *cluA* gene as a probe. This gene, cloned from the sex factor of strain MG1363 (20), contains an *Apa*I site and has been precisely located on the MG1363 genome map (Fig. 3).

Hybridization results, summarized in Table 2, clearly showed that the genomes of strains NCDO2031, LM0230, and LM2301 had a deletion of 50 kb for *Sma*I and *Not*I hybridization and that this event was strictly linked to the chromosomal excision of the entire sex factor. The *Apa*I rearrangement observed for these strains could be explained by the excision of the sex factor and therefore the removal of the *Apa*I site of the *cluA* gene, corresponding to the Ap4-Ap26 fragment junction. The genome of strain NCDO763 had a deletion of 30 kb in or close to the sex factor. In addition, the *cluA* probe revealed the presence of a 50-kb extrachromosomal labile form of the sex factor for strains MG1363 and NCDO712 and a 25-kb labile form for strain NCDO763.

(iv) Genome rearrangement associated with the location of the oligopeptide transport operon (Opp). The Opp system plays a crucial role in the utilization of oligopeptides as a nitrogen source during the growth of *L. lactis* in milk (28, 31). The genes encoding the Opp system are organized in an operon-like structure (*oppDFBCA*), together with a gene (*pepO*) encoding an endopeptidase (74). It was found that the *oppDFCBA-pepO* operon is located on the chromosome in strains NCDO712, NCDO763, MG1363, and LM0230 and either on the chromosome or on the Lac-Prt plasmid in strain C2 (78). In addition, it was shown that strain LM2301 was devoid of the *opp* operon. This operon has been located on Sm6 (105-kb) and Ap10C (75-kb) fragments on MG1363 chromosome (Fig. 3). To study which restriction polymorphism is associated with the various locations of this operon, hybridization experiments were undertaken using different probes. Four regions of the *opp-pepO* operon (*oppFB*, *oppCA*, *pepO* and 5′ end of *oppD*) were used as probes for Southern hybridization with the *Apa*I and *Sma*I fingerprints of the nine strains.

Southern hybridizations using *oppFB*, *oppCA*, or *pepO* as probes (Table 2) revealed that all strains except LM2301 contained a chromosomal copy of the *opp-pepO* operon at the same location as strain MG1363 and confirmed that strain LM2301 lacked the entire operon on its chromosome. IS*S1* hybridization helped us to determine the size of the *opp* deletion (Table 2). Thus, the shift in size of the Sm6 fragment (from 130 to 105 kb) indicated that the removal of the *opp-pepO* operon was correlated to a deletion of 25 kb. Moreover, this deletion removed the *Apa*I site located at the junction of the two adjacent Ap10C (75 kb) and Ap11 (69 kb) fragments, generating a new *Apa*I fragment of 120 kb (Table 2). Hybridization using the 5′ part of the *oppD* gene as a probe revealed an additional *Apa*I fragment of 69 kb for strains NCDO712, NCDO2031, and MG1363, 67 kb for strain LM0230, and 19 kb for strain NCDO763. The weaker hybridization signal of the *Apa*I fragment adjacent to the Ap10C fragment probably indicated a small duplication of part of this gene near the entire *opp* operon.

Furthermore, strains NCDO712, NCDO505, and NCDO2031 contained an additional 55-kb *Apa*I-*Sma*I fragment that strongly hybridized with all of the *opp-pepO* probes, indicating the presence of a second copy of the entire *opp* operon. Since we have shown that these two fragments correspond to the Lac-Prt plasmid, we concluded that the additional *opp-pepO* operon is located on this plasmid, as also observed for strain C2. Another unexpected result was observed for the plasmid-free strain LM0230, where one additional 65-kb *Apa*I fragment and one additional 58-kb *Sma*I fragment hybridized with all of the *opp-pepO* probes, suggesting that strain LM0230 contained two chromosomal copies of the entire *opp* operon. However, we failed to map the location of this second *opp* operon.

Deduction of the physical map of the NCDO712-NCDO505, NCDO763-MMS36, NCDO2031-C2, LM0230, and LM2301 chromosomes. Characterization of genome rearrangements in the nine strains studied allowed the construction of a physical map of each corresponding chromosome. The five genome maps (Fig. 4) were deduced from the map of the MG1363 chromosome with the following assumptions: (i) common restriction fragments between two strains indicate conserved restriction sites on their genome maps, although fortuitous comigration cannot be ruled out; and (ii) the overall similarity of the patterns suggests that the restriction polymorphisms reflect a small number of changes to the map derived from MG1363, as opposed to an entirely different map for each strain.

DISCUSSION

This report describes the macrorestriction genome analysis of clonal variants of *L. lactis* subsp. *cremoris* strains. These strains can be considered adequate candidates for studying genome plasticity in gram-positive bacteria because their biological relationship is known (13). All strains are derivatives of strain NCDO712, an industrial strain that was isolated and recorded at the National Collection of Dairy Organisms in the United Kingdom in the early 1950s. The strain NCDO712 was subcultured under laboratory conditions for two decades before being rerecorded under different names (NCDO505, NCDO763, and NCDO2031) and/or genetically modified in the early 1980s (MG1363, MMS36, LM0230, and LM2301) (Fig. 1).

Comparative analysis of macrorestriction patterns generated with *Apa*I and *Sma*I endonucleases revealed a high degree of genomic similarity among the nine strains studied, with a similarity coefficient (S_D) greater than 0.8. This result implies that comigrating restriction fragments correspond to conserved chromosomal regions at the genetic level (i.e., conservation of the gene order) as well as at a physical level (i.e., conservation of the location of restriction sites). However, at least two examples of fortuitous comigration of unrelated restriction fragments were observed. In the first case, a 105-kb *Sma*I fragment was present in all but LM0230 fingerprints. Southern hybridization analysis (IS*S1* and Sm8 probes on *Sma*I fragments

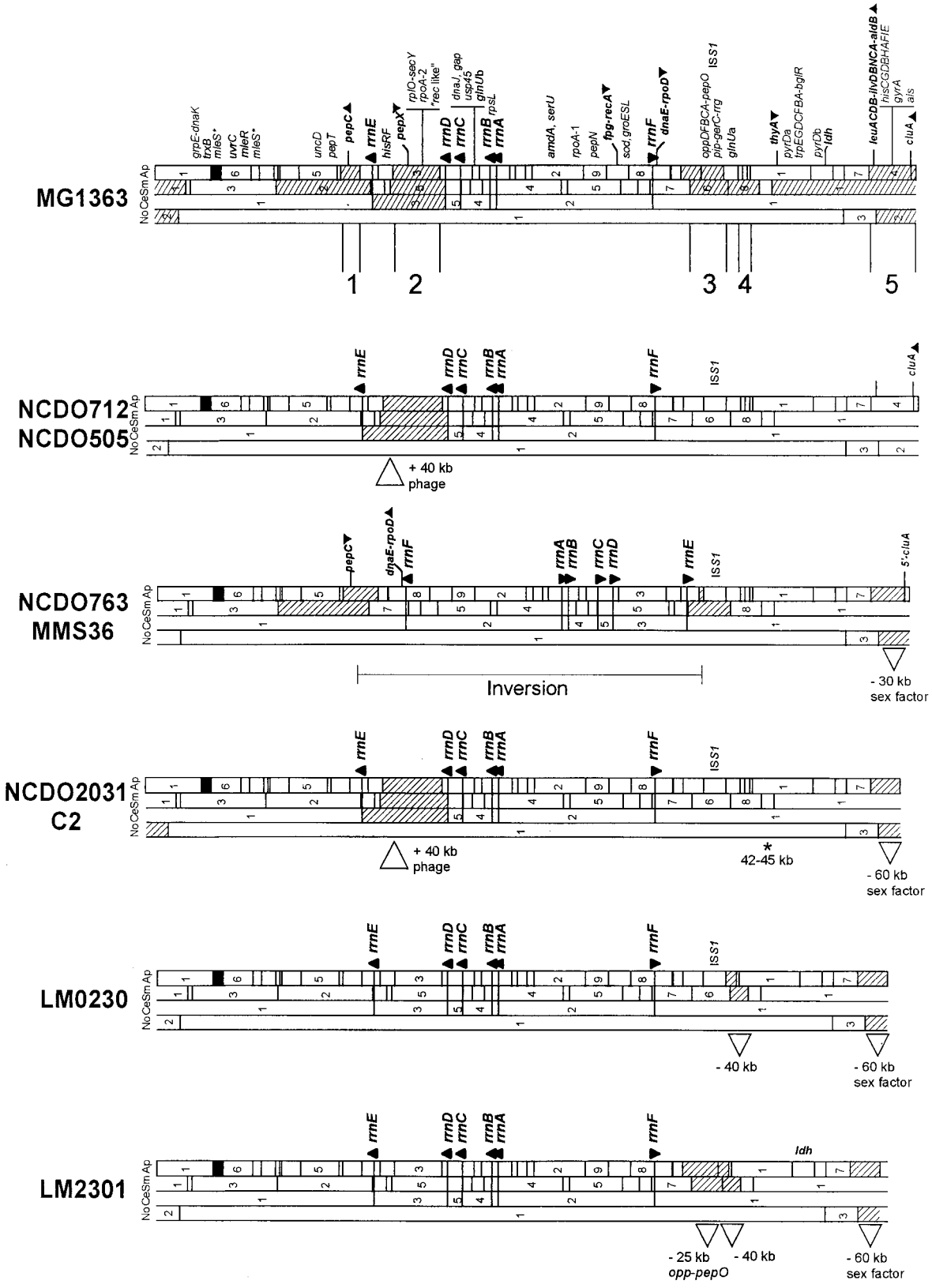

FIG. 4. Chromosome map of the different lactococcal strains deduced from the physical map of the reference strain MG1363. Abbreviations; Sm, *Sma*I; Ap, *Apa*I; No, *Not*I; Ce, I-*Ceu*I.

[Table 2]) clearly demonstrated that the 105-kb *Sma*I fragment in strain LM2301 was unrelated to the 105-kb *Sma*I fragment of other strains but corresponded to the 130-kb *Sma*I fragment in strains NCDO712, NCDO2031, LM0230, and MG1363 and to the 160-kb fragment in strain NCDO763. The second example involved the 120-kb *Apa*I fragment found in the NCDO763 and LM2301 fingerprints. In strain LM2301, this fragment corresponds to the fusion of two fragments (Ap10C and Ap11) due to the deletion of a 25-kb region carrying the *opp-pepO* operon. Strain NCDO763 contains two 120-kb *Apa*I fragments, one that was created by the 30-kb deletion in or near the sex factor region and one that was the result of a large chromosomal inversion that combined the Ap11 and Ap12B fragments (11). Nevertheless, we are confident that fortuitous comigration of independent restriction fragments is rare and that the only consequence may be a slight overestimation of the genomic relatedness between the strains.

The chromosome map of the MG1363 strain was used to construct a genome map of each strain and to present a model that accounts for the observed differences in macrorestriction patterns. From the five polymorphic regions identified, it was possible to correlate a genetic event with each of the RFLPs observed (Fig. 1). Region 1 corresponds to one end of the large inversion of half of the chromosome in strain NCDO763 and its derivative (MMS36). Region 2 corresponds to the presence of a 40-kb unknown element in strains NCDO712, NCDO505, NCDO2031, and C2. Region 3 corresponds to a deletion of 25 kb including the *opp-pepO* operon in strain LM2301 and to the second end of the chromosomal inversion in strains NCDO763 and MMS36. Region 4 corresponds to the integration of the φT712 prophage DNA in strains NCDO712, NCDO505, NCDO763, MMS36, NCDO2031, C2, and MG1363. Region 5 corresponds to the 60-kb deletion of the whole conjugative sex factor region in strains NCDO2031, C2, LM0230, and LM2301 and to a corresponding 30-kb deletion in strains NCDO763 and MMS36.

The nine strains studied differ in their lysogenic status, which is determined by their ability to be lysed after exposure to UV irradiation (13, 46), their lysotypic phenotype, and their genomic content of prophage DNA. Strains NCDO712, NCDO505, NCDO2031, and C2 are all sensitive to UV irradiation, and their chromosome contains one prophage DNA (φT712 or equivalent) located in region 4 and the unknown 40-kb element located in region 2. Strains MG1363, NCDO763, and MMS36 are resistant to UV irradiation as well as to φT712 infection and contain only the prophage DNA located in region 4 of their genome. Strains LM0230 and LM2301 are resistant to UV irradiation but sensitive to spot lysis with φT712 phage and contain neither the prophage DNA in region 4 nor the 40-kb element in region 2. One hypothesis that could correlate the phenotypic and genomic data would be that the φT712 temperate phage is unable to produce infectious particles without the help of the uncharacterized 40-kb element. This unknown element could correspond to a defective prophage that is able to precisely excise itself from the chromosome like the defective phage Rac of *E. coli* (29) and is able to code for some functions that are necessary for the structural integrity of the φT712 virions as does the P2 phage for the P4 phage of *E. coli* (38). Only strains carrying the DNA from the φT712 prophages and the 40-kb element would give φT712 phage particles after UV induction. Strains containing only φT712 prophage would not be UV inducible but would remain resistant to infection by φT712 particles. Only strains devoid of both elements would correspond to authentic phage-cured strains that are not UV inducible and would be sensitive to lysis by φT712 particles.

Another source of genomic rearrangement between the strains studied corresponded to the presence of the chromosomal conjugative sex factor. The strains can be clustered into three groups. The first group, made up of strains NCDO712, NCDO505, and MG1363, contains a complete conjugative factor of 50 kb that is integrated at the target site of the chromosome. This element is able to self-excise from the chromosome with an efficiency high enough to be visualized in PFGE. Strains of the second group, NCDO2031, C2, LM0230, and LM2301, have lost the whole conjugative element from their chromosome and are therefore unable to promote high-efficiency conjugation. Strains of the third group, NCDO763 and MMS36, have lost approximately 30 kb in or near the sex factor. It was found that, in addition to the *attP* site of 24 bp, two additional 13-bp direct repeats (*flip1* and *flip2*) are present in the sex factor and are located 30 kb apart (21). As such, it is tempting to postulate that this deletion arose by accidental recombination between the two *flip* sites.

Analysis of the macrorestriction polymorphism associated with the location of *opp-pepO* gave intriguing findings. All strains except LM2301 contained a chromosomal copy of the *opp-pepO* operon. Strains NCDO712, NCDO505, and NCDO2031 contained an additional copy of this operon located on the Lac-Prt plasmid, whereas strain LM0230 seemed to contain a second chromosomal copy of the operon. The differences in copy number and/or chromosomal location, as well as the size of the deletion in the LM2301 strain, which is three times bigger than the size of the operon, suggest that *opp-pepO* belongs to a larger genetic element with a structure similar to a transposon, as already described for the lactococcal *nis-sac* operon (57). A similar duplication of a chromosomal gene was previously observed in *L. lactis* strain NCDO763, where the *pepF* gene was found to be duplicated and the second copy was located in the Lac-Prt plasmid (48).

In conclusion, we found that a physical chromosome map, combined with comparative analysis of PFGE macrorestriction patterns and Southern hybridization experiments, can be used to characterize the genetic events that are responsible for genome rearrangements between genetically related strains. In addition, they can be used to construct genome maps by comparison of restriction patterns without the need for direct experimental investigations for each chromosome.

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