Physical and Genetic Map of the Chromosome of *Lactococcus lactis* subsp. *lactis* IL1403

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A combined physical and genetic map of the chromosome of *Lactococcus lactis* subsp. *lactis* IL1403 was determined. We constructed a restriction map for the *Not*I, *Apa*I, and *Sma*I enzymes. The order of the restriction fragments was determined by using the randomly integrative plasmid pRL1 and by performing indirect end-labeling experiments. The strain IL1403 chromosome was found to be circular and 2,420 kb in size. A total of 24 chromosomal markers were mapped on the chromosome by performing hybridization experiments with gene probes for *L. lactis* and various other bacteria. Integration of pRC1-derived plasmids via homologous recombination allowed more precise location of some lactococcal genes and allowed us to determine the orientation of these genes on the chromosome. Recurrent sequences, such as insertion elements and rRNA gene (*rrn*) clusters, were also mapped. At least seven copies of IS1076 were present and were located on 50% of the chromosome. In contrast, no copy of ISS1RS was detected. Six ribosomal operons were found on the strain IL1403 chromosome; five were located on 16% of the chromosome and were transcribed in the same direction. A comparison of the physical maps of *L. lactis* subsp. *lactis* IL1403 and DL11 showed that these two strains are closely related and that the variable regions are located mainly near the *rrn* gene clusters. In contrast, despite major restriction pattern dissimilarities between *L. lactis* IL1403 and MG1363, the overall genetic organization of the genome seems to be conserved between these two strains.

Lactococci are lactic acid bacteria that are widely used as starter organisms in the production of fermented dairy products. These gram-positive bacteria are phylogenetically related to the family *Streptococcaceae*, as determined by 16S rRNA sequence comparisons (58). Because of the industrial importance of these organisms, the plasmids and bacteriophages of lactococci have been studied particularly intensively (27, 28), but in recent years there has been an increase in interest in the molecular analysis of chromosomally located markers (8, 40, 41, 50, 53, 65). However, little information is available about the structure and organization of the chromosomes of lactococci (33, 61, 63) and streptococci (21, 43).

The development of pulsed-field gel electrophoresis (PFGE), which gives workers the ability to analyze large fragments of DNA in agarose gels (55) and generates restriction patterns of whole bacterial chromosomes, has provided relevant information on genome size. This technique allows workers to characterize or compare strains at the DNA level (24), as well as to follow the genetic history of a particular strain (34). PFGE is also useful for construction of physical and genetic maps of the chromosomes of bacteria that are poorly understood at the genetic level (6, 12, 14, 56). These maps may yield substantial data which are useful in understanding chromosomal dynamics among bacteria (13, 17).

A physical map of a *Lactococcus lactis* subsp. *lactis* strain has been described previously (63). However, the strain used (strain DL11, a proteinase-negative derivative of *L. lactis* ATCC 11454) was poorly characterized at the genetic level (31, 59). In contrast, plasmid-free strain IL1403 (15), together with strain MG1363 (22), is emerging as the most

widely studied *L. lactis* strain. To assess genetic diversity at the intraspecies level, we constructed the first combined physical and genetic map of *L. lactis* subsp. *lactis* IL1403. A partial map of *L. lactis* subsp. *lactis* MG1363 was also constructed. To do this, we used different techniques, such as hybridization with cloned genes from various bacteria, insertion of rare restriction sites into the chromosome, and indirect end labeling.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. lactis* subsp. *lactis* IL1403 (15) and MG1363 (22), two plasmid-free strains, were grown at 30°C in M17 broth (62) supplemented with 0.4% glucose. *Escherichia coli* TG1 [*supE hsdΔ5 thi Δ(lac-proAB)* [*F'* *traD36 proAB⁺ lacI^q lacZΔM15*]] was grown at 37°C with shaking in LB broth. When required, erythromycin was used at the following concentrations: 300 μg/ml for *E. coli* and 10 μg/ml for *L. lactis* subsp. *lactis*. The plasmids and probes used in this study are shown in Tables 1 and 2.

DNA manipulation. Plasmid isolation, restriction digestion, ligation, and transformation in *E. coli* were performed as described by Maniatis et al. (36). *Lactococcus* strains were transformed by electroporation (46), except that the cells were grown in M17 broth supplemented with 0.4% glucose and 2% glycine. Restriction enzymes and T4 DNA ligase were obtained from Boehringer-Mannheim. DNA restriction fragments were purified from an agarose gel by using the procedure of Vogelstein and Gillespie (66). High-molecular-weight chromosomal DNA from *L. lactis* subsp. *lactis* was purified and digested in agarose blocks as described previously (33). *Apa*I or *Sma*I partial digestions of embedded DNA were carried out by using a modification of the method of Albertsen et al. (1); 75% of an agarose block

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[†] Died 29 December 1991. This paper is dedicated to her memory.

TABLE 1. Plasmids

Plasmid	Characteristics	Reference or source
pBluescript KSII		Stratagene
p5A5	4.4-kb <i>NotI</i> - <i>HindIII</i> chromosomal fragment of strain IL1403 cloned into pBluescript KSII	This study
p5A11	4-kb <i>NotI</i> - <i>HindIII</i> chromosomal fragment of strain IL1403 cloned into pBluescript KSII	This study
p5A32	1.2-kb <i>NotI</i> - <i>HindIII</i> chromosomal fragment of strain IL1403 cloned into pBluescript KSII	This study
p5A39	0.5-kb <i>NotI</i> - <i>HindIII</i> chromosomal fragment of strain IL1403 cloned into pBluescript KSII	This study
pRC1	Cloning vector, <i>Em</i> ^r	32
pRL1	1.47-kb <i>HindIII</i> fragment of ISSIRS cloned into pRC1	32
pRL2A	3.16-kb <i>EcoRI</i> insertion of <i>hisBFHA</i> cloned into pRC1 (clockwise)	32
pRL3A	1.8-kb <i>EcoRI</i> insertion of <i>ilvD</i> cloned into pRC1 (clockwise)	This study
pRL4B	2.3-kb <i>EcoRI</i> insertion of <i>mleR</i> cloned into pRC1 (counterclockwise)	This study
pRL5	1.2-kb <i>XbaI</i> - <i>BamHI</i> insertion of <i>thyA</i> cloned into pRC1	This study
pRL6A	2.3-kb <i>EcoRI</i> insertion of <i>uvrC</i> cloned into pRC1 (clockwise)	This study
pRL7A	0.71-kb <i>XbaI</i> insertion of <i>trpC</i> cloned into pRC1 (clockwise)	This study
pRL8A	1.15-kb <i>BamHI</i> - <i>EcoRI</i> insertion of <i>usp45</i> cloned into pRC1	This study

was digested overnight with 20 U of *NotI* and then treated for 1 h at 4°C in 1 ml of TE 10/1 (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and two times for 1 h each at 4°C in 1 ml of the appropriate buffer without Mg²⁺. The agarose block was cut into three pieces, and each of the pieces was incubated for 1 h at room temperature in 200 µl of the appropriate buffer lacking Mg²⁺ but containing 2.5 U of *SmaI* or 5 U of *ApaI*. For each enzyme, digestions were performed for 1 h at 30°C in the presence of 0.1, 0.05, or 0.02 mM Mg²⁺; digestion was stopped by adding 1 ml of TE 10/100 (10 mM Tris-HCl [pH 7.5], 100 mM EDTA) and incubating the preparation for 1 h on ice. *L. lactis* chromosomal DNA was isolated by using a mini-isolation procedure (35). Briefly, this method involved lysis of the cells with lysozyme, proteinase K treatment, a phenol-chloroform step, and ethanol precipitation of total DNA.

Electrophoresis. Agarose (1%) gels were prepared in 0.1 × TBE (1 × TBE is 1 M Tris base, 1 M boric acid, and 20 mM EDTA). PFGE was performed by using a CHEF system

(Pulsaphor Plus; LKB-Pharmacia) and 0.05 × TBE, as described previously (33). Phage λ DNA concatemers, which were used as DNA ladders, were obtained by using the procedure of Waterbury and Lane (68).

DNA transfer and hybridization. DNA was transferred from conventional gels to a nylon membrane (Hybond N⁺; Amersham) by using a vacuum blotting device (model 2016 Vacugene device; LKB-Pharmacia) in the presence of alkali (19). PFGE gels were treated with 0.5 N NaOH–0.15 M NaCl for 30 min with gentle shaking and then for 30 min with 0.5 M Tris-HCl [pH 7.5]–0.15 M NaCl and were dried at 60°C for 1 h by using a model 483 slab dryer (Bio-Rad). The dried gels were stored between two sheets of Whatman 3MM filter paper at room temperature. For high-stringency hybridizations, the membranes or dried gels were prehybridized for 2 h at 42°C in hybridization buffer containing 50% formamide, 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 2.5% skim milk in a hybridization oven (Appligene). The

TABLE 2. Probes used for localizing genes

Plasmid	Gene(s)	Source	Stringency conditions ^a	Reference(s)
pIL727	<i>hisB</i> , ORF8, <i>hisHAF</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	18
pIL510	<i>ilvD</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	23
pIL480	<i>trpC</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	7
pIL800	<i>pepXP</i>	<i>L. lactis</i> subsp. <i>lactis</i> NCDO 763	H	40
pIL1230	<i>trxB</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	49
pNG2	<i>mleR</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	50
pR302	<i>thyA</i>	<i>L. lactis</i> subsp. <i>lactis</i> MG1363	H	52
pUCB423	IS1076	<i>L. lactis</i> subsp. <i>lactis</i> Z270	H	26
pUCB483	ISSIRS	<i>L. lactis</i> subsp. <i>lactis</i> Z270	H	25
pIL560	<i>uvrC</i>	<i>L. lactis</i> subsp. <i>lactis</i> IL1403	H	23
pHC2.2	<i>usp45</i>	<i>L. lactis</i> subsp. <i>lactis</i> MG1363	H	65
pSA136	<i>rpoA</i>	<i>Bacillus subtilis</i>	L	12, 60
pCPS9	<i>rpoD</i>	<i>Bacillus subtilis</i>	L	47
p5403	<i>tag-3</i> , <i>gtaD1</i> , <i>gtaA12</i>	<i>Bacillus subtilis</i>	L	37
pBSC505	<i>uncD</i>	<i>E. coli</i>	L	67
pORF266	<i>groE</i>	<i>E. coli</i>	L	12
pNO1523	<i>rpsL</i>	<i>E. coli</i>	L	12
pBC15	16S <i>rrnA</i>	<i>Clostridium perfringens</i>	H	20
pBC23S	23S <i>rrnI</i>	<i>Clostridium perfringens</i>	H	11
pGYRA	<i>gyrA</i>	<i>Clostridium perfringens</i>	L	12
pTG2215	<i>rec</i> -like	<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>	L	57
pSP61	<i>ung</i>	<i>Streptococcus pneumoniae</i>	L	38
pR244	<i>amiA</i>	<i>Streptococcus pneumoniae</i>	L	3
pR272	<i>hexA</i> , <i>hexB</i>	<i>Streptococcus pneumoniae</i>	L	48

^a H, high stringency; L, low stringency.

TABLE 3. Sizes of PFGE-separated restriction fragments of strain IL1403 genomic DNA

Restriction enzyme	Fragment	Size (kb) ^a
<i>ApaI</i>	Ap1	215
	Ap2	215
	Ap3	190
	Ap4	180
	Ap5	150
	Ap6	140
	Ap7	130
	Ap8	110
	Ap9	105
	Ap10	100
	Ap11	100
	Ap12	92
	Ap13	88
	Ap14	80
	Ap15	72
	Ap16	55
	Ap17	50
	Ap18	45
	Ap19	43
	Ap20	41
	Ap21	33
	Ap22	33
	Ap23	27
	Ap24	23
	Ap25	22
	Ap26	18
	Ap27	18
	Ap28	16
	Ap29	13
	Ap30	9
	Ap31	2
<i>SmaI</i>	Sm1	765
	Sm2	235
	Sm3	145
	Sm4	135
	Sm5	112
	Sm6	108
	Sm7	104
	Sm8	95
	Sm9	82
	Sm10	77
	Sm11	77
	Sm12	74
	Sm13	68
	Sm14	67
	Sm15	49
	Sm16	49
	Sm17	48
	Sm18	42
	Sm19	38

Continued

probe was added, and hybridization was allowed to proceed overnight at 42°C. The washing steps were as follows: two times in 2× SSC–0.1% SDS for 20 min at room temperature and two times in 0.1× SSC–0.1% SDS for 20 min at 60°C. For low-stringency hybridizations at 42°C the hybridization buffer contained 30% formamide, 6× SSC, 5 mM EDTA, 0.1% SDS, and 2.5% skim milk, and the preparations were washed two times in 2× SSC–0.1% SDS for 20 min at room temperature and two times in 2× SSC–0.1% SDS for 20 min at 55°C. DNA probes were labeled with [α -³²P]dATP by using the Megaprime DNA labeling system (Amersham).

TABLE 3—Continued.

Restriction enzyme	Fragment	Size (kb) ^a
	Sm20	22
	Sm21	18
	Sm22	5
	Sm23	4
<i>NotI</i>	No1	1,370
	No2	555
	No3	495

^a Averages from two to four determinations. The maximal deviation in size was estimated to be 5%. The total for the *ApaI* fragments was 2,415 kb, the total for the *SmaI* fragments was 2,420 kb, and the total for the *NotI* fragments was 2,420 kb.

RESULTS

Construction of the physical map of the strain IL1403 chromosome. (i) **Identification of the chromosomal *NotI*, *SmaI*, and *ApaI* fragments.** A previous analysis of lactococcal genomes showed that *NotI*, *SmaI*, and *ApaI* are the most appropriate enzymes to produce distributions of DNA fragments that are easily resolved by PFGE (33); for *L. lactis* subsp. *lactis* IL1403 these enzymes generate 3, 23, and 31 restriction fragments, respectively. The sizes of these fragments are shown in Table 3. The genome size of strain IL1403 deduced from these values is 2,420 kb.

SmaI and *ApaI* restriction fragments were allocated to the different *NotI* fragments as follows: each PFGE-separated *NotI* fragment was excised from the gel, digested until completion with *SmaI* or *ApaI*, and subjected to PFGE again. However, the lower resolution of restriction fragments after the second electrophoresis did not allow unambiguous location of fragments smaller than 50 kb.

The relative positions of the *ApaI* and *SmaI* fragments overlapping the *NotI* sites were determined by using the following strategy: *NotI*-*HindIII* chromosomal fragments of strain IL1403 DNA were cloned into plasmid pBluescript KSII. Four of six different fragments were isolated and characterized (Table 1).

The recombinant plasmids described above were used as probes in hybridization experiments with PFGE-separated chromosomal restriction fragments produced by *NotI*, *ApaI*, *SmaI*, *NotI* plus *SmaI*, and *NotI* plus *ApaI* (Table 4). For example, the hybridization data obtained when plasmids p5A5 and p5A11 were used as probes are shown in Fig. 1, and these data demonstrate that fragments Ap29 and Sm1 overlapped the *NotI*-B site (located between fragments No2 and No3). The relative positions of these fragments with respect to the *NotI*-B site were deduced from the sizes of the hybridizing fragments in single and double digests.

(ii) **Mapping of randomly integrated rare restriction sites.** Genetic tools that facilitate the construction of chromosomal maps of lactococcal strains have been described recently (32). Plasmid pRL1 contains a functional insertion element (ISS/RS) and is not able to replicate in *Lactococcus* strains. When introduced into *L. lactis*, this plasmid can be integrated into the chromosome via transposition. The structure resulting from plasmid integration is a duplication of the insertion element sequence that brackets the plasmid DNA (Fig. 2). This integration leads to the generation of one additional *NotI*, *SmaI*, and *ApaI* restriction site at the insertion point. Hybridization experiments with chromosomal restriction digests in which pRL1 was used as the

TABLE 4. Localization of the *NotI* site overlapping fragments

Chromosomal restriction digestion with:	Restriction fragments that hybridized with the following probes:			
	p5A5	p5A11	p5A32	p5A39
<i>NotI</i>	No2 (550) ^a	No3 (500)	No3 (500)	No1 (1,370)
<i>ApaI</i>	Ap29 (13)	Ap29 (13)	Ap1 (215)	Ap25 (22)
<i>SmaI</i>	Sm1 (765)	Sm1 (765)	Sm17 (48)	Sm20 (22)
<i>NotI</i> plus <i>SmaI</i>	No2-Sm1 (480)	Sm1-No3 (285)	No3-Sm17 (10)	No1-Sm20 (18)
<i>NotI</i> plus <i>ApaI</i>	No2-Ap29 (8)	Ap29-No3 (5)	No3-Ap1 (130)	No1-Ap25 (18.5)

^a The numbers in parentheses are the sizes of the restriction fragments (in kilobases).

probe allowed rapid and accurate localization of the integrated plasmid into a restriction fragment, determination of the relative positions of *NotI*, *SmaI*, and *ApaI* sites close to the insertion point, and orientation of the integrated plasmid. For example, in strain CL148-7 (Table 5 and Fig. 3B), restriction fragments Ap3 (190 kb), Sm6 (108 kb), and No1 (1,370 kb) were missing, and each of these fragments was replaced by two new fragments, whose estimated sizes were 45 and 150, 65 and 50, and 500 and 875 kb, respectively. The sum of the two fragment sizes was always slightly greater than the original size because of the presence of the 5-kb plasmid pRL1 in one fragment and the presence of the 0.8-kb ISS/RS in the other fragment (Fig. 2). Because of the asymmetric distribution of the rare restriction sites in plasmid pRL1, the fragment containing pRL1 always gave a stronger signal than the fragment containing only the insertion element sequence, when pRL1 was used as the probe. This difference in signal intensity allowed the location of the restriction sites close to the integration site (Fig. 3B).

An analysis of 35 recombinant clones (Table 5) allowed us to map 65% of the *ApaI* and *SmaI* sites on the chromosome. With 35 random insertions, we expected insertion to occur

every 70 kb along the chromosome. The locations of the 35 integration events on the strain IL1403 physical map showed that pRL1 integrations were distributed equally along the chromosome (Fig. 4), but there was at least one large region in which no insertions were identified (between 960 and 1,300 kb) (Fig. 5).

(iii) Physical mapping by indirect end labeling. The un-

FIG. 1. Hybridization of PFGE-separated chromosomal DNA of strain IL1403 with p5A5 as the probe (A) and with p5A11 as the probe (B). The running conditions were as follows: 275 V, 8 s, 10 h. The gel was treated as described in Materials and Methods. Lanes L₁, phage λ DNA concatemers; lanes L₂, *Hind*III-digested λ DNA and *ApaI*-digested λ DNA; lanes 1, *NotI*-*ApaI* digestion; lanes 2, *ApaI* digestion; lanes 3, *SmaI* digestion; lanes 4, *NotI*-*SmaI* digestion.

FIG. 2. Schematic representation of the chromosomal structure after insertion element-mediated integration of plasmid pRL1. Abbreviations: IS, insertion element; A, *ApaI*; S, *SmaI*; N, *NotI*; Er^R, erythromycin resistance gene. The open boxes indicate the positions of the ISS/RS element. The restriction fragments that gave strong or weak signals when they were hybridized with pRL1 are indicated. The arrow at the bottom indicates the direction of chromosome mapping by partial restriction digestion (indirect end labeling).

TABLE 5. Analysis of the insertion element-promoted integration of pRL1 into the strain IL1403 chromosome

Clone	<i>Apa</i> I fragments (kb)		<i>Sma</i> I fragments (kb)		<i>Not</i> I fragments (kb)	
	Absent	Present ^a	Absent	Present ^a	Absent	Present ^a
CL148-1	105	58 + 52	68	49 + 24	550	60 + 495
CL148-2 ^b	190	170 + 25	135	68 + 67	1,370	380 + (995) ^c
CL148-3	88	70 + 28	765	195 + 575	500	395 + 110
CL148-4	100	30 + 74	104	3 + 107	1,370	95 + (1,280)
CL148-5 ^b	215	27 + 195	235	30 + 210	1,370	75 + (1,300)
CL148-6 ^b	88	27 + 70	765	240 + 530	500	435 + 55
CL148-7 ^b	190	45 + 150	108	65 + 50	1,370	500 + (875)
CL148-8 ^b	190	56 + 140	135	30 + 110	1,370	(955) + 420
CL148-9 ^b	50	45 + 10	765	430 + 340	550	490 + 55
CL148-10	100	33 + 72	67	67 + 5	1,370	90 + (1,285)
CL148-11 ^b	180	62 + 123	765	350 + 420	550	430 + 130
CL148-12 ^b	72	30 + 50	104	50 + 60	1,370	(1,220) + 155
CL148-13 ^b	43	33 + 15	42	14 + 35	1,370	690 + 690
CL148-14	180	20 + 165	765	400 + 370	550	480 + 75
CL148-15 ^b	16	10 + 12	765	570 + 200	550	280 + 275
CL148-16 ^b	150	55 + 100	765	685 + 85	500	220 + 270
CL148-17 ^b	92	33 + 70	765	700 + 70	550	420 + 135
CL148-18 ^b	45	5 + 45	82	34 + 53	500	365 + 135
CL148-19 ^b	180	40 + 145	765	520 + 250	550	200 + 355
CL148-20	105	70 + 40	765	760 + 10	550	470 + 85
CL148-22	215	115 + 105	74	60 + 22	500	475 + 30
CL148-23 ^b	130	50 + 85	135	5 + 135	1,370	310 + (1,065)
CL148-24	215	170 + 50	95	65 + 35	1,370	610 + (765)
CL148-26	215	140 + 70	145	80 + 70	1,370	510 + (865)
CL148-28	190	155 + 40	108	45 + 75	1,370	(855) + 520
CL148-29	41	15 + 30	112	25 + 95	1,370	210 + (1,165)
CL148-30	41	3 + 45	112	45 + 80	1,370	230 + (1,145)
CL148-35 ^b	100	50 + 55	67	57 + 15	1,370	80 + (1,295)
CL148-37 ^b	215	8 + 210	145	140 + 12	1,370	(925) + 450
CL148-38 ^b	23	8 + 20	95	20 + 85	1,370	(710) + 665
CL148-39 ^b	215	100 + 110	145	110 + 45	1,370	550 + (825)
CL148-41	215	70 + 150	49	30 + 25	1,370	20 + (1,355)
CL148-43	190	40 + 155	135	55 + 85	1,370	(975) + 400
CL148-47	100	90 + 13	104	85 + 25	1,370	(1,260) + 115
CL148-48 ^b	140	130 + 15	38	9 + 35	1,370	(945) + 430

^a The second fragment of each pair gave a strong signal when it was hybridized with pRL1.

^b Strain used for indirect end labeling.

^c Parentheses indicate that the fragment size was deduced from the total size of fragment No1.

mapped restriction sites were located by using the indirect end-labeling strategy (12), which allows the order of restriction fragments to be established relative to a fixed point. To do this, chromosomal DNAs from strains containing integrated pRL1 in pertinent positions were analyzed by hybridization, using pRC1 as a probe, after partial digestions. An illustration of the strategy is given by the analysis of strain CL148-7 described below. Chromosomal DNA from strain CL148-7 was first totally digested with *Not*I and subjected to partial *Apa*I or *Sma*I digestion, and PFGE-separated fragments were hybridized to the pRC1 probe (Fig. 3A and B). For *Apa*I partial restriction digestion, five fragments (approximately 45, 63, 145, 190, and 210 kb) hybridized with pRC1. This seemed to indicate that the order of *Apa*I fragments from the insertion site was Ap3(190 kb)-Ap26(18 kb)-Ap14(80 kb)-Ap19(42 kb)-Ap24(23 kb) in the clockwise direction according to the map shown in Fig. 5. For *Sma*I partial restriction digestion, the sizes of the hybridizing fragments (50, 70, and 145 kb) suggested that the adjacent fragments were Sm6(108 kb)-Sm21(18 kb)-Sm11(77 kb) in the same direction. These results are in agreement with the results obtained in an analysis of the chromosomal DNAs of strains CL148-28 and CL148-13 (Table 5). The results of indirect end-labeling experiments performed with other in-

tegration sites (Table 5) yielded a physical map of the strain IL1403 chromosome, except for the locations of fragments Ap31 (2 kb), Sm22 (5 kb), and Sm23 (4 kb).

Construction of the genetic map of the strain IL1403 chromosome. In order to combine a genetic map with the physical map, different chromosomally encoded markers (Table 2) were located by hybridizing lactococcal probes or heterologous probes from various bacteria to PFGE-separated restriction fragments.

(i) **Mapping of some recurrent chromosomal sequences.** (a) *rrn* operons. *L. lactis* strains contain six *rrn* operons on their chromosomes (29, 63). A restriction analysis of one lactococcal rRNA operon showed that it contains one *Sma*I site at the 3' end of the 16S rRNA gene and that its organization is similar to that described in the general model proposed for eubacteria (i.e., 16S rRNA-23S rRNA-5S rRNA gene order) (8). The six *rrn* operons were mapped and oriented on the chromosome of strain IL1403 by hybridization with probes specific for the 5' end of the 16S gene (pBC15) and for the 3' end of the 23S gene (pBC23S) derived from *Clostridium perfringens*. Chromosomal DNA was separated by PFGE after single or double digestions with *Not*I plus *Apa*I, *Apa*I, *Apa*I plus *Sma*I, *Sma*I, and *Not*I plus *Sma*I and was hybridized with each *rrn* probe. Six fragments hybridized

FIG. 3. Physical map of the strain CL148-7 chromosome in the region of the pRL1 insertion. (A) Hybridization of partially digested chromosomal DNA in which pRC1 was used as the probe. DNA was digested overnight with *NorI* and for 1 h with *ApaI* (lanes 2 to 4) or *SmaI* (lanes 5 to 8) in the presence of different concentrations of Mg^{2+} . Lane 1, *NorI-ApaI* total digestion; lane 5, *NorI-SmaI* total digestion; lanes 2 and 6, 0.1 mM $MgCl_2$; lanes 3 and 7, 0.05 mM $MgCl_2$; lanes 4 and 8, 0.02 mM $MgCl_2$; lane L_2 , λ DNA concatemers; lane L_1 , *HindIII*-digested λ DNA and *ApaI*-digested λ DNA. (B) Restriction map of the insertion region of the strain CL148-7 chromosome. Abbreviations: A, *ApaI*; S, *SmaI*; N, *NorI*. The arrowhead indicates the position of the pRL1 integration site.

with each probe, demonstrating that six operons are present on the chromosome (Table 6). The fact that the hybridization patterns of the *ApaI* fragments were different when we used 16S or 23S probes suggested that one *ApaI* site could be located in or very close to each *rrn* operon. The locations of fragments Sm22, Sm23, and Ap31 on the physical map were deduced from the ribosomal operon position (Fig. 5). Since fragments Sm22 and Ap31 hybridized only with the 16S probe and fragment Sm23 hybridized only with the 23S probe, the locations of these fragments could be between fragments Sm11 and Sm18 for fragment Sm22, between fragments Sm3 and Sm19 for fragment Sm23, and between fragments Ap3 and Ap26 for fragment Ap31.

(b) **Location of lactococcal insertion elements.** We attempted to map two different lactococcal insertion elements

FIG. 4. Genomic locations of the integrated pRL1 plasmid. The arrows indicate the directions of indirect end labeling on the chromosome. IS- x corresponds to CL148- x in Table 5, where x is a number from 1 to 48.

on the chromosome. First, the *ISS/RS* insertion sequence, which was cloned from lactose protease plasmid pUCL22 of *L. lactis* subsp. *lactis* Z270 and was closely related to lactococcal *ISS1* (25), was used as a probe. In contrast to previous results (54), no hybridization signal was obtained, demonstrating the absence of the *ISS/RS* element on the strain IL1403 chromosome. The second insertion element probed was lactococcal insertion element *IS1076*, which was cloned from plasmid pUCL22 and was closely related to the lactococcal *IS904* insertion sequence (26). Hybridization data from PFGE-separated chromosomal fragments revealed that at least seven copies of *IS1076* are present on the chromosome (Table 6) and are located on 50% of the chromosome (Fig. 5). One of the chromosomal *ApaI* fragments gave a weak signal when it was hybridized with the probe, suggesting the presence of one *IS1076* sequence or of a DNA segment with less sequence homology. Southern blots of chromosomal DNA digested with *HindIII*, *EcoRI*, or *PstI* and hybridized to the *IS1076* probe revealed the presence of nine copies of *IS1076* on the chromosome (data not shown).

(ii) **Mapping of lactococcal genes.** Most of the available lactococcal genes (Table 2) were mapped on the chromosome by performing hybridization experiments under high-stringency conditions. The results are shown in Table 6. For some genes, mapping was achieved by using another procedure. We have shown previously that when a lactococcal gene is introduced into plasmid pRC1, the resulting plasmid allows the accurate location of the gene on the chromosome by integration via homologous recombination (32). In addition, if the transcription direction of the gene is known, the gene orientation (5'-3') on the chromosome can be deduced from hybridization data. The positions of the lactococcal *his*,

FIG. 5. Physical and genetic map of the *L. lactis* subsp. *lactis* IL1403 chromosome. Restriction fragment designations are indicated in some cases. The *Apa*I restriction fragment order is as follows: Ap25-Ap10-Ap15-Ap20-Ap7-Ap3-Ap31-Ap26-Ap14-Ap19-Ap24-Ap2-Ap6-Ap11-Ap8-Ap1-Ap18-Ap21-Ap30-Ap5-Ap13-Ap27-Ap29-Ap17-Ap4-Ap22-Ap28-Ap16-Ap27-Ap12-Ap9. The *Sma*I restriction fragment order is Sm20-Sm10-Sm7-Sm5-Sm4-Sm6-Sm21-Sm11-Sm22-Sm18-Sm8-Sm3-Sm23-Sm19-Sm14-Sm16-Sm2-Sm17-Sm12-Sm9-Sm15-Sm1-Sm13. In order to facilitate comparisons with other maps, the strain IL1403 chromosome was oriented as described by Tulloch et al. (63), and the scale was drawn in percentage of the total size. Abbreviations: No, *Nor*I fragment; Sm, *Sma*I fragment; Ap, *Apa*I fragment. The arrows indicate the 5'-3' orientations of the genes. Parentheses indicate genes which hybridized weakly with the corresponding probe.

ilvD, *mleR*, *thyA*, *trpC*, and *uvrC* genes and their orientations on the chromosome were determined (Table 6 and Fig. 5) with this method by using the recombinant plasmids derived from pRC1 (Table 1).

(iii) **Mapping of heterologous genes.** Since very few genes involved in the metabolism of lactococci have been isolated, the presence of some genes that code for housekeeping functions was tested by using probes from other bacterial species (Table 2). These heterologous probes originated from genes involved in aspects of DNA and RNA metabolism, including DNA mismatch repair (*hexA*, *hexB*, and *ung*), DNA recombination (*rec*-like), DNA topology (*gyrA*), mRNA synthesis (*rpoA* and *rpoD*), and ribosomal proteins (*rpsL*). The presence of ATPase (*uncD*), the oligopeptide transport system (*ami*), the heat shock protein (*groE*), and enzymes involved in the biosynthesis of teichoic acids (*gtaA*, *gtaD*, and *tag*) was also determined. The gene probes were preferentially chosen from bacteria which have low DNA G+C contents (*Streptococcus pneumoniae*, *Clostridium perfringens*, *Streptococcus salivarius* subsp. *thermophilus*, and *Bacillus subtilis*). A comparison of nucleotide sequences in bacteria revealed that codon composition correlates with overall genomic composition and that differences in codon composition occur to the greatest extent in the third base position (39). We believed that the level of DNA sequence divergence between the genes of two unrelated bacteria might be minimized if the two bacteria have approximately the same G+C content. The experiments performed to assign loci on the *L. lactis* chromosome, in which we used heterologous genes and low-stringency Southern hybridization, allowed us to identify related DNA sequences, but could not ensure the identity (in coding

function) of the hybridizing sequences and the heterologous gene probe. Our results are summarized in Table 6 and Fig. 5.

Comparison of the genetic organizations of the strain IL1403 and MG1363 chromosomes. A partial physical and genetic map (980 kb) of the *L. lactis* MG1363 chromosome was constructed by using the same strategy as that used for the strain IL1403 genome map. The *trpC* gene was located on the strain MG1363 chromosome only when hybridization was done under low-stringency conditions. A nucleotide sequence comparison of the strain IL1403 and MG1363 *trpC* genes revealed large differences (7).

A comparison of the two chromosomal maps, representing about 45% of the total chromosome size, showed that there is no similarity between the two physical maps. In contrast, the genetic organization seemed to be conserved between the two strains, except for the location of insertion sequence *IS1076* (Fig. 6).

DISCUSSION

In this paper we describe the construction of the first combined physical and genetic chromosomal map of a lactococcal laboratory strain. A physical map of *L. lactis* subsp. *lactis* DL11 is available (63), but this strain, which was derived from strain ATCC 11454, has not been well characterized and is not widely used for genetic studies. The construction of the restriction map was greatly facilitated by the use of integrative plasmid pRL1. Our method, which consisted of the insertion of additional rare restriction sites at random in the chromosome, enabled us to construct the physical map rapidly. The relatively high integration effi-

TABLE 6. Locations of chromosomal genes

Marker	Restriction fragment sizes (kb)					Map position (kb)	Map position (%)
	<i>NotI-ApaI</i>	<i>ApaI</i>	<i>ApaI-SmaI</i>	<i>SmaI</i>	<i>NotI-SmaI</i>		
Homologous genes ^a							
<i>hisF</i> , ORF8, <i>hisHAF</i>	150	150	150	765	285	1,680	69.3
<i>ihvD</i>	150	150	150	765	285	1,670	69
<i>trpC</i>	130	215	74	74	74	1,445	59.7
<i>pepXP</i>	215	215	72	95	95	717–788	29.6–32.5
<i>trxB</i>	180	180	180	765	480	1,920–2,035	79.3–84.1
<i>mleR</i>	180	180	180	765	480	2,035	84.1
<i>uvrC</i>	180	180	180	765	480	2,075	85.7
<i>thyA</i>	85	215	48	48	38	1,340	55.4
<i>usp45</i>	80	80	77	77	77	570–646	23.5–26.7
Heterologous genes ^b							
<i>rpoA</i>	130 (215) ^c	130 (215)	75 (72)	112 (95)	112 (95)	232–307 (717–788)	9.6–12.7 (29.6–32.5)
<i>rpoD</i>	100	100	77	77	77	19–86	0.8–3.5
<i>uncD</i>	100	100	35	49	49	1,052–1,065	43.5–44
<i>ung</i>	80	80	77	77	77	570–646	23.5–26.7
<i>ami</i>	72 ^d	72 ^d	(72)	(104)	(104)	119–191	4.9–7.9
<i>hexA</i>	80	80	77	77	77	570–646	23.5–26.7
<i>gyrA</i>	88	88	88	765	285	1,740–1,828	71.9–75.5
<i>rec</i> -like	23	23	23	95	95	694–717	28.6–29.6
<i>rpsL</i>	18	18	18	18	18	552–570	22.8–23.5
Recurrent sequences							
IS1076							
A	190	190	106	108	108	442–550	18.3–22.7
B	190	190	80	135	135	362–442	14.9–18.3
C	72	72	72	104	104	119–191	4.9–7.9
D	105	105	35	765	480	2,312–2,348	95.5–97
E	(180)	(180)	(180)	(765)	(480)	(1,920–2,097)	(79.3–86.6)
F	23	23	23	95	95	694–717	28.7–29.6
G	215	215	75	95	95	717–788	29.6–32.5
16S <i>rm</i>							
A	2	2	2	108	108	552	22.8
B	18	18	18	18	18	570	23.5
C	80	80	4.5	5	5	651	26.9
D	43	43	41	42	42	693	28.6
E	215	215	140	145	145	932	38.5
F	3.5	22	22	22	4	2,416	99.8
23S <i>rm</i>							
A	18	18	18	18	18		
B	80	80	77	77	77		
C	43	43	41	42	42		
D	23	23	23	95	95		
E	140	140	4	4	4		
F	105	105	68	68	68		

^a There is no copy of the ISS/RS marker on the strain IL1403 chromosome.

^b For the *hexB*, *groE*, and *tag-gtaDA* markers there is no homology at the DNA level.

^c Parentheses indicate chromosomal fragments which gave weak signals when they were hybridized to the probe.

^d Signal not observed but deduced from the physical map.

ciency of pRL1 (32) permitted us to obtain all of the insertions analyzed in only one electroporation experiment. A total of 35 pRL1 insertions around the genome allowed us to identify the overlapping restriction fragments at each insertion site and to determine the fragment order in this region by indirect end-labeling experiments. This approach, combined with hybridization data, minimized the risk of mistakes in the restriction map, especially in the case of fragments which have similar sizes.

An accurate genetic map was constructed by inserting rare restriction sites in the gene to be mapped via homologous recombination, using recombined plasmid pRC1. The amino acid biosynthesis genes are not clustered on the chromosome. Lactococcal gene *usp45* was not located precisely on the strain IL1403 chromosome; no recombinant clones were obtained after electroporation of plasmid pRL8. No *L. lactis*

strains defective in the synthesis of Usp45 protein have been isolated (65). One explanation is that, since an internal fragment of the *usp45* gene was used for the homologous recombination, the insertion of plasmid pRL8 led to gene disruption, which had a lethal effect. Our results showed that the lactococcal genes that were precisely mapped were transcribed in the same direction as the *rm* operons. These orientations may be in agreement with the results observed in *E. coli* and *Bacillus subtilis*, in which 66% (5) and 96% (71) of the genes, respectively were oriented with their 5' ends proximal to the replication origin. Some heterologous genes were not mapped on the strain IL1403 chromosome because no hybridization signal was obtained, indicating either a lack of the corresponding gene in *Lactococcus* strains or too much sequence divergence between the two genes. However, in the case of the *groE* gene, previous immunological

FIG. 6. Genomic comparison of strains IL1403 and MG1363. Restriction fragment designations are shown. Abbreviations: No, *Not*I fragment; Sm, *Sma*I fragment; Ap, *Apa*I fragment. The arrowheads indicate the 5'-3' orientations of the genes.

data have demonstrated the existence of heat shock proteins GroEL and DnaK in lactococcal strains (69).

The availability of the *Sma*I restriction maps of *L. lactis* subsp. *lactis* IL1403 and DL11 and the positions of the rRNA operons allowed a precise comparison of the physical maps of the two strains in order to assess genomic diversity at the intraspecies level. A good correspondence in the *Sma*I restriction site positions was observed on approximately 60% of the chromosome (between positions 40 and 100 in Fig. 5); fragments Sm13, Sm1, Sm15, Sm17, Sm2, and Sm16 might be strain DL11 fragments Sm1, SmA, SmJ3, SmJ2, SmB, and SmJ1, respectively. Fragments Sm9 and Sm12 of strain IL1403 might result from insertion into strain DL11 fragment SmF of genetic element(s) containing a unique *Sma*I site with a size of approximately 60 kb. The 40% of the chromosome (between positions 0 and 40 in Fig. 5) that contains the six rRNA operons seems to be subject to more genetic variation, since only the *Sma*I fragments close to the rRNA operons were conserved in the two strains. Thus, fragments Sm20, Sm21, Sm22, Sm18, and Sm23 might correspond to strain DL11 fragments SmL, SmM, SmO, SmK, and SmN, respectively.

A striking similarity between the two strains was observed in the chromosomal positions of the rRNA operons; for strain IL1403 five operons were clustered on 16% of the chromosome and were transcribed in the same direction (clockwise according to Tulloch et al. [63]). The six operons were localized on 40% of the genome. This rRNA location is analogous to that found in many bacteria, such as *Brucella melitensis* (2), *Bacillus subtilis* (44), *Campylobacter jejuni* (42), *Clostridium perfringens* (12), *E. coli* (5), *Haemophilus influenzae* (10), *Streptococcus pneumoniae* (21), and *Salmonella typhimurium* (70), in which rRNA operons are located on 37 to 52% of the chromosome. However, when the strain IL1403 and DL11 maps were compared meticulously, the chromosomal region located between the rRNA clusters seemed to be variable in size. The distance between *rnmF* and *rnmA* is shorter by at least 50 kb on the strain DL11 chromosome, whereas the distance between *rnmB* and *rnmC* is larger by at least 50 kb in strain DL11. *rnmD* and *rnmE* are approximately the same distance apart in the two strains.

The variation in size observed in the chromosomal DNA segments between the two strains might be due to insertions of DNA elements rather than deletions, since, as has been described for other organisms, deletions are much less frequent, probably because they have deleterious consequences (30). An unexpected finding was that most of the variable regions of the chromosomes of the two strains were located between the *rnm* gene clusters. This suggests that one mechanism for genomic diversification in *L. lactis* could involve horizontal transfer of DNA and homologous recombination between *rnm* genes, leading to segmental exchange, as has been found in *E. coli* and *Salmonella typhimurium* (4). A similar observation has been made in *Clostridium* strains (13). The nature of the variable regions in these two strains is currently under investigation. Strain IL1403 is known to have at least two prophages (16), whereas strain DL11 is able to ferment sucrose and to produce nisin (31).

Our preliminary results for strain MG1363 indicated that the restriction maps of strains IL1403 and MG1363 differ from one another, but that the overall gene positions seem to be preserved. Such analogous genetic organization has been observed previously for unrelated *E. coli* (17) and *Pseudomonas* (30) strains and for the related species *E. coli* and *Salmonella typhimurium* (51). However, no correspondence in the genetic maps of *L. lactis* and *Streptococcus pneumoniae* (21) was obvious, on the basis of the chromosomal locations of the *ami*, *hexA*, *rpsL*, *ung*, and *rnm* loci.

The location of insertion elements on the bacterial chromosome is a critical parameter for understanding chromosomal dynamics, since it has been demonstrated that insertion elements are predominant Hfr integration sites (64) and are involved mainly in rearrangements, such as large inversions, on the *E. coli* chromosome (9). The difference in the *IS1076* copy numbers found by classical gel electrophoresis (nine copies) and by PFGE (seven copies) might be explained by the presence of composite transposons on the strain IL1403 chromosome, as shown for lactose protease plasmid pUCL22, in which two *IS1076* elements are organized in a composite transposon (26). Another possibility is that there are hot spots of insertion sequence target sites. It should be noted that five of the seven *IS1076* integration events and 20 of the 35 *ISS1* integration events are located on the 40% of the chromosome that contains the variable regions.

Interestingly, in contrast to other observations (45), we found that either insertion sequence *ISS1* was not present on the strain IL1403 chromosome or one copy of *ISS1* was present on the strain MG1363 chromosome (32). This observation suggests that insertion sequence *ISS1* might not be primarily responsible for possible genomic rearrangements in these two *Lactococcus* strains.

The genetic map of *L. lactis* subsp. *lactis* has to be improved by precise mapping of every lactococcal gene that will be cloned in the future. This work could be facilitated by using pRC1 as a general cloning vector. This genetic map will also provide relevant information concerning the structural relationships among the chromosomes of strains belonging to the genera *Lactococcus* and *Streptococcus*.

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