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Presence of Lactose Genes and Insertion Sequences in Plasmids of Minor Species of the Genus *Lactococcus*

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The type strains of all known species and biovars of the *Lactococcus* genus were tested for the presence of plasmids, lactose genes, and insertion sequences cloned from the lactose plasmid of *Lactococcus lactis* subsp. *lactis*. Only the biovar xylosus of this subspecies is plasmid free. The lactose plasmid is present only in lactose-positive strains except in *Lactococcus plantarum*. The distribution of insertion sequences varies within the type strains of the *Lactococcus* genus.

Mesophilic lactic streptococci are the most widely used bacteria in the dairy industry. The taxonomy of the *Streptococcus* genus has been reconsidered in the light of physiological and biochemical data: lipoteichoic acid structure, fatty acid and menaquinone composition of the membrane, and especially DNA-DNA hybridization findings. The result was the definition of a new genus, *Lactococcus* (18). This genus includes the dairy species *Streptococcus lactis*, *S. diacetylactis*, *S. cremoris*, and *S. raffinolactis*; other unclassified species of *Streptococcus* such as *S. plantarum* and *S. garvieae* (4); and some misidentified lactose-negative lactobacilli, for example, *Lactobacillus xylosus*, which proved to be a biovar of *Lactococcus lactis* and *Lactobacillus hordniae* (16, 21).

The genetics of *Lactococcus lactis* subsp. *lactis*, the type strain of the genus *Lactococcus*, its biovar diacetylactis, and the subspecies *cremoris* and in particular their plasmid-borne genes have been extensively studied (for reviews, see references 7 and 12). However, no studies of the genetics of the other *Lactococcus* species, subspecies, or biovars have been reported.

In this work, we screened the type strains (19) of these taxa, generally the only isolated strain, for the presence of plasmid DNA and of cloned plasmid genes and insertion sequences by DNA hybridization using probes previously constructed in our laboratory (2, 6, 10, 11, 14).

Strains are listed in Table 1. Cells were grown at 30°C on M17 medium (22) supplemented with 0.5% lactose or glucose (for lactose-negative strains). Strains were tested for fermentation of each of 49 substrates at 30°C with API 50CH galleries (Bio-Mérieux, Craponne, France), and results were recorded after 48 h (Table 2). Plasmid DNA was extracted as described by Anderson and McKay (1) and electrophoresed in 0.7% agarose gels in Tris-acetate buffer (13). A total plasmid preparation of *Lactococcus lactis* ML3 (NCDO763) was used as the molecular weight reference. Covalently closed circular configuration of plasmid bands was checked by the method of Hinterman et al. (8).

Plasmids were electrophoresed and transferred onto Hybond-C Extra membranes (Amersham International) (20) which were then heated to 80°C for 2 h and incubated in a prehybridization medium containing Denhardt's solution (13) for 2 h at 60°C.

Four different probes were constructed from (i) the entire lactose-protease plasmid pUCL22 of *Lactococcus lactis* CNRZ270 (11), (ii) the pUCB25 recombinant plasmid constituted by the 4.4-kb *Xho*I fragment containing specific genes of the lactose operon inserted in pAT153 (2), (iii) the 1.46-kb *Hin*dIII fragment of the recombinant plasmid pUCB470 which contains the IS*1*RS element of 0.81 kb from pUCL22 and 0.65 kb from pVA797 corresponding to the insertion target region (10), and (iv) the 0.8-kb *Hin*dIII-*Pst*I internal fragment of IS*1076* obtained from pUCB412 recombinant plasmid (11). Plasmids were purified by cesium chloride gradient centrifugation with ethidium bromide, and DNA fragments were isolated from agarose gel by electroelution (13). Both plasmids and DNA fragments were nick translated with [α-^{32}P]dCTP (Amersham International).

The labeled plasmids and DNA fragments were used to probe the membranes for hybridization at 60°C for 16 h. Membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) during 5 min at room temperature, with 2× SSC–1% SDS during 30 min at 60°C, and then with 0.1× SSC–1% SDS during 30 min at 60°C. The membranes were then exposed at −20°C for at least 24 h to X-ray film (Amersham International) with intensifier screens.

Fermentation patterns. Our results (Table 2) generally confirm those of Collins et al. (4) for *Lactococcus lactis* subsp. *hordniae* and those of Rogosa (15) for *Lactococcus lactis* subsp. *lactis* biovar xylosus (except for esculin, which was fermented by our biovar xylosus strain). Our findings differed from previous reports as follows: ribose, galactose, and lactose were fermented by *Lactococcus plantarum*; cellobiose was fermented by *Lactococcus lactis* subsp. *cremoris*; sucrose and inulin were not fermented by *Lactococcus lactis* subsp. *lactis*; and ribose, mannitol, α-methyl-D-glucoside, amygdalin, inulin, melezitose, and turanose were not fermented by *Lactococcus raffinolactis*. These discrepancies could be explained by use of various fermentation tests or by a different evolution of this strain in collections or laboratories.

Plasmid content. Plasmid DNA was prepared from all strains of *Lactococcus* species and was analyzed by agarose gel electrophoresis. Plasmid bands were detected in all strains, except in the biovar xylosus of *Lactococcus lactis* subsp. *lactis* (Table 3). One to seven plasmid bands were confirmed as covalently closed circular DNA by two-dimensional electro-

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TABLE 1. Strains and plasmids used

Lactococcus strains and plasmids	Characteristic(s)[a]	Source or reference[b]
Strains		
Lactococcus lactis subsp. *lactis* CNRZ270	Lac^+	CNRZ
Lactococcus lactis subsp. *lactis* ATCC 19435^T	Lac^+	ATCC
Biovar diacetylactis NCFB176^T	Lac^+	NCFB
Biovar xylosus DSM20175^T	Lac^-	DSM
Lactococcus lactis subsp. *cremoris* NCDO607^T	Lac^+	NCDO
Lactococcus lactis subsp. *hordniae* DSM20450^T	Lac^-	DSM
Lactococcus raffinolactis NCFB617^T	Lac^+	NCFB
Lactococcus garvieae NCDO2155^T	Lac^+	NCDO
Lactococcus plantarum NCDO1869^T	Lac^+	NCDO
Plasmids		
pUCL22	Lac^+, 54 kb	11
pAT153	Amp^r Tet^r, 3.6 kb	2
pVA797	Cam^r Tra^+, 30.2 kb	10
pUCB25	(pAT153), P-Gal^+, 8 kb	2
pUCB470	(pVA797), IS*S1*RS, 5.1 kb	10
pUCB412	(pUC19), IS*1076*, 6 kb	11

[a] Lac^+, lactose utilization; Tra^+, presence of transfer genes; P-Gal^+, presence of P-β-galactosidase; Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Cam^r, chloramphenicol resistance.

[b] CNRZ, Centre National de Recherches Zootechniques, Institut National de la Recherche Agronomique, Jouy en Josas, France; ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

phoresis (8). Their sizes ranged from 2.2 to 55.2 MDa (Table 3). Five to seven plasmids were present in all subspecies of *Lactococcus lactis* whereas only one or two plasmids are present in other species (Table 3).

Detection of various plasmid-borne sequences. A ^{32}P-DNA probe from entire pUCL22 hybridized with one or two plasmids in all lactose-positive strains but with no plasmid in the lactose-negative *Lactococcus lactis* subsp. *hordniae* (Table 4). A positive response was always obtained with the largest plasmid of the multiplasmid strains (from 25.2 to 55.2 MDa) (Table 4) except with *Lactococcus garvieae*. In this lactose-negative species, only the smaller plasmid (5.1 MDa) hybridized with pUCL22 probe. Consistent with previous reports (10), it also hybridized in some *Lactococcus lactis* strains with a second smaller plasmid (from 7.1 to 14.1 MDa) (Table 4).

The pUCB25 (*lac* genes) hybridized with the largest plasmid in all the multiplasmid *Lactococcus lactis* strains and with the sole plasmid of *Lactococcus raffinolactis* but not with that of *Lactococcus plantarum*. The 4.4-kb *Xho*I fragment was also found in various *Lactococcus lactis* strains and contained *lacE*, *lacG*, and a part of *lacX* (5), specific genes of the lactose operon. Control experiments conducted with labeled pAT153

TABLE 2. Fermentation patterns of *Lactococcus* species, subspecies, and biovars

Substrate from which acid produced[a]	*L. lactis*	*L. diacetylactis*	*L. cremoris*	*L. raffinolactis*	*L. garvieae*	*L. plantarum*	*L. hordniae*	*L. xylosus*
Ribose	+	+	−	−	+	+	−	+
D-Xylose	−	−	−	+	−	−	−	+
Mannitol	+	−	−	−	+	+	−	+
Sorbitol	−	−	−	−	−	+	−	−
Amygdalin	−	−	−	−	+	+	−	+
Arbutin	+	+	−	+	+	+	+	+
Salicin	+	+	−	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	−	+
Maltose	+	+	−	+	+	+	−	+
Lactose	+	+	+	+	+	+	−	−
Melibiose	−	−	−	+	−	−	−	−
Sucrose	−	−	−	+	+	+	+	+
Trehalose	+	+	−	+	+	+	+	+
Inulin	−	−	−	−	−	+	−	−
Melezitose	−	−	−	−	−	+	−	−
D-Raffinose	−	−	−	+	−	−	−	−
Starch	−	+	−	+	−	−	−	+
β-Gentiobiose	+	+	−	+	+	+	−	+
D-Turanose	−	−	−	−	−	+	−	−
D-Tagatose	−	−	−	−	+	−	−	−
Gluconate	−	−	−	−	+	−	−	+

[a] Acid from carbohydrates was determined by using the API 50CH system; readings were made at 48 h. All strains produced acid from galactose, D-glucose, D-fructose, D-mannose, *N*-acetyl-glucosamine, and esculin; acid was not produced from adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, glycerol, glycogen, inositol, 2-ketogluconate, 5-ketogluconate, D-lyxose, α-methyl-glucoside, α-methyl-mannoside, β-methyl-xyloside, rhamnose, L-sorbose, xylitol, and L-xylose.

TABLE 3. Plasmid profiles of *Lactococcus* species, subspecies, and biovars[a]

Lactococcus strain	No. of plasmid bands	CCC DNA plasmids	Approx size(s) (MDa)
L. lactis subsp. *lactis* ATCC 19435^T	11	5	55.2, 14.1, 4.2, 3.5, 3.2
Biovar diacetylactis NCFB176^T	11	5	27.4, 21.6, 8.4, 5.1, 4.45
Biovar xylosus DSM20175^T	ND	ND	
L. lactis subsp. *cremoris* NCDO607^T	14	7	25.2, 7.1, 3.3, 3.1, 2.9, 2.3, 2.2
L. lactis subsp. *hordniae* DSM20450^T	10	5	22.2, 9, 5.7, 3.4, 3.2
L. raffinolactis NCFB617^T	1	1	17.3
L. garvieae NCDO2155^T	4	2	12.5, 5.1
L. plantarum NCDO1869^T	1	1	14.9

[a] The molecular mass standard was a total plasmid preparation from *Lactococcus lactis* ML3 (NCDO763) (33, 4.2, 2.7, and 1.8 MDa). ND, not detectable. CCC, covalently closed circular.

showed no hybridization with all lactococcus plasmids tested (data not shown). This demonstrates the presence of an equivalent of the well-characterized lactose plasmid (11) in dairy strains of the genus *Lactococcus* and its absence from *Lactococcus plantarum* and *Lactococcus garvieae*. The absence of lactose sequences from plasmids of *Lactococcus lactis* subsp. *hordniae* isolated from an insect, and *Lactococcus garvieae* isolated from mastitis, is consistent with their inability to ferment lactose.

The pUCB470 probe containing the insertion sequence ISS*1*RS and a pVA797 fragment (11, 14) hybridized with the lactose plasmid of all the strains in which this plasmid was present except *Lactococcus raffinolactis* (Table 4). In *Lactococcus raffinolactis*, the lactose plasmid (17.3 MDa) is smaller than those previously described, whose size is ca. 33 MDa. This negative result could be due to the loss of this genetic element from the lactose plasmid of *Lactococcus raffinolactis* in the course of evolution. Alternatively, the lack of this insertion sequence could result from the absence of a transposition phenomenon by this sequence into this plasmid. Control experiments conducted with labeled pVA797 showed no hybridization with all lactococcus plasmids tested (data not shown). This ISS*1*-like sequence was found in all well-characterized lactose plasmids, in some cryptic plasmids of several *Lactococcus lactis* strains (10), in two plasmids from *Lactococcus plantarum* (10), and in *Lactococcus casei* (10).

The IS*1076* (IS*904*) insertion sequence is not so widely distributed as ISS*1* in lactococci (17). We confirm this narrow distribution: the pUCB412 (IS*1076*) probe only hybridized with lactose plasmids in the *Lactococcus lactis* subsp. *lactis* ATCC 19435 type strain and NCDO763 reference strain and the *Lactococcus lactis* subsp. *cremoris* NCDO607 type strain. No hybridization occurred between insertion sequence probes and total DNA from the plasmid-free strain of *Lactococcus lactis* subsp. *lactis* biovar xylosus, suggesting that this sequence was not integrated into the chromosome of this strain.

TABLE 4. Hybridization of plasmids from *Lactococcus* species, subspecies, and biovars with ^{32}P-labeled probes[a]

Lactococcal strain	pUCL22	pUCB25 (*lac* genes)	pUCB470 ISS*1*RS	pUCB412 IS*1076*
L. lactis subsp. *lactis*	55.2, 14.1	55.2	55.2, 14.1	55.2
Biovar diacetylactis	27.4, 8.4	27.4	27.4, 8.4	ND
Biovar xylosus	ND	ND	ND	ND
L. lactis subsp. *cremoris*	25.2, 7.1	25.2	25.2, 7.1	25.2
L. lactis subsp. *hordniae*	ND	ND	ND	ND
L. raffinolactis	17.3	17.3	ND	ND
L. garvieae	5.1	ND	ND	ND
L. plantarum	14.9	ND	14.9	ND

[a] Sizes are given in megadaltons. ND, not detected.

The hybridization of the whole pUCL22 probe with plasmids other than the lactose plasmid of *Lactococcus lactis* strains and with the unique plasmid of *Lactococcus plantarum* which most probably lacks the phospho-β-galactosidase gene since its DNA shows no hybridization signal with pUCB25 (*lac* genes) may be due, at least in part, to the presence of an ISS*1*RS-like sequence on these plasmids (Table 4). The absence of specific genes of the lactose operon from the *Lactococcus plantarum* plasmid suggests that this strain (lactose positive in our hands), like the atypical 7962 strain of *Lactococcus lactis* subsp. *lactis*, possesses a β-galactosidase rather than a phospho-β-galactosidase to metabolize lactose (3). The lack of ISS*1*RS in *Lactococcus raffinolactis*, and in *Lactococcus lactis* subsp. *lactis* INA45 (14), suggests that the lactose plasmid in these strains is not conjugative or mobilizable. In contrast to ISS*1*RS, IS*1076* is found only on lactose plasmids from *Lactococcus lactis* and *Lactococcus cremoris* subspecies and could be associated with nisin or *clp* genes (9).

Finally, the positive signal obtained between pUCL22 and the sole plasmid of *Lactococcus garvieae* could be due to the sequence similarities between the replication origins of these two plasmids (6).

In conclusion, we showed that lactose utilization was determined by plasmid-borne genes in different species, subspecies, and biovars of the *Lactococcus* genus, as it was already demonstrated in *Lactococcus lactis*, the type species of this genus (7), with one exception, *Lactococcus plantarum*, in which lactose genes are probably chromosomal. The distribution of ISS*1* parallels that of the lactose plasmid. These observations argue for horizontal dissemination of this genetic material in these strains sharing at least an ecological niche.

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