Mosaic structure of large regions of the *Lactococcus lactis* subsp. *cremoris* chromosome

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***Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* are closely related phenotypically and genetically. Here we report that certain regions of their chromosomes diverge considerably more than others. Conserved regions differ by less than 20%, whilst variable regions differ by more than 60%. This mosaic structure may have arisen by horizontal gene transfer from distantly related bacteria since in a particular region of the *L. lactis* subsp. *cremoris* chromosome the G + C content and the codon bias are not typical for lactococci. Such an exchange, which conserves the function of the gene and cannot be achieved under selective pressure, may be of considerable importance in the evolution of bacteria.**

Keywords: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, horizontal gene transfer, chromosome structure

INTRODUCTION

The genus *Lactococcus* is a distinct and homogeneous group of lactic acid bacteria (Collins *et al.*, 1989) isolated from dairy products, plants, and the mucosa and digestive tract of animals. Within the genus, the status of *Lactococcus lactis* subsp. *lactis* (*Streptococcus lactis* Lister 1873) and *Lactococcus lactis* subsp. *cremoris* (*Streptococcus cremoris* Orla-Jensen 1919) as separate species or subspecies has been the subject of some debate, since their phenotypic differentiation is difficult. Garvie & Farrow (1982) proposed that both organisms belong to a single species because of similarities in their phenotype and DNA homology, which was reported to be 80% for the type strains and to range between 75 and 100% (Jarvis *et al.*, 1981). This classification is supported by the high degree of homology of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* 16S rRNA (Collins *et al.*, 1989; Salama *et al.*, 1991). However, it has been argued that 16S rRNA sequence identity may not be sufficient to guarantee species identity (Fox *et al.*, 1992) and the *lactis/cremoris* classification was questioned by the recent evidence that certain genes of one subspecies hybridize only very weakly with those of the other (Godon *et al.*, 1992b).

To extend the previous observations we undertook

hybridization analysis of the entire tryptophan, histidine and branched-chain amino acid (BCAA; isoleucine, leucine and valine) operons. Interestingly, a mosaic structure of the operons was found, with alternating conserved and divergent regions.

METHODS

Bacterial strains, plasmids, phage and media. The bacterial strains, plasmids and the phage used in this work were described previously (Delorme *et al.*, 1992; Godon *et al.*, 1992a). Strains earlier misclassified as *L. lactis* subsp. *lactis* are here termed *L. lactis* subsp. *cremoris* as proposed previously (Godon *et al.*, 1992b). Lactococcal strains were grown in M17 medium (Terzaghi & Sandine, 1975).

Cloning of *L. lactis* subsp. *cremoris* NCDO 763 DNA in *Escherichia coli*. Total DNA from lactococci was isolated by standard procedures (Loureiro Dos Santos & Chopin, 1987). Subcloning of total DNA was carried out in pBluescript plasmid (Stratagene). A ligation mix was used to transform competent *E. coli* cells, as described by Sambrook *et al.* (1989). To isolate the 2.5 kb segment, previously detected in *L. lactis* subsp. *cremoris* DNA by hybridization with the *his* operon of *L. lactis* subsp. *lactis* (Godon *et al.*, 1992b), a partial *L. lactis* subsp. *cremoris* genomic library was prepared. *EcoRI* DNA fragments ranging from 2.2 to 2.7 kb were purified by gel electrophoresis and ligated to a dephosphorylated pBluescript vector. The relevant clones were identified by colony hybridization at low stringency (25%, v/v, formamide) with pIL704 (Delorme *et al.*, 1992) as probe. Three positive clones harboured the same plasmid, as judged by its size and by restriction analysis. One, designated pIL732, was chosen for further study.

Abbreviation: BCAA, branched-chain amino acid.

The GenBank accession number for the nucleotide sequence reported in this paper is L35562.

Fig. 1. Organization of the 30 kb *L. lactis* subsp. *lactis* chromosomal region covering the histidine and the BCAA operons and the 8 kb region covering the tryptophan operon. Top: genetic organization. Middle: the *L. lactis* subsp. *lactis* probes used for the Southern experiments. The heavy and light hatched boxes denote a strong and weak hybridization signal, respectively, at high stringency. The white boxes denote the absence of a signal. Bottom: results of the hybridization experiments at high stringency. *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* DNA are in left and right lanes, respectively, in each experiment. Raoul size markers are shown in the leftmost lane (15.0, 9.0, 7.3, 5.6, 4.3, 4.0, 3.6, 2.9, 2.3, 1.8, 1.4, 1.2, 0.75 kb).

A 1.5 kb chromosomal fragment of the *L. lactis* subsp. *cremoris* *his* operon was amplified by the polymerase chain reaction (PCR) using the primers His1 (CCAATATCAA-CAATCCATG, nucleotides 1809–1790 of NCDO 763) and His2 (ATTTCTGATTGGTTACGGAT, nucleotides 329–348). The amplified fragment was either rendered blunt-ended with T4 polymerase and inserted into pBluescript in the *EcoR* I site or partially cut with *Alu*I and cloned in the M13mp18 vector cleaved with *Sma*I.

Southern hybridization analysis. Standard procedures were used (Sambrook *et al.*, 1989) with 2 µg of chromosomal DNA. DNA segments were transferred to a nylon membrane (Pal Biotec) and hybridized with probes that were labelled with [³²P]dCTP by the random priming procedure (Boehringer). Stringency of hybridization was controlled by varying the formamide concentration as previously described (Godon *et al.*, 1992). The filters were washed twice in 2× SSC at 42 °C and then twice at 50 °C.

DNA sequence analysis. Nested deletions were produced by the action of DNase I on pBluescript plasmids containing the segments to be sequenced. Single-stranded DNA was prepared as described by Vieira & Messing (1987) and sequenced essentially according to the Applied Biosystems protocol accompanying the 370A DNA sequencer. The DNA was used in dideoxynucleotide chain-termination sequencing reactions with *Taq* DNA polymerase and fluorescent dye-coupled primers (Applied Biosystems). The reported sequence was determined on both DNA strands. Errors arising from gene amplification were resolved by sequencing at least two independently cloned segments.

The DNA and protein sequences were analysed using the BISANCE and the University of Wisconsin GCG packages, implemented at the Centre InterUniversitaire d'Informatique à Orientation Biomédicale, Paris.

RESULTS

Heterogeneity of the *L. lactis* subsp. *cremoris* chromosome

In a previous study, certain *L. lactis* subsp. *lactis* DNA segments were found to hybridize only very weakly with the chromosomal DNA of *L. lactis* subsp. *cremoris* (Godon *et al.*, 1992b). To determine whether the level of divergence between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* DNA varies significantly along the chromosome we undertook the following Southern hybridization analysis. Segments of two previously sequenced *L. lactis* subsp. *lactis* chromosomal regions of ~30 and ~8 kb, covering a total of 1.6% of the chromosome and containing *his* (Delorme *et al.*, 1992), *leu-ilv-ald* (Godon *et al.*, 1992a) and *trp* (Bardowski *et al.*, 1992) operons (Fig. 1), were hybridized with *L. lactis* subsp. *cremoris* DNA at a stringency low enough to detect homology of >80%. Hybridization was observed with some, but not all, probes (Fig. 1). Control experiments indicated that all probes hybridized with *L. lactis* subsp. *cremoris* DNA at a lower stringency (25% formamide, data not shown). We conclude that *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* diverge considerably in certain chromosomal regions and less in others.

Sequence of a DNA segment from the *L. lactis* subsp. *cremoris* *his* operon

To determine more precisely the level of divergence between the poorly hybridizing segments, the sequence of the 2.5 kb segment from the *L. lactis* subsp. *cremoris* *his* operon, carried on pIL732 (third box in Fig. 1, see Methods), was determined and compared with the sequence of the corresponding segment of *L. lactis* subsp.

lactis NCDO 2118 (Fig. 2). The sequence of this fragment was previously compared in two *L. lactis* subsp. *lactis* strains and was found to be more than 98% identical (Delorme *et al.*, 1993). The *L. lactis* subsp. *cremoris* sequence is ~200 bp longer, due to an insertion extending from position 1384 to 1597. Excluding the insertion, alignment of the two sequences is possible, the mean divergence being ~27%. Surprisingly, divergence is much greater in certain regions than in others, as seen by comparing 70 bp intervals of the two sequences (Fig. 3). It is relatively high, up to 65% with a mean of ~45%, in the central region (positions 480–1700), with only one better conserved interval (15% divergence, see below for possible explanation). In contrast, divergence is relatively low, 10–30% with a mean of ~15%, at the 3' end (beyond position 1750). Some short divergent stretches are interspersed at the conserved 5' end (up to position 480). Interestingly, the G+C content of the entire divergent central region of the *L. lactis* subsp. *cremoris* segment is quite high, 48 mol%, whereas that of the corresponding region of *L. lactis* subsp. *lactis* is only 36 mol% G+C, which is close to the 38 mol% found for total *L. lactis* DNA (Garvie & Farrow, 1982). In contrast, the G+C content of both 5' and 3' conserved regions is not significantly different.

The insertion present in the highly divergent central region of the *L. lactis* subsp. *cremoris* *his* segment is composed of (i) three quasi-perfect repeats of 59 bp (position 1400–1582) and (ii) two flanking non-repeated and non-coding stretches, of 22 and 28 bp, respectively (Fig. 2). A remarkable feature of this insertion is its high G+C content (54 mol%), which is higher than that of the flanking region (48 mol%).

Analysis of the coding sequences

Variations in the extent of divergence found in the *his* operon could be due to rapid accumulation of mutations or to acquisition of DNA from phylogenetically distant species by horizontal gene exchange. In the first case, the divergent regions would almost certainly conserve codon usage (assuming that the gene was functional after it diverged) whilst in the second the chances are that the codon usage would be different. These considerations led us to compare the coding properties of the previously sequenced *L. lactis* subsp. *lactis* and *cremoris* segments. The former was previously shown to carry the 3' end of *hisC*, the entire *orf3* and *hisG* and the 5' end of *hisD* (Fig. 2; Delorme *et al.*, 1992), which encode enzymes involved in histidine biosynthesis (HisC, HisG, HisD) and a protein homologous to histidyl tRNA synthetases (Orf3). The same proteins are encoded by the *L. lactis* subsp. *cremoris* segment, except that frameshift mutations are present in the four genes (at positions 117, 1043, 1945 and 2424). The four gene products are therefore probably inactive, which is expected from the observation that all the *L. lactis* subsp. *cremoris* strains are auxotrophic for histidine (17 strains tested, Delorme *et al.*, 1993). When corrected for the frameshifts, the potential *L. lactis* subsp. *cremoris* gene products share 78, 51, 83 and 73% homology,

respectively, with their *L. lactis* subsp. *lactis* counterparts. Interestingly, codon usage in the better-conserved terminal regions of the *L. lactis* subsp. *cremoris* sequence is as expected for lactococci, whereas in the highly divergent central region, which encodes ~60% of Orf3 (the carboxy end) and ~20% of HisG (the amino end), codon usage is quite atypical (Table 1; only the significantly deviant codons are shown). For instance, codons GTG for valine, GCG for alanine, GAG for glutamine, and CGG and CGC for arginine are preferentially used in this region whereas they occur rarely in other regions of the *Lactococcus* chromosome (Chopin, 1993). Unusual codon usage, together with the high G+C content and the presence of additional DNA (see above) suggest that the central *L. lactis* subsp. *cremoris* region might originate from a taxonomically distant bacterial species.

Divergence is not due to the loss of function

Sequence analysis (see above) and phenotype tests (Delorme *et al.*, 1993) show that the *his* operon of *L. lactis* subsp. *cremoris* NCDO 763 is inactive. The question arises whether the divergence might be due to a rapid drift of genes which are no longer used, notwithstanding the atypical codon usage and G+C content of the central region. In this case, the corresponding region from different *L. lactis* subsp. *cremoris* strains might be poorly conserved. To test this possibility a 1.4 kb segment from two different *L. lactis* subsp. *cremoris* strains, IL182 and AM2, was amplified by PCR and its sequence determined (Fig. 4). The two coding sequences differ by less than 0.8% and differ only slightly more, ~3%, from the sequence of the NCDO 763 strain. Moreover, most of the differences between NCDO 763 and the two other strains are either silent (22 and 20 for IL182 and AM2, respectively), or conservative (5 and 7), as expected for functional diverging genes. In addition, all the changes which affect the protein sequence (10 and 9 for IL182 and AM2, respectively) are present at positions which are not well conserved, suggesting that they do not affect protein function. Finally, the frameshift found in position 1043 of *orf3* from NCDO 763 is not present in the other two strains, which implies that this change occurred relatively recently when the environmental conditions allowed the fixation of this mutation. These results suggest that the region was functional over a long period of time since strain NCDO 763 diverged from IL182 and AM2, and argue against its rapid drift due to the loss of gene function.

It should be noted that the domains of the highly divergent central region which are better conserved than the adjacent domains (see, for instance, the domain close to position 600, which diverges only ~15%, Fig. 3), code either for the motifs which are typical for class II tRNA synthetases, to which Orf3 belongs (M1 and M2), or for the signature regions (SR1, SR2a and b), specific for histidyl tRNA synthetases (Figs 2 and 3; Raben *et al.*, 1992; Eriani *et al.*, 1990). It is possible that the constraints of preserving protein function might have limited the nucleotide sequence divergence of these domains.

hisC

I	R	L	G	V	A	Y	G	S	A	E	A	I	S	H	L	Y	D	V	K	N	F	N	S	Y	P	I	D			
GA	ATT	CGT	TTG	GGT	GTA	GCT	TAT	GGC	TCT	GCT	GAA	GCA	ATT	GCT	TAT	TAT	GAT	GTG	AAA	AAT	TCA	TTT	AAT	TCC	TAT	CCA	ATT	GAT		
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S	L	A	Q	I	I	G	E	A	S	L	M	D	E	H	Y	F	Q	K	N	I	Q	K	I	I	K	T	R	E	V	
AGT	TTG	GCA	CAA	ATT	ATT	GGA	GAA	GCT	TTA	ATG	GAT	GAA	CAT	TAT	TTT	CAA	AAA	AAC	ATT	CAG	AAA	ATC	ATT	AAG	ACA	AGA	GAA	GTT		
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F	K	D	N	L	V	N	L	G	F	E	V	T	D	S	K	A	N	F	V	F	V	H	H	P	K	V	K	A	E	
TTT	AAA	GAT	AAT	CTG	TTT	AAT	TTA	GGA	TTT	GAA	GTG	ACT	GAT	TCA	AAG	GCT	AAC	TTT	GTT	TTT	GTT	CAT	CAT	CCA	AAA	GTA	AAA	GCA	GAA	
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D	L	F	K	A	L	Y	E	A	K	I	I	V	R	H	W	N	Q	P	R	I	D	S	D	W	L	R	I	T	I	G
GAC	CTT	TTT	AAG	GCG	CTT	TAT	GAG	GCA	AAA	ATT	ATT	GTC	AGG	CAT	TGG	AAT	CAA	CCA	CGG	ATT	GAT	GAT	TGG	TTA	CGG	ATA	ACT	ATT	GGA	
ACA	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	

orf3

T	N	K	E	M	N	K	V	I	E	F	L	K	G	Y	L	K	N	N	E	E	I	S	D	E	W	K	K	I	N	Y
ACT	AAT	AAA	GAA	ATG	AAC	ATA	GTG	ATT	GAA	TTT	TTA	AAA	GGC	TAT	TTA	AAA	AAG	AAT	GAG	GAA	ATT	GAC	GAA	TGG	AAA	AAA	TAA	ATT	AT	
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Motif 1

L	L	P	E	E	S	A	E	M	T	L	N	Q	V	K	S	L	R	Q	I	E	G	R	L	R	K	L	F	M	L
CTG	CTT	CCT	GAA	GAA	TCG	GCA	GAA	ATG	ACC	TTG	AAT	CAA	GTT	ATT	AGT	CTA	CGG	CAG	ATA	GAA	GGG	CGT	TTA	AGA	AAA	TTA	TTT	AGC	TTG
T-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	

Motif 2

K	N	Y	Q	E	V	M	P	P	S	F	E	Y	T	Q	L	Y	T	A	L	E	S	N	G	K	T	F	N	Q	E
QAA	AAT	TAT	CAG	GAA	GTC	ATG	CCC	CCA	AGC	TTT	GAA	TAT	ACA	VAA	CTC	TAT	ACA	GCA	CTT	GAA	AGT	AAT	GGA	AAA	ACT	TTT	AAT	CAA	GAA
C-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	

Signature Region 1

K	M	F	Q	F	I	N	H	E	G	Q	S	I	T	A	L	R	Y	D	F	T	L	P	L	V	R	L	N	Y	S	Q	I	L
AAA	ATG	TTT	CAG	TTT	ATC	AAT	CAT	GAG	GGA	CAA	TCA	ATT	ACA	CTT	CGT	TAT	GAT	TTT	ACA	CTT	CCT	TTA	GTT	AGA	CTC	TAT	TCG	CAA	ATC			
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Signature Region 2

K	D	S	T	S	A	R	Y	S	Y	F	G	K	I	F	R	K	E	K	R	H	K	G	R	S	T	E	N	Y	Q	
AAG	GAT	TCT	ACT	AGT	GCC	CGT	TAT	TCA	TAT	TTT	---	GGA	AAA	ATA	TTT	AGA	AAA	GAA	AAA	CGG	CAT	AAA	GGT	CGT	TCA	ACG	GAA	AAT	TAT	CAG
TCA	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		

Signature Region 2a

F	S	E	K	L	S	M	I	K	P	I	I	I	D	L	F	G	P	M	V	P	K	A	M	D	Y	Y	T	D	L	M	F	K	Q
N	L	P	D	K	T	A	A	C	Q	H	Y	L	F	G	L	G	A	G	A	G	A	A	G	A	G	A	G	A	G	A	G	A	G
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Signature Region 2b

A	Y	S	C	A	N	A	Q	P	I	L	S	G	G	R	Y	D	Q	L	L	S	N	F	Q	E	E	A	V	A	I			
V	V	E	G	V	A	A	C	A	T	I	A	T	I	A	V	R	E	H	M	I	K	I	A	I	T	K	G	R	I	Q	K	Q
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hisG

V	T	K	L	L	E	A	D	Y	V	E	P	I	L	N	L	G	R	E	L	Q	I	K	T	K	D	L	D
GTG	ACC	AAA	CTT	TGA	AAT	GCG	GAT	GAT	TTT	GAA	CCA	ATT	CTA	AAT	CTA	GGA	CGT	GAA	TTA	CAA	ATT	AAA	ACG	AGA	GAT	GAT	TTA
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orf3 (cont. NCDO2118)

G	F	C	C	H	M	D	T	I	L	K	A	L	E	R	Q	E	...	(cont.)
GGT	TTT	TGT	TGT	CAT	ATG	GAT	ACT	ATT	TTA	AAG	GCA	CTG	GAA	AGA	CAA	GA	*****	
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1395

GATAGAAAGCTTGGCGAAGCAAGCTGTCTAAAGGGCAGCAAGGGGGAGCTTTTTCAGGTTTGGCG

1436

ATAGAAGCTTGGCTAAGCAAGCTATCTGCTGCCCTTTGGGCGAG

1451

V	T	K	L	L	E	A	D	Y	V	E	P	I	L	N	L	G	R	E	L	Q	I	K	T	K	D	L	D
GTG	ACC	AAA	CTT	TGA	AAT	GCG	GAT	GAT	TTT	GAA	CCA	ATT	CTA	AAT	CTA	GGA	CGT	GAA	TTA	CAA	ATT	AAA	ACG	AGA	GAT	GAT	TTA
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1541

Q	I	I	F	G	K	A	N	D	V	I	T	F	L	E	H	G	I	V	D	I	G	F	V	G	K	D	T	L	D
GAA	ATC	ATT	TTT	GGA	AAA	GCT	AAT	GAT	GTC	ATT	ACT	TTT	TGA	GAA	CAT	GGA	ATT	GTT	GAT	ATT	GGC	TTT	GTT	GGT	AAA	GAT	ACG	CTT	GAT
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1631

ATAGAAGCTTGGCTAAGCAAGCTATCTGCTGCCCTTTGGGCGAG

1653

V	T	K	L	L	E	A	D	Y	V	E	P	I	L	N	L	G	R	E	L	Q	I	K	T	K	D	L	D
GTG	ACC	AAA	CTT	TGA	AAT	GCG	GAT	GAT	TTT	GAA	CCA	ATT	CTA	AAT	CTA	GGA	CGT	GAA	TTA	CAA	ATT	AAA	ACG	AGA	GAT	GAT	TTA
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1743

Q	I	I	F	G	K	A	N	D	V	I	T	F	L	E	H	G	I	V	D	I	G	F	V	G	K	D	T	L	D
GAA	ATC	ATT	TTT	GGA	AAA	GCT	AAT	GAT	GTC	ATT	ACT	TTT	TGA	GAA	CAT	GGA	ATT	GTT	GAT	ATT	GGC	TTT	GTT	GGT	AAA	GAT	ACG	CTT	GAT
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1831

Fig. 2. For legend see facing page.

E	N	D	F	D	D	Y	Y	E	L	L	D	L	K	I	G	Q	C	I	F	A	L	A	S	Y	P	D	F	S	N	
GAA	AAT	GAT	TTT	GAT	GAT	TAT	TAT	GAA	TTA	TTG	GAT	TTA	AAA	ATT	GGG	CAG	TGT	ATT	TTT	GCC	CTT	GCT	TCC	TAT	CCT	GAC	TTT	TCA	AAT	1721
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K	N	F	Q	R	R	K	R	I	A	S	K	Y	P	R	V	T	K	K	Y	F	A	Q	K	Q	E	D	I	E	I	
AAA	AAT	TTT	CAA	AGA	CGT	AAA	CGA	ATT	GCT	TCA	AAA	TAT	CCA	AGA	GTG	ACA	AAA	AAA	TAT	TTT	GCT	CAA	AAG	CAA	GAA	GAT	ATT	GAA	ATT	1811
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I	K	L	E	G	S	V	E	L	G	P	V	V	G	L	A	D	A	I	V	D	I	V	E	T	G	N	T	L	S	
ATC	AAG	TTG	GAA	GGT	TCT	GTT	GAG	CTT	GGA	CCA	GTT	GTT	GGT	TTA	GCT	GAT	GCA	ATT	GTT	GAC	ATT	GTC	GAA	CAA	GGA	AAT	ACT	TTA	TCT	1901
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A	N	G	L	E	V	I	E	K	I	S	D	I	S	T	R	M	I	V	N	K	S	S	F	K	F	K	K	D	K	
GCA	AAT	GGT	TTA	GAG	GTC	ATT	GAA	AAA	ATC	AGT	GAC	ATT	TCA	ACA	CGG	ATG	ATT	GTC	AAT	AAA	TCT	AGT	TTC	AAA	TTT	AAA	AAA	GAT	AAA	1991
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hisD																														
I	I	E	M	V	E	R	L	E	D	A	Q	T	N	END																
ATT	ATA	GAA	ATG	GTG	GAG	AGG	TTA	GAA	GAT	GCT	CAA	ACA	AAT	TGAT	TAT	CAA	GGA	AAG	CTT	GAA	GAA	ATT	GCT	GAA	AAA	TTT	CAA	GGT	CGA	2082
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	2283
K	T	E	V	S	K	E	V	N	K	T	V	Q	Q	I	V	A	D	I	Q	K	N	G	D	T	A	L	F	N	Y	
AAA	ACA	GAA	GTA	TCA	AAA	GAA	GTT	AAT	AAA	ACT	GTT	CAA	CAG	ATT	GTA	GCA	GAC	ATT	CAA	AAA	AAT	GGA	GAT	ACT	GCC	TTA	TTT	AAC	TAT	2172
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	2373
A	K	K	F	D	G	Y	D	V	N	T	S	N	L	L	V	T	R	M	E	R	E	A	G	L	E	Q	I	D	E	
GCC	AAA	AAG	TTC	GAC	GGT	TAT	GAT	GTG	AAT	ACT	AGT	AAT	TTA	CTG	GTC	ACG	CGC	ATG	GAA	CGT	GAA	GCA	GGA	CTA	GAA	CAA	ATT	GAT	GAG	2262
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	2462
D	Y	F	R	I																										
GAT	TAT	TTT	AGA	ATT	C																									
A	---	---	---	---	---																									

Fig. 2. Comparison of the nucleotide sequences and the putative translation products from *L. lactis* subsp. *lactis* NCDO 2118 (first and third lines) and *L. lactis* subsp. *cremoris* NCDO 763 (second and fourth lines). Conserved motifs 1 and 2 of class II tRNA synthetases, and signature regions 1 and 2 of histidyl tRNA synthetases (Eriani *et al.*, 1990; Raben *et al.*, 1992) are indicated. Stars indicate the missing nucleotides or amino acids. The translation products are shown irrespective of the frameshifts, to allow comparison over their entire length.

DISCUSSION

The level of divergence between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* varies considerably along a large region of the chromosome. It is low (10–20%) in conserved regions, which agrees with comparisons of the 16S rRNA sequences (Salama *et al.*, 1991) and with global DNA–DNA homology measurements (Jarvis *et al.*, 1981), but is very high (35–65%) in variable regions. The conserved and variable regions are interspersed, which suggests that the chromosomal region studied has a mosaic structure.

The divergence appears not to be due to an abnormally rapid genetic drift, as indicated by analysis of a region of the *his* operon which is highly divergent between *L. lactis* subsp. *lactis* and *cremoris*, but is remarkably well conserved among different *L. lactis* subsp. *cremoris* strains. Several features of this region in *L. lactis* subsp. *cremoris* are not representative of the genus *Lactococcus*, such as a high G + C content, unusual codon bias and the presence of a repeated DNA element, suggesting that it might have been acquired by horizontal gene transfer from distantly related bacteria. This interpretation is supported by the existence of a rather sharp boundary between this atypical region and the adjacent lactococcus-like 3' region. Genes acquired by horizontal transfer often have unusual G + C content and codon bias, as shown by extensive analysis of *E. coli* sequences (Médigue *et al.*, 1991). Comparable results have also been found in other bacterial genera

(Groisman *et al.*, 1992; Shanley *et al.*, 1994). It is not known at present from which species the divergent *L. lactis* subsp. *cremoris* region was derived.

The postulated horizontal gene transfer is certainly not restricted to the *his* operon, since interspersed of conserved and divergent regions was also detected in the BCAA and the *trp* operons. The sequence of these regions of the *L. lactis* subsp. *lactis* chromosome is known (Delorme *et al.*, 1992) and its analysis did not reveal any distortion in the G + C content or codon bias from other lactococcal genes. Our results do not permit the conclusion that all *L. lactis* subsp. *cremoris* divergent regions deviate from the lactococcal model (as does the divergent *his* region) but point to the mosaic structure of the *L. lactis* subsp. *cremoris* chromosome. The genes analysed here are grouped in two regions. The *his* and the BCAA operons are adjacent and ~ 200 kb from the *trp* operon (Tulloch *et al.*, 1991; Le Bourgeois *et al.*, 1992). Future work should establish whether the *L. lactis* subsp. *cremoris* chromosome is mosaic only in these two regions or whether the mosaicism extends further along.

DNA acquired by heterospecific gene transfer may be associated with extrachromosomal elements. For instance, sequences showing a high similarity with the *incFII-oriT* region of plasmid R100 and with a region of the 145 kb plasmid harbouring the gene for the heat-stable toxin (*estAI*) were found in an *E. coli* strain 350 and 400 bp upstream of the *phoN* and *sinR* loci, respectively (Groisman *et al.*, 1992, 1993). Similarly, the *bin* and *estAI*

Fig. 3. Divergence between the *L. lactis* subsp. *lactis* NCDO 2118 and *L. lactis* subsp. *cremoris* NCDO 763 sequences. The squares connected by the solid line represent the mean divergence over a 70 bp sequence and the horizontal dashed line represents the mean of the region covered. Nucleotide positions in the *L. lactis* subsp. *cremoris* NCDO 763 sequence are indicated on the abscissa. M and SR denote conserved motifs and signature regions present in *orf3* (indicated in Fig. 2) and the arrows show their position. The ORFs indicated at the top are delimited by vertical dashed lines; DIR, direct repeat.

regions are associated with a transposon-like element (Simon *et al.*, 1980) or an insertion sequence (Groisman *et al.*, 1993). Interestingly, a conjugative element has been mapped at a position less than 50 kb away from the *his* operon carrying the mosaic genes in *L. lactis* subsp. *cremoris* chromosome (P. Le Bourgeois, personal communication). Furthermore, the *L. lactis* subsp. *cremoris* *his* region contains an element that is reminiscent of prokaryotic repetitive DNA sequences such as REP and ERIC (Higgins *et al.*, 1988; Hulton *et al.*, 1991; Gilson *et al.*, 1991). It is therefore possible that the mosaicism is restricted to only a portion of the *L. lactis* subsp. *cremoris* chromosome, which would permit reconciliation of the reported overall DNA homology (Jarvis *et al.*, 1981) with the high local divergence observed in the present work.

Interestingly, the operons resulting from the postulated gene transfer may all have been functional. The *trp* operon is known to be active (Reiter & Oram, 1962) and the *his* operon was presumably active, as deduced from the conservative base change pattern among different *L. lactis* subsp. *cremoris* strains. It has been reported that intra-specific gene exchange may generate functional genes, such as those involved in penicillin resistance of *Neisseria* (Spratt *et al.*, 1992; Maynard Smith *et al.*, 1991) or *Streptococcus* (Martin *et al.*, 1992; Dowson *et al.*, 1989). However, the exchange that occurred in these cases, as well as the extensive recombination in the *rfb* O-antigen locus in *Salmonella enterica* (Reeves, 1993) were generated under intense selection. Our results represent the first indication that a hetero-specific gene exchange might

Table 1. Codon usage in lactococci

Codon*	Total codon number		Codon proportion (%)		
	Central region†	Terminal regions‡	Central region†	Terminal regions‡	Total lactococci§
TTA (Leu)	8	15	19	44	31
TTG (Leu)	14	8	34	23	23
GTT (Val)	3	11	14	52	48
GTG (Val)	8	4	38	19	13
GCT (Ala)	4	9	17	37	39
GCG (Ala)	8	1	43	4	11
CAA (Gln)	6	13	37	93	82
CAG (Gln)	10	1	63	7	18
AAA (Lys)	13	33	54	77	77
AAG (Lys)	11	10	46	23	23
GAA (Glu)	16	28	57	76	83
GAG (Glu)	12	9	43	24	17
CGG (Arg)	3	5	19	6	6
CGC (Arg)	5	1	31	12	9
AGA (Arg)	1	5	6	29	29

* Only the codons showing significant differences between central and terminal regions are shown.

† Positions 481–1356 and 1600–1740 from *L. lactis* subsp. *lactis* NCDO 763.

‡ Positions 1–480 and 1741–2478 from *L. lactis* subsp. *lactis* NCDO 763.

§ From Chopin (1993).

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