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Gene Inactivation in *Lactococcus lactis*: Branched-Chain Amino Acid Biosynthesis

JEAN-JACQUES GODON,* CHRISTINE DELORME, JACEK BARDOWSKI, MARIE-CHRISTINE CHOPIN, S. DUSKO EHRLICH, AND PIERRE RENAULT

Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, 78352 Jouy en Josas Cedex, France

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The *Lactococcus lactis* subsp. *lactis* strains isolated from dairy products are auxotrophs for branched-chain amino acids (leucine, isoleucine, and valine), while most strains isolated from nondairy media are prototrophs. We have cloned and sequenced the *leu* genes from one auxotroph, IL1403. The sequence is 99% homologous to that of the prototroph NCDO2118, which was determined previously. Two nonsense mutations and two small deletions were found in the auxotroph sequence, which might explain the branched-chain amino acid auxotrophy. Nevertheless, the *leu* genes from the auxotroph appear to be transcribed and regulated similarly to those from the prototroph.

It is well known that many microorganisms isolated from natural habitats have nutritional requirements which make them unable to grow on simple mineral media supplemented with a carbon source. Examples include amino acid and vitamin requirements of various *Salmonella* species (25), amino acid requirements of *Pasteurella pestis* (12), amino acid, vitamin, and nucleic acid base requirements of *Neisseria gonorrhoeae* (4, 19) as well as a 12-amino-acid requirement of *Lactobacillus casei* (34). Inability to use a given carbon source has been particularly well documented in members of the family *Enterobacteriaceae* with respect to β-glucosides (for a review, see reference 26). The observed deficiencies might confer a selective advantage, as shown for auxotrophic mutants of *Bacillus subtilis* and *Escherichia coli* competing with prototrophic strains in glucose-limited chemostats (11, 50), but the reasons for this advantage are not entirely clear. Whichever the reason, it might be expected that a change of the ecological niche of a microorganism from a less to a richer environment may lead to the loss of functions which have become superfluous.

Several types of genetic defects might cause the inability of a microorganism to synthesize or to degrade a given substance. (i) The required genes might be present and encode functional proteins, but the level of gene expression might be insufficient. For example, *bgl* and *cel* operons of *E. coli* can be activated by integration of insertion sequences in the vicinity of their promoters (26, 35, 39) or by modification of a repressor (35). (ii) The required genes might be adequately expressed but encode nonfunctional proteins. An example is the *ilvG* gene of *E. coli*, which contains a frameshift site (23, 24). Mutant alleles which specify an active protein can, however, be easily isolated. Such easily activable genes were termed cryptic (17). (iii) Genes might be present but be poorly expressed and encode nonfunctional proteins, as in the case of the *trp* operon of *Shigella dysenteriae* (30, 32). (iv) Finally, the genes might not be present at all, as a consequence of deletions. This is expected for microorganisms with a particularly low genome size, such as mycoplasmas (36).

It is conceivable that a cryptic gene might accumulate mutations, which would render its reactivation progressively more difficult. Such a gene would thus evolve first to a silent gene and might even be eventually lost from the bacterial population by deletion (21). In an attempt to study evolution of genetic defects which underlie nutritional deficiencies, we decided to characterize the inability of *Lactococcus lactis* to synthesize certain amino acids.

L. lactis dairy fermentations strains are believed to derive from plant strains, which were introduced in a novel and relatively rich ecological niche, milk (38). Dairy strains seem to have acquired features which adapted them to milk, such as capacity to utilize lactose via a phosphotransferase system (10) and to degrade casein by a cell wall protease (22). In parallel, they have lost some functions, including the ability to synthetize branched-chain amino acids (BCAA) and histidine (2, 5, 7, 13, 37). We have recently characterized the BCAA and histidine biosynthetic operons of a prototrophic, plant-derived *L. lactis* subsp. *lactis* strain (8, 16). Here we describe the BCAA genes from an auxotrophic dairy strain. A similar study of the inactive histidine biosynthetic genes is reported in the accompanying paper (9).

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and media. The bacterial strains, plasmids, and phage used in this work are listed in Table 1. *L. lactis* strains were grown on M17 medium (44) or on CDM (chemically defined medium [41]) at 30°C. Their auxotrophy was tested by growing cells overnight in M17 medium, washing them twice in CDM, and spotting dilutions ranging from 10^{-4} to 10^{-6} on CDM agar plates.

B. subtilis and *E. coli* strains were grown in Luria-Bertani medium (29) at 37°C. When needed, erythromycin (5 μg/ml for *L. lactis*; 0.3 μg/ml for *B. subtilis*) or ampicillin (50 μg/ml for *E. coli*) was added to the culture medium. *B. subtilis* amino acid requirements were tested on Spizizen minimal medium (42).

Molecular cloning. Plasmids and total DNA were prepared as previously described (27, 29, 40). Procedures for DNA manipulations, transformation of *E. coli* cells, and cloning were essentially as described by Maniatis et al. (29). Competent *B. subtilis* cells were prepared and transformed as described by Anagnostopoulos and Spizizen (1).

* Corresponding author.

TABLE 1. Strains, plasmids, and phage

Strain, plasmid, or phage	Relevant markers and characteristics	Reference or origin[a]
Strains		
E. coli TG1	*supE Δthi* (*lac-proAB*) *hsdD5* (F'$^+$ *traD36 proAB lacI^{q}ZΔM15*)	14
B. subtilis		
MT119	*leuB6 trpC2* r$^-$ m$^-$	43
CU740	*leuA5 trpC2* (SPβ)	49
CU741	*leuC7 trpC2*	49
CU315	*leuD117 trpC2* (SPβ)	28
IL3151	*ilvD4 leuB6* r$^-$ m$^+$	16
L. lactis subsp. *lactis*[b]		
Dairy strains		
CNRZ148	1954	
CNRZ151	1954	
CNRZ167	1964	
CNRZ430	1972	
IL186	Isolated from starter, France, 1972	
IL427	Isolated from starter, France, 1965	
IL561	Isolated from starter, Belgium	
IL562	Isolated from starter, France, 1965	
IL564	Isolated from starter, France, 1965	
IL636	Isolated from starter, France, 1960	
IL894	Isolated from raw milk, France, 1958	
IL907	Isolated from cheese, France, 1962	
IL978	Isolated from starter, France, 1966	
IL982	Isolated from starter, France, 1965	
IL985	Isolated from starter, France, 1965	
IL1305		
IL1403	Plasmid free	6
NCDO966	Isolated from long milk, Swedish, 1957	
NCDO1966	Isolated from starter, Bulgarian, 1969	
Nondairy strains		
NCDO1867	Isolated from frozen peas, England, 1966	
NCDO2091	Isolated from seeds of Chinese radish, Japan	
NCDO2108	Isolated from frozen beans, 1978	
NCDO2110	Isolated from frozen peas, 1978	
NCDO2111	Isolated from frozen peas, 1978	
NCDO2118	Isolated from frozen peas, 1978	
NCDO2125	Isolated from termite gut, United States, 1978	
NCDO2146	Isolated from mastitis, 1979	
NCDO2633	Isolated from rectum of cow, 1981	
NCDO2727	Isolated from mung bean, China, 1983	
NCDO2738	Isolated from anchu mash, Germany, 1984	
Plasmids		
pBluescript	Amp^r M13*ori* pBR322*ori*	Stratagene
pIL253	Em^r pAMβ1*ori*	40
pIL334	10-kb *Sau*3A from IL1403 in pIL253	This work
pIL384	14-kb *Sau*3A from NCDO2118	16
Phage MK07	Helper phage for single-strand production with pBluescript vector	48

[a] IL strains are from our laboratory collection; CNRZ strains are from the Institut National de la Recherche Agronomique, Jouy en Josas, France; NCDO strains are from the Reading Laboratory, Agriculture and Food Research Laboratory, Reading, England.
[b] Year, medium, or place of isolation are given when known.

Induction of gene expression and hybridization procedures. RNA was prepared from cells grown in CDM supplemented with BCAA to exponential stage, washed three times, and transferred to CDM with and without BCAA for 20 min. Total RNA was prepared as previously described for *B. subtilis* (15). Total RNA samples (50 μg) were incubated in glyoxal buffer. Northern (RNA) and Southern hybridizations were done as described by Maniatis et al. (29) with DNA probes prepared with a random priming kit and ^{32}P-labeled dCTP. The hybridization control with pBluescript vector (Stratagene) as the probe against *L. lactis* DNA does not give a signal.

DNA sequence analysis. *E. coli* clones for sequencing were obtained by subcloning specific DNA fragments in pBluescript and by generating a series of overlapping deletions. Single-stranded DNA was prepared as described by Vieira and Messing (48) and sequenced essentially according to the Applied Biosystems protocol for the 370A DNA sequencer (Applied Biosystems, San Jose, Calif.). The reported sequence was determined on both strands. The DNA and protein sequences were analyzed by using the BISANCE package, implemented at the Centre Inter-Universitaire d'Informatique à Orientation Biomédicale (Paris, France).

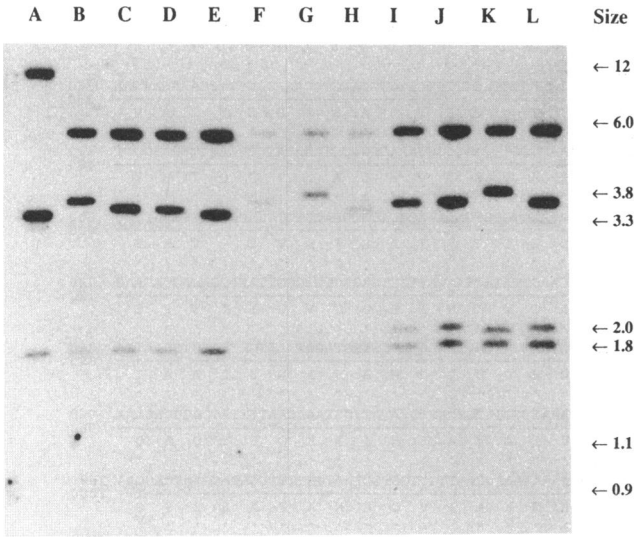

FIG. 1. Hybridization analysis of different *Lactococcus* strains. Total bacterial DNA was cleaved with *Eco*RI and hybridized with a 14.5-kb DNA segment encoding the *L. lactis* subsp. *lactis* NCDO2118 *ilv* operon. Molecular sizes are given in kilobases. Lanes: A to F, nondairy strains NCDO2091, NCDO2118, NCDO2146, NCDO2108, NCDO2738, and NCDO2727, respectively; G to L, dairy strains IL564, CNRZ430, IL636, CRNZ148, IL1403, and IL562, respectively.

Nucleotide sequence accession number. The GenBank accession number for the NCDO2118 sequence is M90761.

RESULTS

BCAA auxotrophy. Different *L. lactis* subsp. *lactis* strains, isolated from either dairy products or nondairy sources (Table 1), were tested for growth in the absence of BCAA. For this purpose, serial dilutions of exponentially growing cells were plated on solid CDM lacking one of the three amino acids or all of them and incubated at 30°C. Formation of large colonies within 24 h was scored as growth, whereas absence of detectable colonies within 24 h and appearance of very small heterogeneous colonies after >3 days was scored as no growth. Two distinct phenotypes were observed. All of the 17 dairy strains tested but only 2 of the 11 nondairy strains (NCDO2108 and NCDO2110) were unable to grow when the three BCAA were missing, which confirms and extends the previous reports of BCAA auxotrophy among the dairy strains (2, 5, 7, 37).

Auxotrophic strains possess genes required for BCAA biosynthesis. BCAA auxotrophy of the dairy strains could be due to the absence of the required biosynthetic genes. To test this possibility, we performed Southern hybridization analysis of the DNA from six prototrophs and six auxotrophs, using as a probe plasmid pIL584, which carries the *leu* and *ilv* genes of the prototrophic strain NCDO2118 (16). Hybridizing bands, resulting from *Eco*RI cleavage, were detected in all cases (Fig. 1). All patterns are very similar to that of NCDO2118 (lane B), which contains two strong bands (6 and 3.8 kb) and four weak bands (3.3, 1.8, 1.1, and 0.9 kb; the last two cannot be seen on Fig. 1 but were clearly visible on the original autoradiogram). The other patterns can be explained by a loss of a single *Eco*RI site (between two 6-kb segments [lane A] and between 1.1- and 0.9-kb segments [lanes G and I to L]) and by the presence of various numbers of 72-bp direct repeats carried on the 3.8-kb segment (see below). These results suggest that genes required for BCAA biosynthesis are present in auxotrophic *L. lactis* subsp. *lactis* strains.

Cloning of the BCAA region from an auxotrophic strain. The presence of the BCAA biosynthetic genes in the auxotrophic strains indicates that the genes are not functional, possibly because of either mutations or inadequate expression. To test these hypotheses, we decided to clone the BCAA region from our laboratory strain IL1403, which is a BCAA auxotroph. Anticipating the presence of at least some functional genes in this region, we used a cloning strategy based on complementation of a *leuC* mutation present in *B. subtilis* MT119 (the mutation is known as *leuB6* but is carried in the gene which corresponds to *E. coli leuC*; the *E. coli* nomenclature [46] was previously followed for *L. lactis* BCAA genes [16] and is used throughout this text).

Chromosomal IL1403 DNA was partially digested with endonuclease *Sau*3A to obtain a majority of fragments larger than 5 kb, ligated with *Bam*HI-cleaved cloning vector pIL253, and used to transform *B. subtilis* MT119 competent cells to leucine independence and erythromycin resistance (a vector-specified character). Twelve independent transformants were obtained with 5 μg of *L. lactis* DNA. All transformants contained a plasmid. The plasmids carried a common region, which corresponds to the previously analyzed *leu* genes of the *L. lactis* BCAA prototroph NCDO2118 (16), as judged by restriction analysis with several multicutting enzymes. A Southern hybridization analysis, using as a probe plasmid pIL584, which carries the NCDO2118 BCAA region, confirmed this conclusion. The auxotrophic strain IL1403 therefore contains at least one functional BCAA gene, *leuC*.

Complementation assays. To test whether other BCAA genes from the auxotrophic strain are functional, we used plasmid pIL334 for complementation experiments in *B. subtilis*. This plasmid contains the largest IL1403 insert, of 10 kb, encoding the *leuABCD* and *ilvD* genes, as deduced by comparing its restriction pattern with that of the BCAA region from the prototrophic strain NCDO2118. It complemented *leuC* and *ilvD* mutants but not *leuA*, *leuB*, and *leuD* mutants. In contrast, plasmid pIL584, which carries the genes from the prototroph, complemented all of the mutants except *leuD*. This finding suggests that the auxotrophic strain contains two functional (*leuC* and *ilvD*) and two inactive (*leuA* and *leuB*) BCAA genes. No conclusion could be drawn about *leuD*, which was not complemented even with the prototroph gene (16).

Nucleotide sequences of the *leu* genes. To further characterize the BCAA genes from the auxotrophic strain, we determined the nucleotide sequence of a 6,140-bp region carried on plasmid pIL334. This region contains all *leu* genes and an open reading frame of unknown function, designated ORF2 (16). The sequence and its counterpart from the prototrophic strain NCDO2118 are shown in Fig. 2. Comparison of the two sequences is summarized in Table 2.

The overall homology between the auxotroph and prototroph sequences is very high, only ~1% of bases being different. Noncoding regions contain a higher proportion of base substitutions (2.3%) than do coding regions (0.9%), and most substitutions in the coding regions (59%) occur in the third codon position. Transitions are more frequent than transversions in the noncoding region but not in the coding region (Table 2). Two small deletions are present in the auxotroph, in the *leuA* gene (24 bp) and upstream of the *leuC* gene (21 bp). They most probably occurred by recombina-

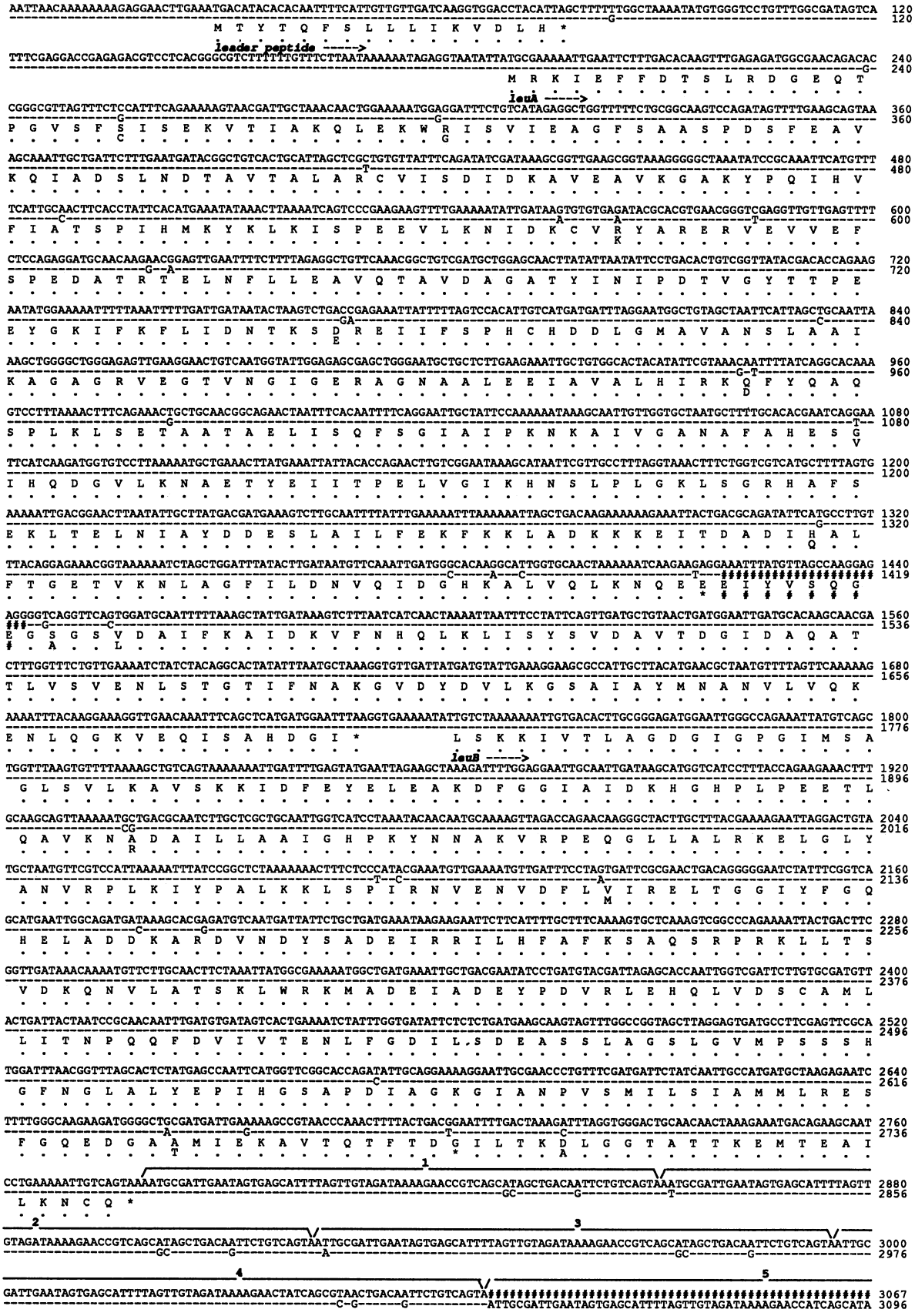

FIG. 2. Nucleotide and derived amino acid sequences of the *L. lactis* subsp. *lactis leu* operon. NCDO2118 (prototroph) and IL1403 (auxotroph) sequences are shown on the top and bottom lines, respectively. Symbols: *, stop codon; #, missing homologous nucleotide; – and ·, identical nucleotides and amino acids, respectively. The 72-bp direct repeats are indicated by brackets above the sequences.

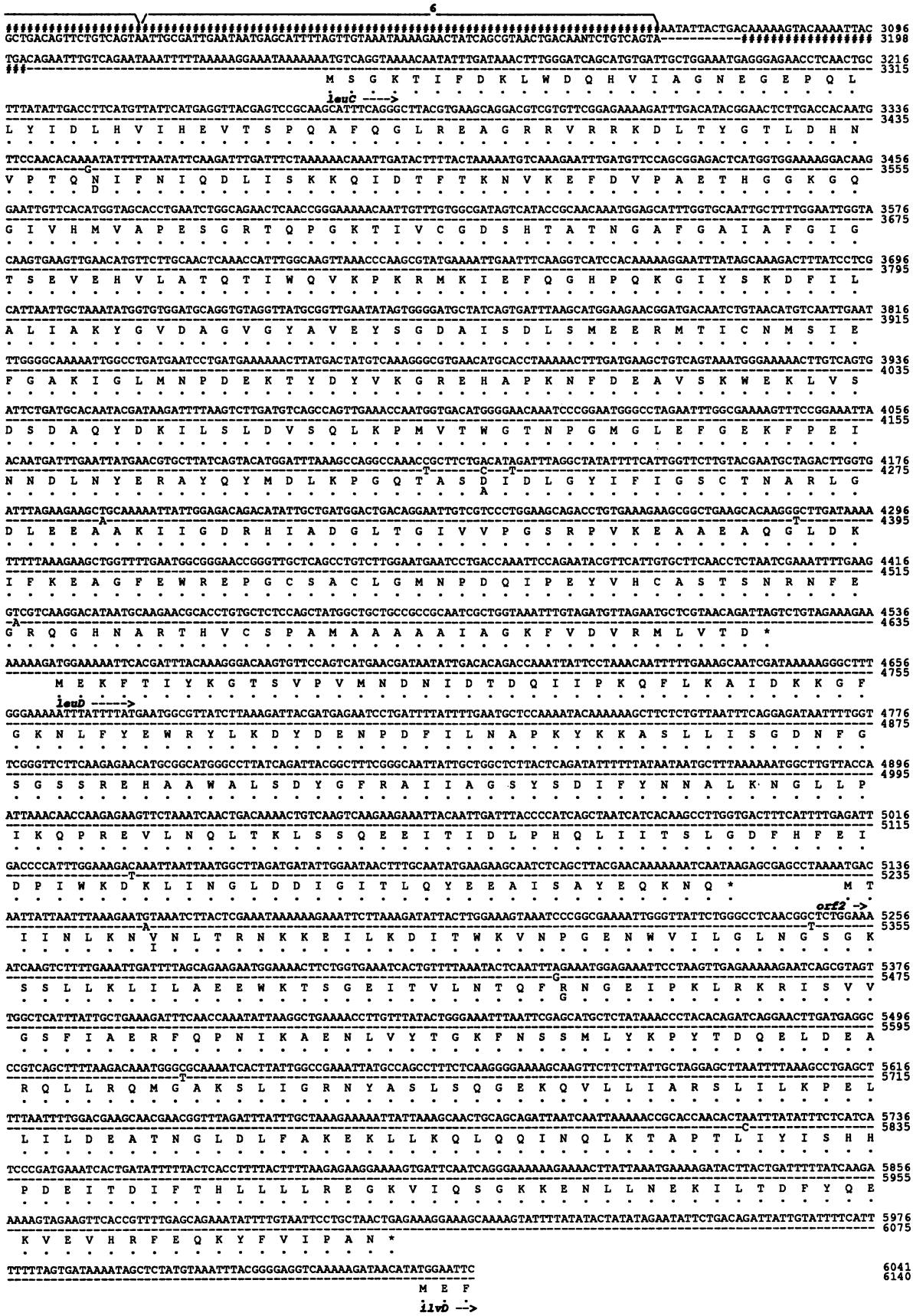

FIG. 2—*Continued*

TABLE 2. Comparison of pairs of genes and noncoding regions from strains IL1403 and NCDO2118

DNA region	No. of substitutions/ total length[a]	Distribution of difference			No. of transitions/ no. of transversions	No. of silent substitutions	No. of amino acid changes	Observation in strain IL1403
		1[b]	2	3				
Leader	0/51	0	0	0	0/0	0	0	
leuA	24/1,542	7	3	14	11/13	13	10	24-bp deletion between positions 1399 and 1422; stop in position 1395
leuB	12/1,038	4	2	6	7/5	6	5	Stop in position 2677
leuC	7/1,383	1	1	5	2/5	5	2	
leuD	1/576	0	0	1	1/0	1	0	
orf2	5/780	2	0	3	4/1	3	2	
Coding region[c]	49/5,370	14	6	29	25/24	28	19	
Noncoding region	15/671				12/3			2 additional 72-bp direct repeats; 21-bp deletion between positions 3181 and 3201
Total	64/6,041				37/27			

[a] NCDO2118 sequence is used; deletions are excluded; length is in nucleotides.
[b] Number refers to codon position.
[c] Including the beginning of *ilvD*.

tion between two short direct repeats of 5 and 8 bp, respectively. In addition, six copies of the 72-bp direct intergenic repeat are present between *leuB* and *leuC* genes in the auxotroph, whereas only four such repeats are found in the prototroph. The number of repeats appears to vary in other *L. lactis* subsp. *lactis* strains as well, as judged from the difference in size of the *Eco*RI segment which carries the repeats (between 3.6 and 4.0 kb; Fig. 1).

Two base substitutions created stop codons in the *leuA* and *leuB* genes. The two corresponding proteins are thus truncated, LeuA being 409 amino acid long instead of 513 in the prototroph and LeuB being 320 amino acids long instead of 345. These changes could have made the proteins inactive. The deletion in the *leuA* gene is another event that might inactivate the protein, although it is localized downstream from the new stop codon. The auxotrophy of the IL1403 strain is therefore likely to be the consequence of mutational gene inactivation. Interestingly, the auxotroph *leuD* gene product is identical to that of the prototroph, which indicates that at least three of the BCAA auxotroph genes (*leuC*, *leuD*, and *ilvD*) are functional.

RNA analysis. The three functional BCAA genes of the auxotroph could nevertheless be inactive if they were not transcribed. To test this possibility, RNA was prepared from the auxotrophic IL1403, and the prototrophic NCDO2118 cells were incubated in the absence or presence of the three BCAA (see Materials and Methods). The RNA was analyzed by Northern hybridization with a 1.8-kb probe containing the *ilvD* gene (Fig. 3). Two major RNA bands, of 14,500 and 7,700 nucleotides, were found in samples prepared from prototrophic cells incubated without BCAA. These might result from transcription initiated at two putative promoters revealed by sequence analysis in front of the *ilv* and *leu* genes, respectively (16). The hybridization pattern with RNA from the auxotroph is comparable, and the intensity of transcription not significatively different. Hybridization was not detected in samples from cells incubated with BCAA (Fig. 3), which indicates that the transcription is controlled in a similar way in the auxotrophic and prototrophic *L. lactis* strains.

DISCUSSION

The analysis reported here is the first to establish the nature of lesions affecting amino acid biosynthesis in lactic acid bacteria at the molecular level. The extent of the lesion in the *leu* genes does not allow the reversion to prototrophy, which is different from observations for lactobacilli, enterococci, and pediococci, in which several biosynthetic pathways could be reverted, presumably by single mutations (7, 33, 34). These mutations generally affect specific genes of the pathways, but it was also suggested that a mutation in the RNA polymerase may reactivate a pleiotropic gene such as a transaminase, which could be involved in several pathways (7).

It is striking that both the *leu* and *ilv* operons of *L. lactis* subsp. *lactis* contain active and inactive genes. Two hypotheses might be considered to explain these observations. One is that the operon is still in an early stage of inactivation and that additional mutations will accumulate with time. This hypothesis is consistent with a view of progressive modification and an eventual loss of cryptic genes from the cell (21). The auxotroph that we analyzed was isolated in a dairy factory 35 years ago and has been maintained since then in the laboratory collection. We do not know for how long its putative prototroph ancestor was used for milk fermentation. However, it is surprising that another inactive operon from the same strain, *his*, which is described in the accompanying report (9), contains no functional genes. It is nevertheless possible that the two operons evolve with different rates. The other hypothesis is that the BCAA genes which

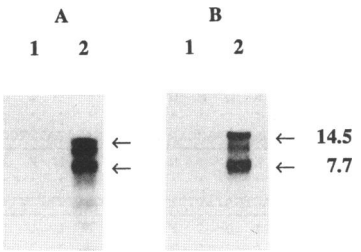

FIG. 3. Transcription of the BCAA operon. A 1.8-kb DNA probe from NCDO2118 containing the *ilvD* gene was used. (A) Prototroph NCDO2118 RNA; (B) auxotroph IL1403 RNA. Lanes: 1, cells incubated with BCAA; 2, cells incubated without BCAA. The intermediate band is presumably a processing of the larger one, and its intensity varies significantly according to the extraction. Sizes are indicated in kilobases.

remained active have a role other than amino acid biosynthesis in *L. lactis* subsp. *lactis*. This role is not essential, since it was possible to delete the entire BCAA operon of the prototrophic strain without affecting its viability (unpublished results). We cannot distinguish at present between the two hypotheses.

The reasons for the systematic BCAA deficiency in *L. lactis* subsp. *lactis* dairy strains are not known. The only other systematic requirement of dairy strains is for histidine (9); arginine and methionine are occasionally required, and other amino acids are rarely necessary (2, 5, 7, 37). In contrast, lactobacilli, which are other major dairy bacteria, are generally auxotrophic for 7 to 15 amino acids (33, 34). BCAA are the most frequent constituents of *L. lactis* subsp. *lactis* proteins (45) and are not particularly abundant in milk (18, 31). These observations suggest the existence of a selective pressure for auxotrophy, as previously observed with *B. subtilis* and *E. coli* laboratory strains (11, 50). It is conceivable that an intermediate of the BCAA pathway may be toxic for the cell or might perturb regulation of some other pathways, since (i) endogenous 2-ketobutyrate, an intermediary of BCAA synthesis, is toxic in *S. typhimurium* (47) and (ii) the BCAA pathway is linked to two other pathways, an anabolic one for pantothenate (3) and a catabolic one for 2,3-butanediol (20). Regulation of these pathways might be affected by BCAA synthesis. Further analysis of cell metabolism might allow testing of these hypotheses.

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