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

> > Received 23 November 1992/Accepted 11 May 1993

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 μ g/ml for L. lactis; 0.3 μ g/ml for B. subtilis) or ampicillin (50 μ g/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.

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

give a signal.

^b Year, medium, or place of isolation are given when known.

were done as described by Maniatis et al. (29) with DNA probes prepared with a random priming kit and ³²P-labeled dCTP. The hybridization control with pBluescript vector (Stratagene) as the probe against L. lactis DNA does not (Paris, France).

strains are from the Reading Laboratory, Agriculture and Food Research Laboratory, Reading, England.

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

Strain, plasmid, or phage	Reference or origin ^a	
Strains		
E. coli TG1	supE Δ thi (lac-proAB) hsdD5 (F' ⁺ traD36 proAB lacPZ Δ M15)	14
B. subtilis		
MT119	leuB6 trpC2 r ⁻ m ⁻	43
CU740	leuA5 trnC2 (SPB)	49
CU741	leuC7 trnC2	49
CU315	leuD117 trnC2 (SPB)	28
11.3151	$ilvD4 levB6 r^- m^+$	16
L. lactis subsp. lactis ^b		10
Dairy strains		
CNRZ148	1954	
CNRZ151	1954	
CNRZ167	1964	
CNR7430	1972	
II 186	Isolated from starter France 1072	
IL 427	Isolated from starter, France, 1972	
IL 561	Isolated from starter, Flance, 1905	
IL 562	Isolated from starter, France, 1965	
IL 564	Isolated from starter, France, 1965	
IL 636	Isolated from starter, France, 1960	
II 804	Isolated from raw milk France, 1959	
11 007	Isolated from cheese France, 1950	
11.078	Isolated from starter France, 1966	
11.082	Isolated from starter, France, 1965	
11.762	Isolated from starter France, 1965	
IL 1205	Isolated from statter, Flance, 1905	
IL 1303	Diasmid free	6
NCD0966	Isolated from long milk Swedish 1057	0
NCDOJ066	Isolated from starter Bulgarian 1960	
Nondairy strains	Isolated Holli Statter, Bulgarian, 1909	
NCDO1967	Isolated from frazen nees. England 1066	
NCDO2001	Isolated from seeds of Chinese radish Jonan	
NCDO2091	Isolated from frozen beens, 1079	
NCDO2106	Isolated from frozen pees, 1978	
NCDO2110 NCDO2111	Isolated from frozen peas, 1978	
NCDO2111 NCDO2118	Isolated from frozen peas, 1978	
NCDO2116 NCDO2125	Isolated from formits and United States 1079	
NCDO2125 NCDO2146	Isolated from mostifie 1070	
NCDO2140	Isolated from masters of new 1001	
NCD02033	Isolated from rectum of cow, 1961	
NCDO2727 NCDO2729	Isolated from mung bean, China, 1983	
NCDU2/30 Plaamida	Isolated from anchu mash, Germany, 1984	
		C 4 4
poluescript	Amp Milon pBK3220n	Stratagene
p1L233	EIII μ Amp 10π 10 kb Sau2 A from II 1402 in all 252	40 This a 1
p1L334	10-KU SauSA from 1L1403 in piL253	I his work
PILJO4 Dhaga MK07	14-kD Sausa Irom NUDU2118 Ushan nhana fan single strand nas dustien with a Diversitie	10
rnage MNU/	neiper phage for single-strand production with pBluescript vec- tor	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



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 NCD02118 (16). Hybridizing bands, resulting from *Eco*RI cleavage, were detected in all cases (Fig. 1). All patterns are very similar to that of NCD02118 (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 BCAA GENE INACTIVATION IN L. LACTIS 4385

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 Sau3A to obtain a majority of fragments larger than 5 kb, ligated with BamHI-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 NCD02118. 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 $\sim 1\%$ 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-

AATTAACAAAAAAAAGAGGAACTTGAAATGACATACAACACAATATTCATTGTTGTTGATCAAGGTGGACCTACATTAGCTTTTTTGGCTAAAATATGTGGGTCCTGTTTGGCGATAGTCA G M T Y T Q F S L L L I K V D L H *	120 120
leader peptide>	
TTTCGAGGACCGAGAGACGTCCTCACGGGGGTCTTTTTTGTTTCTTAATAAAAAATAGAGGTAATATTATGCGAAAAAATTGAATTCTTTGACACAAGATGGCGAACAGACAG	240
M R K I E F F D T S L R D G E Q T	
${\tt Loca} {\tt} correct and the construction of the construct$	360
––––––––––––––––––––––––––––––––––––––	360
C	
AGCAAATTGCTGATTCTTTGAATGATACGGCTGTCACTGCATTAGCTCGCTGTGTTATTTCAGATAAGCGGTTGAAGCGGTAAAGGGGGCTAAATATCCGCAAATTCATGTTT T	480
KQIADSLNDTAVTALARCVISDIDKAVEAVKGAKYPQIHV	
TCATTCC & ACTTC & CCT&TTC & CATCA & TATA & A ATT & CTCCCCC & A C& A C	600
	600
CTCCAGAGGATGCAACAAGAAGGAGTGCAAGTTCAATTTCTTTTAGAGGCTGTTCAAACGGCTGTCGATGCAGGGGGGCGACCTTTAATATTTAATATTCCTGACACTGCGGTTCAATATACGACCACGAGA	720
S P E D A T R T E L N F L L E A V Q T A V D A G A T Y I N I P D T V G Y T T P E	120
$ = \begin{bmatrix} a_1 & a_2 & a_3 \\ a_4 & a_5 & a_6 &$	840
EYGKIFKFLIDNTKSDREIIFSPHCHDDLGMAVANSLAAI	
AAGCTGGGGCTGGGAAGTTGAAGGAACTGTCAATGGTATTGGAGAGCGAGC	960
KAGAGRVEGTVNGIGERAGNAALEEIAVALHIRKQFYQAQ	960
· · · · · · · · · · · · · · · · · · ·	
GTCCTTTAAAACTTTCAGAAACTGCTGCAACGGCAGAACTAATTTCACAATTTTCAGGAATTGCTATTCCAAAAAAAA	1080 1080
S P L K L S E T A A T A E L I S Q F S G I A I P K N K A I V G A N A F A H E S G	
TTCATCAAGATGGTGTCCCTTAAAAATGCTGAAACTTATGAAAATTATTACACCAGAACTTGTCGGAATAAAGCATAATGCGTGCCCTTTAGGTAAACTTTCTGGTCGGCGCATGCCTTTAGG	1200
	1200
AAAAATTGACGGAACTTAATATTGCCTTATGACGATGAAAGTCTTGCAATTTTATTTGAAAAAATTTAGACAGAAAAATTAGCTGACAAAAAAATAACGAACATAACGAACATGACGAAAATTCAA	1320
EKLTELNIAYDDESLAILFEKFKKLADKKKEITDADI HAL	1320
· · · · · · · · · · · · · · · · · · ·	
TTACAGGAGAAACGGTAAAAAATCTAGCTGGATTTATCTTGATAATGTCAAATTGATGGCACAGGCATTGGCAACGAAATCAAGAAGGAAATTTATGTTACCCAAGGAG	1440 1419
FIGETVKNLAGFILDNVQIDGHKALVQLKNQEEEIYVSQG 	
AGGGGTCAGGTTCAGTGGATGGATGCAATTTTAAAGCTATTGATAAAGTCTTTAATCATCAACTAAATTAATT	1560
###~GCCCCCC	1536
Ŧ. Ă L	
CTTTGGTTTCTATAAAATCTATCTACAGGCACTATATTTAATGCTAAAGGTGTTGATTATGATGATGAAGGGAGCGCCATTGCTTACATGAACGCTAATGTTTAGTTCAAAAAG	1680
T L V S V E N L S T G T I F N A K G V D Y D V L K G S A I A Y M N A N V L V Q K	1000
	1900
	1776
TGGTTTAAGTGTTTTAAAAGCTGTCAGTAAAAAAATTGATTTTGAGTATGAATTAGAAGCTAAGGTTTTGGAGGAATTGCAATTGAATAAGCATGGTCATCCTTTAACCAGAAGAAACTTT	1920
G L S V L K A V S K K I D F E Y E L E A K D F G G I A I D K H G H P L P E E T L	1990
	2040
Q A V K N A D A I L L A A I G H P K Y N N A K V R P E Q G L L A L R K E L G L Y	
TGCTAATGTTCGTCCATTAAAAATTTATCCGGCTCTAAAAAAACTTTCTCCCATACGAAATGTTGAAAATGTTGATTTCCTAGTGATTCGCGAACTGACAGGGGGAATCTATTTCGGTCA	2160
ANVRPLKIYPALKKLSPIRNVENVDFLVIRELTGGIYFGQ	2136
· · · · · · · · · · · · · · · · · · ·	
GCARTGAATTGGCAGATGATAAAGCACGAGAATGTCAATGATTATTCTGCTGATGAAATAAGAAGAATTCTTCAATTTGGCTTTCAAAAGTGCTCAAAGTCGGCCCAGAAAATTACTGACTTC 	2280
HELADDKARDVNDYSADBIRRILHFAFKSAQSRPRKLLTS	
GGTTG 3/78 3 8/7 8 3 8 3 7 GTTTTTTTTG C 8 6/7 TTTTTT 3 8 8/7 7 8/7 GGCTG C 6 8 8 8 8 7 GGCTG 2 7 G 8 7 7 8 7 G 7 7 G 8 7 8 8 7 7 7 7 G 8 7 8 8 7 7 7 7	2400
	2376
actgattactaatccgcaacaatttgatgtgatagtcactgaaaatttggtgatattctctctgatgaaggcaagtagtttggccggtagcttaggaggtgatgccttcgagttccca	2520
L I T N P Q Q F D V I V T E N L F G D I L,S D E A S S L A G S L G V M P S S S H	2490
TIGET TARCAC TATAGAGCAATTATGTTCGCACCAATAAGAAAAGAA	2640 2616
G F N G L A L Y E P I H G S A P D I A G K G I A N P V S M I L S I A M M L R E S	
TTTTGGGCAAGAAGATGGGGCTGCGATGATGAAAAAGCCGTAAACCCTATACTGACGGAATTTTGACTAAAGATTTAGGTGGGACTGCAACAACTAAAGAAATGACAGAAGCAAC	2/60
TTTTGGGCAAGAAGATGGGGCTGCGATGATTGAAAAAGCCGTAACCCAAACTTTTGACGGACTTTTGACTAAAGATTTAGGTGGGACTGCAACAACTAAAGAAATGACAGAAGCAAT 	2736
TTTTGGGCAAGAAGATGGGGCTGCGATGATTGAAAAAGCCGTAACCCAAACTTTTACTGACGGAATTTTGACTAAAGATTTAGGTGGGACTGCAACAACTAAAGAAATGACAGAAGCAAA F G Q E D G A A M I E K A V T Q T F T D G I L T K D L G G T A T T K E M T E A I 	2736
$\text{TTTTGGGCAAGAAGATGGGGCTGCGATGAAAAAGCCGTAAAGCGTAACCCAAACTTTTACTGACGGAATTTTGACTAAAGATTTAGGTGGGACTGCAACAACTAAAGAAATGACAGAAGCAATTTTAGTGGGAACTAAAGAAACGAACCGTCAACAACTAAAGAAATGGCGATTGCAATAAAAGGACCGTCACTAAAGAAATGGCGATTGCAATAAAAGGACCGTCACTAAAGAAACGAACCGCTCACCATTGTCACTAAAAAGGACCGTCACTAAAGAAATGGCGATTGCAATAAAAGGACCGTCACTAAAAAGAACCGCTCACTAAAGAAATGGCGATTGCAATAAAAGGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAGAAATGGCGATTGCACTAAAAAGGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGATTGCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAAAGAACGACCGTCACTAAAAAAATGCGATGCACTAAAAAAAA$	2736
$\begin{array}{c} \begin{array}{c} TTTGGGCAAGAAGATGGGGCTGCGATGATTGAAAAAGCCGTAAACCCAAACTTTTACTGACGGAATTTTGACTAAAGATTAGGTGGGGACTGCAACAACTAAAGAAATGACAGAAGCAATTTTGACTAAAGAAATTTTGACTAAAGAAACTAAAGAAATGACAGAAGCAATTTTGACTAAAGAAATTGTCAGTAAAATGCGATTGAATAGTGAGCATTTTAGTTGTGAGAAAAAGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTGTGAGAAAAAGCGGCGATGAAATGCGATTGAATAGTGAGCATTTTAGTTGTGTGTG$	2736 2736 2880 2856
$\text{TTTTGGGCAAGAAGATGGGGCTGGCGATGATTGAAAAAGCCGTAACCCAAACTTTTACTGACGGAATTTTGACTAAAGATTAGGTGGGGACTGCAACAACTAAAGAAATGACAGAAGCAATTTTGACGAACTAAAGAAATGCGAATGCGAACAACTAAAGAAATGCGAATGCGATTGAATAGTGAGCATTTTAGTTGTAGAAAAGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTAGATAAAAGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAAAAGGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAAAAGGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAAAAGGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAAAAGGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAAAAGGAACCGTCAGCATAGCTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTCAGTAGAATGCGATTGAATAGTGAGCATTTTAGTTGTGTGAGAAAGGAACCGTCAGCATGCGACGATGCGACGTGACAATTCTGTCAGTAAATGCGATTGAATAGTGAGCATTTTAGTTGTGTGTG$	2736 2736 2880 2856
$\text{TTTTGGGCAAGAAGATGGGGCTGGCAGGATGGAAAAAGCCGTAAAGCCGTAAGCCGAAAGCCGTAGGGAATTTTGGGAATTTTGGGAATTTTGGGGACTGGAACAACTAAAGAAATGGCAAGAAGCAAATGCGAACAACTAAAGAAATGGCAATTTGGGAATAGTGAGGAATTTTGGGAATAGTGGGGACTGGAAAATGGGACTGAAAATGGGACTGAAAATGGGACTGAAAATGGGACTGAAAATGGGACTGAAAATGGGACTGAAAAAGAACCGTCAGGATGAAATGGGACTGAAATAGTGGGATTGGAATAATGGGATTGAAATAGTGAGGATTGAATAGTGAGGATTGAATAGTGAGGATTGAATAGTGGGGATGGACGATTGTAGAATAGTGAGGATTGAAATGGCGATGGCATTGGGATGAAATGGCGATGGACGATGGCATTGGGATGAAAAGAACCGTCAGGATGAAATGGCGGCAGGATGGCGATGGACGATGGACGATGGCGATGGACGATGGCGATGGACGATGGCGATGGACGATGGCGATGGACGATGGCGATGGAGGATGGCGGCAGGATGGAGGATGGAGGATGGCGGCAGGATGGCGGATGGAGGATGGCGGCAGGATGGCGGATGGAGGATGGGATGGGGATGGGGATGGCGGCAGGATGGCGGCATGGCGGATGGGATGGAGGATGGGGATGGAGGATGGGGATGGGATGGGGATGGGATGGGGATGGGATGGGATGGGATGGGATGGGGATGGGGATGGGATGGGGATGGAGGA$	2736 2736 2880 2856
$\begin{array}{c} \begin{tabular}{c} \begin{tabular}{c} \begin{tabular}{c} \begin{tabular}{c} \begin{tabular}{c} \begin{tabular}{c} \end{tabular} \\ \end{tabular} \\ \end{tabular} \\ \end{tabular} \\ \end{tabular} \\ \end{tabular} \\ \begin{tabular}{c} \end{tabular} \\ \begin{tabular}{c} \end{tabular} \\ $	2736 2736 2880 2856 3000 2976
$\begin{array}{c} TTTTGGGCAAGAAGATGGGGCTGGCGATGATTGAAAAAGCCGTAACCCAAACTTTTACTGACGGAATTTTGGCGAATTTTGGCGGGGGGGG$	2750 2736 2880 2856 3000 2976

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 \cdot , identical nucleotides and amino acids, respectively. The 72-bp direct repeats are indicated by brackets above the sequences.

6	
######################################	3096 3198
TGACAGAATTTGTCAGAATAAATTTTTTAAAAAGGAAATAAAAAATGTCAGGTAAAACAATATTTGATAAACTTTGGGATCAGCATGTGATTGCTGGAAATGAGGGAGAACCTCAACTGC	3216
M S G K T I F D K L W D Q H V I A G N E G E P Q L	3313
Leuc > TTTATATTGACCTTCATGTTATTCATGAGGTTACGAGGTCGCAAGGCATTTCAGGGCTTACGTGAAGCAGGACGTCGTGTTCGGGAGAAAAGATTTGACATACGGAACTCTTGACCACAATG	3336
LYIDLHVIHEVTSPQAFQGLREAGRRVRRKDLTYGTLDHN	3435
TTCCAACACAAAAATATTTTTAATATTCAAGATTTGATTTCTAAAAAAAA	3456
VPTQNIFNIQDLISKKQIDTFTKNVKEFDVPAETHGGKGQ	3555
GANTTGETCACATGGTAGCACCTGAATCTGGCAGAAAACTGGGGAAAAAACAATTGGTTGTGGGGGATAGTCATACCGGCAACGAACTGGGGGGGG	3576
GIVHMVAPESGRTQPGKTIVCGDSHTATNGAFGAIAFGIG	3675
	2606
CARGIGARCTICARCACTTTCTCCARCCATTTCGCARGTTAARCCCARGCCTATGAAAATTGAATTCAAGGTCATCCACAAAARGGAATTTATGCAAGACTTTATCCTCG	3795
CATTAATTAGCTAAATATGGTGGGGGGGGGGGGGGGGGG	3816 3915
ALIAKY GVDAGVGYAVEY SGDAISDLSMEERMTICNMSIE ••••••••••••••••••••••••••••••••••••	
TTGGGGCAAAAATTGGCCTGATGAATCCTGATGAAAAAACTTATGACTATGTCAAAGGGCGTGAACATGCACCTAAAAACTTTGATGAAGCTGTCAGTAAATGGGAAAAACTTGTCAGTG	3936 4035
F G A K I G L M N P D E K T Y D Y V K G R E H A P K N F D E A V S K W E K L V S	
ATTCTGATGCACAATACGATAAGATTTTAAGTCTTGATGTCAGCCAGTTGAAACCAATGGTGACATGGGGAACAAATCCCGGAATGGGCCTAGAATTTGGCGAAAAGTTTCCGGAAATTA	4056
D S D A Q Y D K I L S L D V S Q L K P M V T W G T N P G M G L E F G E K F P E I	4155
ACAATGATTTGAATTATGAACGTGCTTATCAGTACATGGATTTAAAGCCAGGCCAAACCGCTTCTGACATAGATTTAGGCTATATTTTCATTGGTTCTTGTACGAATGCTAGACTTGGTG	4176
NNDLNYERAYQYMDLKPGQTASDIDLGYIFIGSCTNARLG	4275
ATTTAGAAGAAGCTGCAAAAAATTATTGGAGACAGAACATATTGCTGATGAGCTGACAGGAATTGTCGTCCCTGGGAAGCAGACCTGTGAAAGAAGCGGCTGAAAGCACAAGGGCTTGATAAAA	4296
D L E E A A K I I G D R H I A D G L T G I V V P G S R P V K E A A E A Q G L D K	4395
TTTTTAAGAACCTGTTTTGAATGGGGGGAACCGGGTGCTCAGCTGTCTGGAATGCATCGACGAATTCCAGAATTCCAGAATTCCAGAATTTGAAG	4515
GTCGTCAAGGACATAATGCAAGAACGCACCTGTGCTCTCCAGCTATGGCTGCTGCCGCCGCGAATCGCTGGTAAATTTGTAGAATGCTCGTAAAAGAATTAGTCTGTAGAAAGAA	4536 4635
G R Q G H N A R T H V C S P A M A A A A A I A G K F V D V R M L V T D *	
AAAAAGATGGAAAAATTCACGATTTACAAAGGGACAAGTGTTCCAGTCATGAACGATAATATTGACACAGACCAAATTATTCCTAAACAATTTTTGAAAGCAATCGATAAAAAGGGCTTT	4656 4755
MEKFTIYKGTSVPVMNDNIDTDQIIPKQFLKAIDKKGF	
I ded> GGGAAAAATTTATTTATGAATGGCGTTATCTTAAAGATTACGATGAGAATCCTGATTTTATTTTGAATGCTCCAAAATACAAAAAAGCTTCTCTGTTAATTTCAGGAGATAATTTTGGT	4776
G K N L F Y E W R Y L K D Y D E N P D F I L N A P K Y K K A S L L I S G D N F G	
TCGGGTTCTTCAAGAGAACATGCGGCCATGGGCCTTATCAGATTACGGCTTTCGGGCCAATTATTGCTGGCTCTTACTCAGATATTTTTTATAATAATGCTTTAAAAAAATGGCTTGTTACCA	4896
SGSSREHAAWALSDYGFRAIIAGSYSDIFYNNALK'NGLLP	4995
ATTAAACAACCAAGAGAAGTCTAAATCAACTGACAAAACTGTCAAGTCAAGAAGAAATTACAATTGATTTACCCCATCAGCTAATCATCATCACCAGGTGACTTTCATTTTGAGATT	5016
I K Q P R E V L N Q L T K L S S Q E E I T I D L P H Q L I I T S L G D F H F E I	5115
	6126
D D T W K D K L T N G L D D T G T T L O Y R R A T S A Y R O K N O * M T	5235
orf2 ->	
AATTATTAAAGAATGTAAATCTTACTCGAAATAAAAAAAGAAATTCTTAAAGATATTACTTGGAAAGTAAATCCCCGGCGAAAATTGGGTTATTCTGGGCCCCCAACGGCTCTGGAAA	5256 5355
IINLKNVNLTRNKKEILKDITWKVNPGENWVILGLNGSGK	
ATCAAGTCTTTTGAAATTGATTTTAGCAGAAGAATGGAAAACTTCTGGTGAAATCACCGTTTTAAATACTCAATTTAGAAATGGAGAAATTCCTAAGTTGAGAAAAAGAATCAGCGTAGT	5376 5475
S S L L K L I L A E E W K T S G E I T V L N T Q F R N G E I P K L R K R I S V V	
TGGCTCATTTATTGCTGAAAGATTTCAACCAAATATTAAGGCTGAAAACCTTGTTTATACTGGGAAATTTAATTCGAGCATGCTCTATAAACCCTACACAGATCAGGAACTTGATGAGGGC	5496
G S F I A E R F Q P N I K A E N L V Y T G K F N S S M L Y K P Y T D Q E L D E A	5555
CCGTCAGCTTTTAAGACAAATGGGGGGCAAAATCACTTATTGGCCGAAATTATGCCAGCCTTTCTCAAGGGGGAAAAGCAAGTTCTTCTTATTGCTAGGAGCTTAATTTTAAAGCCTGAGCT	5616
	5715
R Q L L R Q M G A K S L I G R N Y A S L S Q G E K Q V L L I A R S L I L K P E L	5736
R Q L L R Q M G A K S L I G R N Y A S L S Q G E K Q V L L I A R S L I L K P E L	5835
R Q L L R Q M G A K S L I G R N Y A S L S Q G E K Q V L L I A R S L I L K P E L TTAATTTTGGACGAACGAACGGATTAAGATTAATTGCTAAAGAAAAATTATTAAAGCAACTGCAGCAGATTAATCAATTAAAAACGGACCAACACTAATTTAATATTTCCAACA L I L D E A T N G L D L F A K E K L L K O L O O I N O L K T A P T L I Y I S H H	
R Q L L R Q M G A K S L I G R N Y A S L S Q G E K Q V L L I A R S L I L K P E L <u>TTTAATTTTGGACGAACGAACGAACGAACGGTTTAGATTTATTT</u>	E
$\begin{array}{c} \begin{array}{c} R \\ Q \\ L \\ L \\ R \\ Q \\ M \\ G \\ A \\ K \\ S \\ L \\ I \\ L \\ S \\ S \\ S \\ L \\ I \\ S \\ S$	5856 5955
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	5856 5955
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	5856 5955 5976 6075
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	5856 5955 5976 6075
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	5856 5955 5976 6075 6041 6140
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	5856 5955 5976 6075 6041 6140

FIG. 2-Continued

TADIE 4	 - ·	~	• •				•	~		TT 1 400		NICON	0011	0
	 omnaricon	OT.	naire of	nenec	and	noncoding	Temone	trom	ctrainc	11 1/104	and	NI 11		×
INDLL 4	 Joinparison	O1	Dans Or	POLICE	anu	Inducounie	ICEIOIIS	nom	SILAIIIS	111100	anu	INCD	ULLI	o
			F	0										

DNA region	No. of substitutions/	Distribution of difference			No. of transitions/ no. of transver-	No. of silent	No. of amino acid	Observation in strain IL1403		
	total length	1 ^b	2	3	sions	substitutions	changes			
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 NCD02118 containing the *ihvD* gene was used. (A) Prototroph NCD02118 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.

ACKNOWLEDGMENTS

We thank E. Rygaard for providing valuable assistance in sequencing, R. Raya for help with RNA analysis, and C. Anagnostopoulos for critical reading of the manuscript.

This work was supported in part by BRIDGE contract BIOT-CT91-0263 of the Commission of the European Communities.

REFERENCES

- 1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. 81:741-746.
- Anderson, A. W., and P. R. Elliker. 1953. The nutritional requirements of lactic streptococci isolated from starter cultures. I. Growth in a synthetic medium. J. Dairy Sci. 36:161– 167.
- Brown, G. M., and J. M. Williamson. 1987. Biosynthesis of folic acid, riboflavin, thiamine, and pantothenic acid, p. 521–538. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
- Carifo, K., and B. W. Catlin. 1973. Neisseria gonorrhoeae auxotyping: differentiation of clinical isolates based on growth responses on chemically defined media. Appl. Microbiol. 26: 223-230.
- 5. Chopin, A. Personal communication.
- Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid 11:260–263.
- Deguchi, Y., and T. Morishita. 1992. Nutritional requirements in multiple auxotrophic lactic acid bacteria: genetic lesions affecting amino acid biosynthetic pathways in *Lactococcus lactis*, *Enterococcus faecalis* and *Pediococcus acidilactici*. Biosci. Biotechnol. Biochem. 56:913–918.
- Delorme, C., S. D. Ehrlich, and P. Renault. 1992. Histidine biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 174:6571–6579.
- Delorme, C., J. J. Godon, S. D. Ehrlich, and P. Renault. 1993. Gene inactivation in *Lactococcus lactis*: histidine biosynthesis. J. Bacteriol. 175:4391–4399.
- 10. De Vos, W. M., and M. J. Gasson. 1989. Structure and expression of the Lactococcus lactis gene for phospho-β-galactosidase

(lacG) in Escherichia coli and L. lactis. J. Gen. Microbiol. 135:1833-1846.

- 11. Dykhuizen, D. 1978. Selection for tryptophane auxotrophs of *Escherichia coli* in glucose-limited chemostats as a test of the energy conservation hypothesis of evolution. Evolution 32:125-150.
- 12. Englesberg, E., and L. Ingraham. 1957. Meiotrophic mutants of *Pasteurella pestis* and their use in the elucidation of nutritional requirements. Proc. Natl. Acad. Sci. USA 43:369–372.
- Farrow, J. A. E. 1980. Lactose hydrolysing enzymes in Streptococcus lactis and Streptococcus cremoris and also in some other species of Streptococci. J. Appl. Bacteriol. 49:493-503.
- 14. Gilson, T. J. 1984. Ph.D. thesis. University of Cambridge, Cambridge, England.
- 15. Glatron, M. F., and G. Rappoport. 1972. Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. Biochimie 54:1291–1301.
- Godon, J. J., M. C. Chopin, and S. D. Ehrlich. 1992. Branchedchain amino acid biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. 174:6580–6589.
- 17. Hall, B. G., S. Yokoyama, and D. H. Calhoun. 1983. Role of cryptic genes in microbial evolution. Mol. Biol. Evol. 1:109-124.
- Hugenholtz, J., M. Dijkstra, and H. Veldkamp. 1987. Amino acid limited growth of starter cultures in milk. FEMS Microbiol. Ecol. 45:191–198.
- Juni, E., and G. A. Heym. 1980. Studies of some naturally occurring auxotroph of *Neisseria gonorrhoeae*. J. Gen. Microbiol. 121:85-92.
- 20. Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek 49:209-224.
- Koch, A. L. 1972. Enzyme evolution. I. The importance of untranslatable intermediates. Genetics 72:297-316.
- Kok, J. 1990. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. 87:15-42.
- Lawther, R. P., D. H. Calhoun, C. W. Adams, C. A. Hauser, J. Gray, and G. W. Hatfield. 1981. Molecular basis of valine resistance in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA 78:922–925.
- 24. Lawther, R. P., D. H. Calhoun, J. Gray, G. W. Adams, G. A. Hauser, and G. W. Hatfield. 1982. DNA sequence fine-structure analysis of *ilvG* (*ilvG*⁺) mutations of *Escherichia coli* K-12. J. Bacteriol. 149:294–298.
- Lederberg, J. 1947. The nutrition of Salmonella. Arch. Biochem. 13:287-290.
- Lopilato, J., and A. Wright. 1990. Mechanisms of activation of the cryptic bgl operon of Escherichia coli K12, p. 435–444. In K. Drlica and M. Riley (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
- 27. Loureiro Dos Santos, A. L., and A. Chopin. 1987. Shotgun cloning in *Streptococcus lactis*. FEMS Microbiol. Lett. 42:209-212.
- MacKay, C. J., and S. A. Zahler. 1982. Insertion of bacteriophage SPβ into the *citF* gene of *Bacillus subtilis* and specialized transduction of the *ilvBC-leu* genes. J. Bacteriol. 151:1222–1229.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Manson, M. D., and C. Yanofsky. 1976. Naturally occurring sites within the *Shigella dysenteriae* tryptophan operon severely limit tryptophan biosynthesis. J. Bacteriol. 126:668–678.
- Mills, O. E., and T. D. Thomas. 1981. Nitrogen sources for growth of lactic streptococci in milk. N.Z. J. Dairy Sci. Technol. 15:43-55.
- 32. Miozzari, G., and C. Yanofsky. 1978. Naturally occurring promoter down mutation: nucleotide sequence of the trp promoter operator leader region of *Shigella dysenteriae* 16. Proc. Natl. Acad. Sci. USA 75:5580-5584.
- Morishita, T., Y. Degushi, M. Yajima, T. Sakurai, and T. Yura. 1981. Multiple nutritional requirement of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. J. Bacteriol. 148:64-74.
- 34. Morishita, T., T. Fukada, M. Shirota, and T. Yura. 1974.

Genetic basis of nutritional requirements in *Lactobacillus casei*. J. Bacteriol. **120:**1078–1084.

- Parker, L. L., and B. G. Hall. 1990. Mechanism of activation of the cryptic *cel* operon of *Escherichia coli* K12. Genetics 124: 473–482.
- Pyle, L., and L. R. Finch. 1988. A physical map of the genome of *Mycoplasma mycoides* Y with some functional loci. Nucleic Acids Res. 16:6027–6039.
- 37. Reiter, B., and J. D. Oram. 1962. Nutritional studies on cheese starters. J. Dairy. Res. 29:63-77.
- Sandine, W. E., P. C. Radich, and P. R. Elliker. 1972. Ecology of the lactic streptococci. A review. J. Milk Food Technol. 35:176–185.
- Schnetz, K., and B. Rak. 1992. IS5: a mobile enhancer of transcription in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 89:1244-1248.
- 40. Simon, D., and A. Chopin. 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie 70:559–566.
- 41. Smid, E. J., and W. N. Konings. 1990. Relationship between utilization of proline and proline-containing peptides and growth of *Lactococcus lactis*. J. Bacteriol. 172:5286–5292.
- 42. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA 44:1072-1078.

- 43. Tanaka, T. 1979. Restriction of plasmid-mediated transformation in *Bacillus subtilis* 168. Mol. Gen. Genet. 175:235-237.
- Terzaghi, B., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807–813.
- Thomas, T. D., and G. G. Prichard. 1987. Proteolytic enzymes of dairy starter cultures. FEMS Microbiol. Rev. 46:245–268.
- 46. Umbarger, H. E. 1987. Biosynthesis of the branched-chain amino acids, p. 353-367. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), Escherichia coli and Salmonella typhimurium: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
- VanDyk, T. K., and R. A. LaRossa. 1990. Prevention of endogenous 2-ketobutyrate toxicity in *Salmonella typhimurium*, 123– 130. *In Z.* Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH, Weinheim, Germany.
- Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
- Ward, J. B., and S. A. Zahler. 1973. Genetic studies of leucine biosynthesis in *Bacillus subtilis*. J. Bacteriol. 116:719-726.
- 50. Zamenhof, S., and H. H. Eichorn. 1967. Study of microbial evolution through loss of biosynthetic functions: establishment of defective mutants. Nature (London) 216:456-458.