JOURNAL OF BACTERIOLOGY, July 1993, p. 4391–4399
0021-9193/93/144391-09$02.00/0

Vol. 175, No. 14

Gene Inactivation in *Lactococcus lactis*: Histidine Biosynthesis

CHRISTINE DELORME,* JEAN-JACQUES GODON, 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

Lactococcus lactis **strains from dairy and nondairy sources were tested for the ability to grow in the absence of histidine. Among 60 dairy strains tested, 56 required histidine, whereas only 1 of 11 nondairy strains had this requirement. Moreover, 10 of the 56 auxotrophic strains were able to grow in the presence of histidinol (Hol^+), the immediate histidine precursor. This indicates that adaptation to milk often results in histidine auxotrophy. The histidine operon was detected by Southern hybridization in eight dairy auxotrophic strains tested. A large part of the histidine operon (8 kb, containing seven histidine biosynthetic genes and three unrelated open reading frames [ORFs]) was cloned from an auxotroph, which had an inactive *hisD* gene, as judged by its inability to grow on histidinol. Complementation analysis of three genes, *hisA*, *hisB*, and *hisG*, in *Escherichia coli* showed that they also were inactive. Sequence analysis of the cloned histidine region, which revealed 98.6% overall homology with that of the previously analyzed prototrophic strain, showed the presence of frameshift mutations in three *his* genes, *hisC*, *hisG*, and *hisH*, and two genes unrelated to histidine biosynthesis, ORF3 and ORF6. In addition, several mutations were detected in the promoter region of the operon. Northern (RNA) hybridization analysis showed a much lower amount of the *his* transcript in the auxotrophic strain than in the prototrophic strain. The mutations detected account for the histidine auxotrophy of the analyzed strain. Certain other dairy auxotrophic strains carry a lower number of mutations, since they were able to revert either to a Hol^+ phenotype or to histidine prototrophy.**

Standard laboratory microorganisms, such as *Escherichia coli* and *Bacillus subtilis*, can grow on a simple mineral medium containing only an appropriate carbon source. In contrast, many other microorganisms have more complex nutritional requirements, which probably result from adaptation to a relatively rich ecological niche. The genetic defects which underlie these requirements have been studied in several cases and were found to include inadequate gene expression, mutational gene inactivation, and the combination of the two, as summarized in the accompanying report (8). To further characterize such defects, we decided to study gene inactivation in *Lactococcus lactis*. This bacterium, thought to be naturally associated with plant material, is widely used in milk fermentation. It was therefore introduced relatively recently into a novel, more plentiful ecological niche, milk, and may thus offer the possibility to analyze early stages of gene inactivation.

L. lactis dairy strains can ferment lactose and efficiently degrade caseins. Both functions are most often carried on plasmids (16) and were probably acquired during adaptation to milk. In addition, dairy strains often require branched-chain amino acids (BCAA; isoleucine, leucine, and valine) for growth (1, 2, 4, 21), which might be due to adaptation to milk, since the related strains from nondairy sources are able to synthesize the three amino acids (8). The accompanying paper reports that the BCAA requirement may be the result of only a few mutations, since the entire operon is preserved in all dairy strains analyzed and is actively transcribed and efficiently regulated in the strain studied in more detail (8). In this paper, we report the analysis of the histidine requirement of dairy strains, which was reported to be very common (1, 2, 4, 21) and might also be due to adaptation for growth in milk.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used are listed in Table 1. Growth conditions are described in the accompanying report (8). For complementation studies, *E. coli* was grown in minimal medium supplemented with the necessary components and histidinol or histidine (0.11 μg/ml), as required (17). *L. lactis* auxotrophy was tested in CDM (chemically defined medium) as described in the accompanying report (8). For the reversion tests, $\sim 10^{10}$ cells of an overnight culture washed twice were plated on minimal medium agar plates without histidine or supplemented with histidinol.

Cloning of IL1403 DNA in *E. coli*. Total DNA from *L. lactis* subsp. *lactis* was isolated as described previously (14). Total DNA was subcloned in plasmid pBluescript (pBS; Stratagene). *E. coli* transformation and colony hybridization were performed by standard procedures (17).

Southern hybridization, RNA analysis, and DNA sequencing. The methods used are described in the accompanying report (8). The reported sequence was determined on both strands.

Nucleotide sequence accession number. The GenBank, EMBL, and DDBJ nucleotide sequence accession number for the sequence shown in Fig. 1 is M90760.

RESULTS

Histidine and histidinol auxotrophy. Different *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* dairy strains, as well as *L. lactis* subsp. *lactis* strains isolated from various nondairy sources (mostly plants; Table 1), were tested for growth on a defined medium supplemented with all amino

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant markers and characteristics	Reference or origin[a]
Strains		
E. coli		
TG1	*supE Δthi* (*lac-proAB*) *hsdD5* (F'$^+$ *traD36 proAB lacI^{q}ZΔM15*)	7
Hfr G6	*hisA323* λ$^-$	18
SB3930	*hisB463* λ$^-$	6
JC411	*leuB6 fhuA2 lacY1 supE44 gal-6 hisG1 rfbD1? galP63? argG6 rpsL104 malT1* λ^r *xyl-7 mtl-2 metB1*	15
L. lactis subsp. *cremoris*[b]		
CNRZ105	1957, His$^-$	
CNRZ107	1958, His$^-$	
CNRZ114	1958, His$^-$	
CNRZ119	1958, His$^-$	
CNRZ306[c]	1967, His$^-$	
CNRZ357	1970, His$^-$	
CNRZ482	His$^-$	
IL420	His$^-$	
IL563	Starter, Belgium, His$^-$	
IL675	Starter, France, 1965, His$^-$	
IL680	Starter, France, 1966, His$^-$	
IL737	Starter, France, 1970, His$^-$	
IL746	Starter, France, 1970, His$^-$, $R^{Hol^+} \sim 5 \times 10^{-9}$	
IL963	His$^-$, $R^{Hol^+} < 10^{-9}$	
IL969[c]	His$^-$	
NCDO712[c]	Starter, New Zealand, His$^-$, $R^{Hol^+} < 10^{-9}$	
NCDO763[c]	Starter, New Zealand, His$^-$	
L. lactis subsp. *lactis*[b]		
Dairy strains		
CNRZ144	1958, Hol$^+$, $R^{His^+} < 6 \times 10^{-10}$	
CNRZ148	1954, His$^-$, $R^{His^+} \sim 6 \times 10^{-8}$	
CNRZ151	1954, Hol$^+$, $R^{His^+} < 7 \times 10^{-9}$	
CNRZ157	1955, His$^+$	
CNRZ167	1964, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
CNRZ193[d]	1964, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
CNRZ261	1970, His$^-$, $R^{His^+} \sim 8 \times 10^{-9}$	
CNRZ268	Holt$^+$, $R^{His^+} < 1 \times 10^{-9}$	
CNRZ272	Hol$^+$, $R^{His^+} < 3 \times 10^{-9}$	
CNRZ377	1971, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
CNRZ430	1972, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
CNRZ483	Starter, France, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
IL413	Raw milk, France, 1958, His$^-$, $R^{His^+} \sim 1 \times 10^{-9}$	
IL414	Raw milk, France, 1958, Hol$^+$, $R^{His^+} < 2 \times 10^{-9}$	
IL427	Starter, France, 1965, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
IL561	Starter, Belgium, His$^+$	
IL562	Starter, France, 1965, His$^-$, $R^{His^+} \sim 7 \times 10^{-9}$	
IL564	Starter, France, 1965, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL573	Starter, France, 1974, His$^-$, $R^{His^+} < 2 \times 10^{-9}$	
IL583	Starter, France, His$^-$, $R^{Hol^+} \sim 7 \times 10^{-9}$	
IL635	Milk, France, 1962, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
IL639	Starter, France, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL741	Starter, France, 1970, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL899	Cheese, France, 1962, His$^-$, $R^{Hol^+} \sim 2 \times 10^{-9}$	
IL903	Starter, France, 1959, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL904	Cheese, France, 1962, Hol$^+$, $R^{His^+} < 4 \times 10^{-10}$	
IL907	Cheese, France, 1962, Hol$^+$, $R^{His^+} < 3 \times 10^{-10}$	
IL925	Starter, France, 1959, His$^-$, $R^{His^+} < 5 \times 10^{-10}$	
IL929	Starter, France, 1960, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL933	Raw milk, France, Hol$^+$, $R^{His^+} < 5 \times 10^{-10}$	
IL935	Raw cream, France, 1963, His$^-$, $R^{His^+} < 4 \times 10^{-10}$	
IL942	Raw milk, France, 1962, His$^-$, $R^{His^+} < 7 \times 10^{-10}$	
IL948	Starter, France, 1966, His$^-$, $R^{His^+} < 4 \times 10^{-10}$	
IL960	Hol$^+$, $R^{His^+} < 6 \times 10^{-10}$	
IL977	Starter, France, 1966, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
IL982	Starter, France, 1965, His$^-$, $R^{His^+} < 6 \times 10^{-10}$	
IL985	Starter, France, 1965, His$^-$, $R^{His^+} < 1 \times 10^{-9}$	
IL1403	Plasmid free, His$^-$, $R^{His^+} < 1 \times 10^{-10}$	3
NCDO184	Cheese curd, 1950, Hol$^+$, $R^{His^+} < 2 \times 10^{-9}$	

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant markers and characteristics	Reference or origin[a]
NCDO496	1950, His^-, $R^{His^+} \sim 5 \times 10^{-10}$	
NCDO966	Long milk, Sweden, 1957, His^+	
NCDO1966	Starter, Bulgaria, 1969, His^-, $R^{His^+} \sim 2 \times 10^{-9}$	
NCDO2054	Milk, His^+	
Nondairy strains		
NCDO1867	Frozen peas, England, 1966, His^+	
NCDO2091	Seeds of Chinese radish, Japan, His^+	
NCDO2108	Frozen beans, 1978, Hol^+	
NCDO2110	Frozen peas, 1978, His^+	
NCDO2111	Frozen peas, 1978, His^+	
NCDO2118	Frozen peas, 1978, His^+	
NCDO2125	Termite gut, United States, 1978, His^+	
NCDO2146	Mastitis, 1979, His^+	
NCDO2633	Rectum of cow, 1981, His^+	
NCDO2727	Mung bean, China, 1983, His^+	
NCDO2738	Anchu mash, Germany, 1984, His^+	
Plasmids		
pBS	Amp^r M13*ori* pBR322*ori* plasmid for sequencing	Stratagene
pIL378	9-kb *Sau*3AI fragment from NCDO2118 in pIL253	5
pIL700	9-kb *Sac*I fragment from pIL378 in pBS	5
pIL704	2.2-kb *Eco*RI fragment from pIL378 in pBS, $hisG^+$	5
pIL708	3.1-kb *Eco*RI fragment from pIL378 in pBS, $hisB^+$ $hisA^+$	5
pIL712	1.9-kb *Eco*RI-*Xba*I fragment from pIL708 in pBS, $hisA^+$	5
pIL721	1.3-kb *Eco*RI fragment from IL1403 in pBS	This work
pIL722	0.25-kb *Eco*RI fragment from IL1403 in pBS	This work
pIL723	2.2-kb *Eco*RI fragment from IL1403 in pBS, *hisG*	This work
pIL725	1.7-kb *Eco*RI fragment from IL1403 in pBS	This work
pIL727	3.1-kb *Eco*RI fragment from IL1403 in pBS, *hisB hisA*	This work
pIL728	1.2-kb *Eco*RI-*Xba*I fragment from pIL727 in pBS, *hisB*	This work
pIL730	1.9-kb *Eco*RI-*Xba*I fragment from pIL727 in pBS, *hisA*	This work

[a] IL strains are from our laboratory collection, CNRZ strains are from Station de Recherche Laitière, Institut National de la Recherche Agronomique, Jouy en Josas, France, and NCDO strains are from the Reading Laboratory, Agriculture and Food Research Laboratory, Reading, England.

[b] Place, medium, and year of isolation are given when known. His^+, growth without histidine; Hol^+, growth with histidinol; His^-, no growth without histidine or histidinol; R, rate of obtention of His^+ or Hol^+ revertants.

[c] Previously classified as *L. lactis* subsp. *lactis* (9).

[d] Previously classified as *L. lactis* subsp. *cremoris* (9).

acids except histidine and containing or lacking histidinol (the last intermediate in histidine biosynthesis). Ten of eleven nondairy strains did not require histidine or histidinol, and one could grow on histidinol (Hol^+). In contrast, none of the *L. lactis* subsp. *cremoris* dairy strains (17 were tested; nondairy strains are not known) was able to grow without histidine. An intermediary situation was found among 43 *L. lactis* subsp. *lactis* dairy strains, since 29 required histidine, 10 required histidinol, and 4 were prototrophic for histidine. This finding suggests that dairy use selects for loss of the capacity to synthesize histidine.

Cloning of a silent histidine operon. To study the genetic basis of the histidine requirement, eight auxotrophic strains were compared by Southern hybridization with five prototrophic strains, including strain NCDO2118, which was previously used for sequence analysis of the histidine operon (5). Plasmid pIL700, containing the first 9 kb of the functional histidine operon (5), was used as a probe, and pBS was used as a control. Similar hybridization patterns were observed with all strains (not shown), which indicates that the histidine biosynthetic genes are present in the auxotrophic strains and that they are not grossly rearranged.

To further confirm this conclusion, we decided to clone the histidine biosynthesis genes from the standard *L. lactis* subsp. *lactis* auxotrophic strain IL1403. For this purpose, total IL1403 DNA was cleaved with *Eco*RI, ligated to *Eco*RI-cleaved and dephosphorylated pBS cloning vector, and used to transform *E. coli* to ampicillin resistance. Transformants harboring *L. lactis* histidine genes were revealed by colony hybridization with plasmid pIL378, which contains a 9-kb segment from the histidine region of the prototroph NCDO2118 (5). A total of five different *Eco*RI segments, measuring 3.1, 2.2, 1.7, 1.3, and 0.25 kb, were thus obtained (Table 1). Matching segments are present in the histidine operon of the prototrophic strain NCDO2118 (5). The 3′ end of the operon is not present in these segments and appears to be carried on a segment unstable in *E. coli* (unpublished results).

Complementation analysis. Histidine auxotrophy in IL1403 could be due to inactivation of one or several biosynthetic genes. The failure of the strain to grow in the presence of histidinol suggests that its *hisD* gene is not functional. *hisA*, *hisB*, and *hisG* under the control of the *lac* promoter of pBS also appear to be inactive, as judged from the failure of plasmids pIL730, pIL727, and pIL723 to complement the corresponding *E. coli* mutants (strains HfrG6 *hisA23*, SB3930 *hisB463*, and JC411, which carries the *hisG1* mutation, were used). It was previously found (5), and confirmed here, that these mutants can be complemented by matching plasmids pIL712, pIL708, and pIL704 respectively, carrying genes from the prototroph NCDO2118 strain. Complementation of *hisC* and *hisD* mutants, which was efficient with genes cloned from the prototroph (5), was not tested, because the genes from the auxotroph were interrupted, being

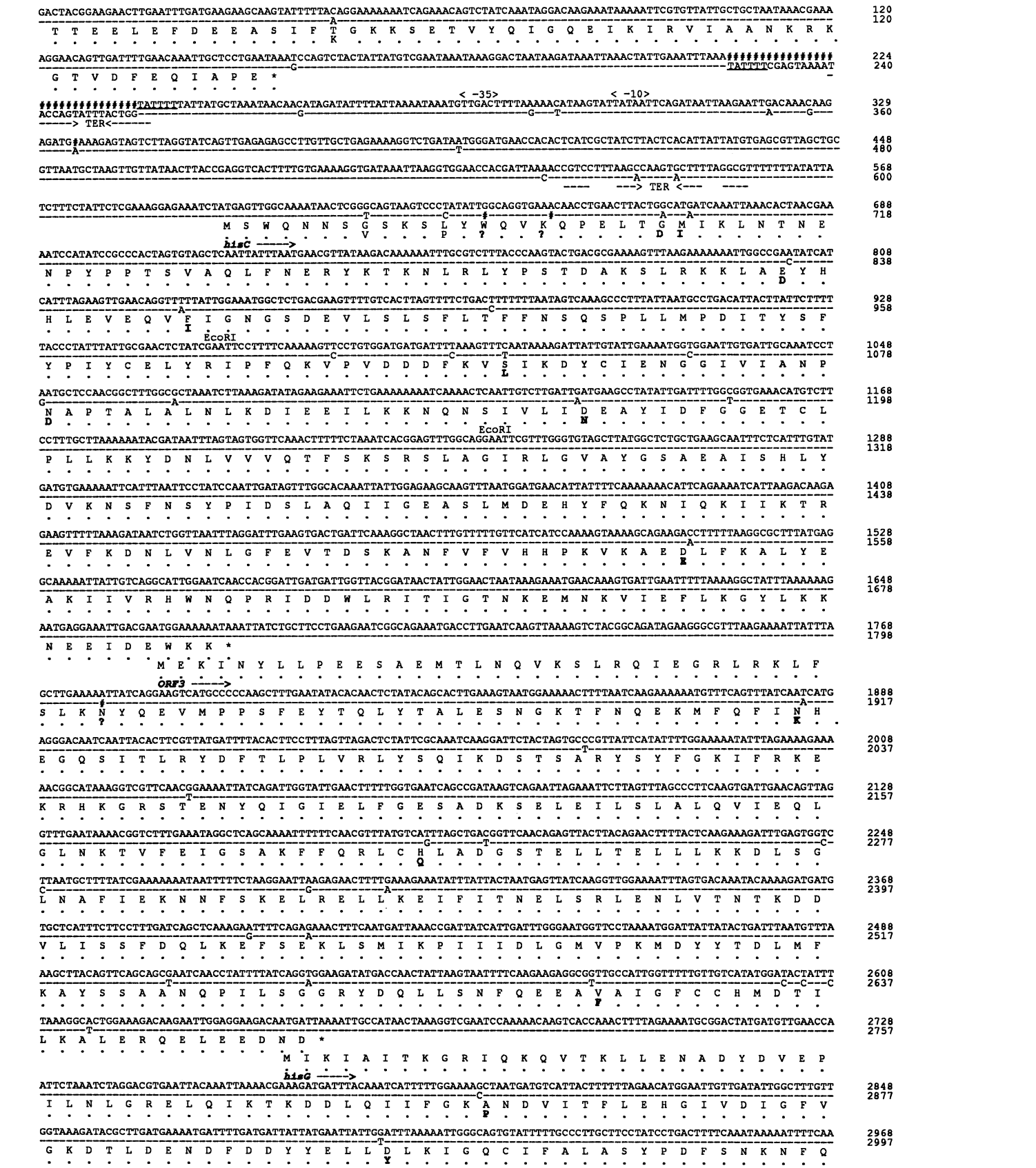

FIG. 1. Comparison of the nucleotide and deduced amino acid sequences of the histidine operon from the *L. lactis* subsp. *lactis* prototrophic strain NCDO2118 and auxotrophic strain IL1403. The first line is the nucleotide sequence from NCDO2118, the second line is that from IL1403, the third line is the amino acid sequence from NCDO2118, and the fourth line is that from IL1403. Numbers on the right refer to nucleotides. Gene names are indicated at the beginning of each amino acid sequence. The −35 and −10 boxes of the promoter and the two rho-independent terminators are indicated. Symbols: *, stop codon; #, base deletion.

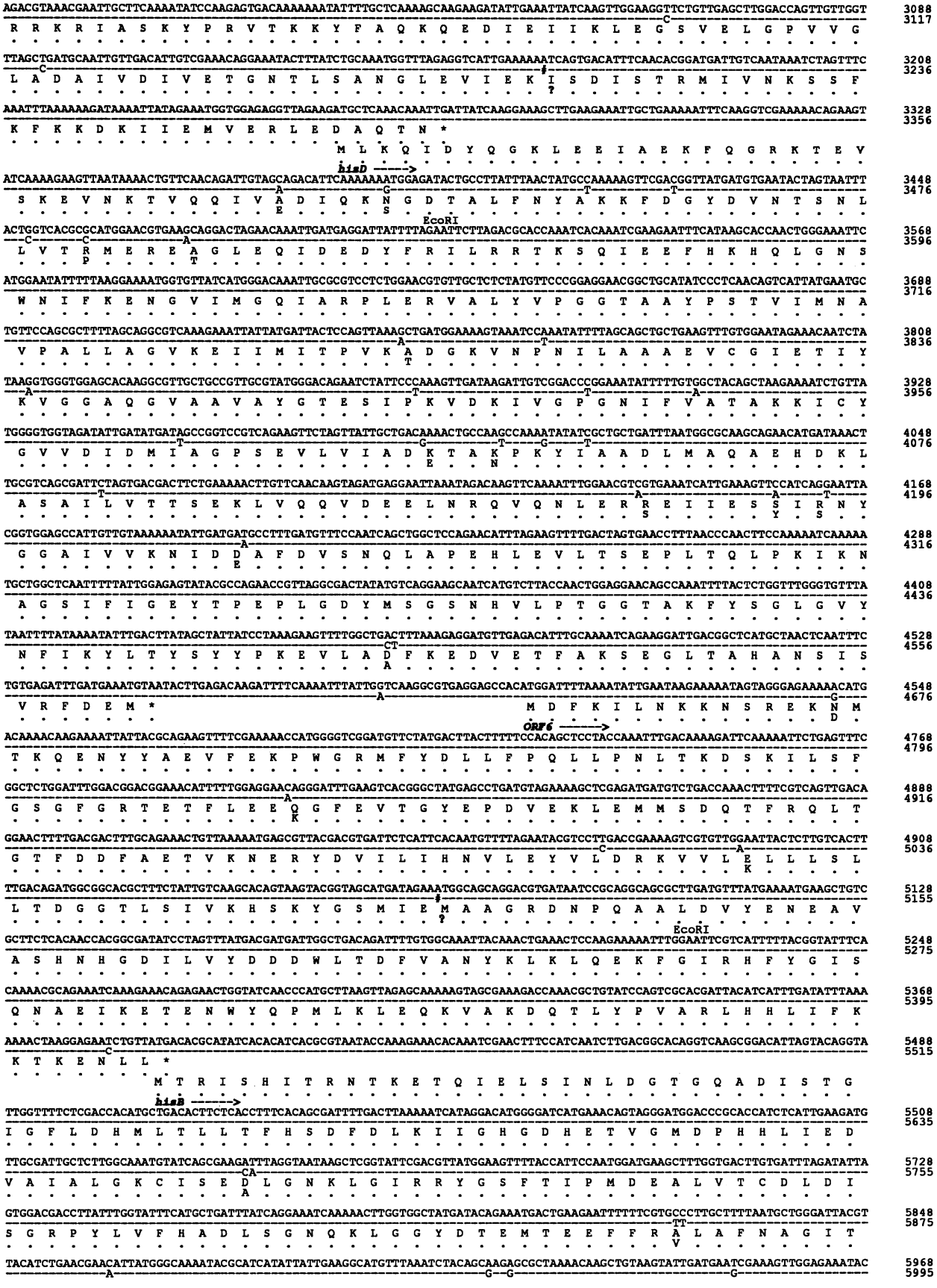

FIG. 1—*Continued*

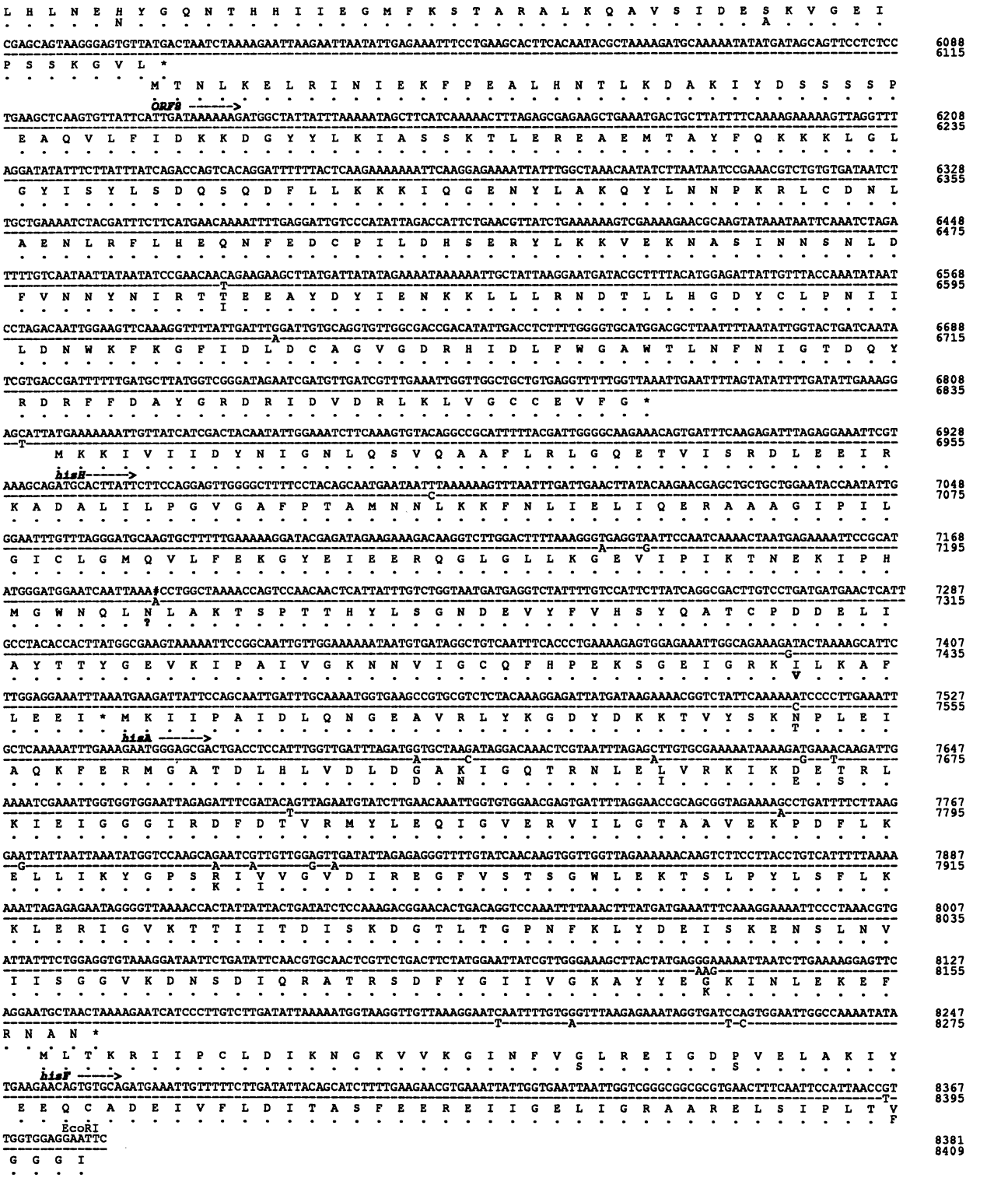

FIG. 1—*Continued*

carried on two separate IL1403 DNA segments. These results indicate that at least four histidine biosynthetic genes were inactive in the auxotrophic strain.

Nucleotide sequence. To further characterize the histidine biosynthesis genes from the auxotrophic strain, the sequence of the cloned IL1403 segment was determined (Fig. 1). Its total length is 8,409 bp, and it covers most of the *his* operon: six complete (*hisCGDBHA*) and one truncated (*hisF*) biosynthetic gene, three open reading frames (ORFs) of unknown function (ORF3, ORF6, and ORF8), which are

probably not related to histidine biosynthesis, and the region upstream of the operon, encoding the 3′ end of an unrelated ORF. A part of the operon expected to carry the end of *hisF*, *hisIE*, and ORF13 has not been sequenced because we failed to clone it (see above). Comparison of the 8,381-bp-long sequence of the histidine region of the auxotroph with the prototroph counterpart is summarized in Table 2.

Overall sequence homology is very high, since only ~1.4% of bases differ. Divergence is somewhat higher in the 377-bp noncoding region (2.7%) than in the coding region (1.3%). About half of the substitutions in the coding region are silent, and one-third of those which are not result in a conservative amino acid change (Fig. 1 and Table 2). However, besides base substitutions, five deletions and one insertion of a single base were detected within the coding region, resulting in frameshift mutations (see below). A single larger deletion was found, eliminating 31 bp from the noncoding region of the prototrophic strain, probably by recombination between the flanking 6-bp repeats (Fig. 1). A similar level of sequence conservation was observed in the *leu* region of the auxotrophic strain (8).

What might the phenotypic consequences of the observed differences be? The six frameshift mutations found in the coding region should affect five IL1403 proteins, three of which belong to the histidine biosynthetic pathway. As a result, the HisC, HisG, and HisH proteins are expected to contain 16, 173, and 127 amino acids, respectively, while the prototrophic counterparts should contain 359, 208, and 202 amino acids, respectively. Similarly, the products of ORF3 and ORF6 from the auxotroph should contain 36 and 185 amino acids rather than the 327 and 263 amino acids, respectively, found in the prototroph. The truncated proteins are most probably inactive, as shown for HisG by the failure to complement a corresponding *E. coli* mutation (see above). Furthermore, the amino acid changes detected in *hisA*, *hisB*, and *hisD* (Table 2) also generate inactive proteins, as judged by the lack of complementation of *E. coli* mutants and the inability to grow on histidinol. These results suggest that none of the six complete *his* genes that we analyzed are functional.

Changes present in the noncoding region could also affect the *his* operon. Two base substitutions are between the −35 and −10 boxes of the promoter, another is at −76, and two are at positions +8 and +14. Some of these substitutions might reduce the efficiency of the auxotrophic promoter (see below). Two mismatches at positions 571 and 577 might destabilize a rho-independent-like terminator involved in attenuation of transcription. The 31-bp deletion, which presumably occurred in the prototrophic strain, eliminates a terminator-like structure, composed of a hairpin followed by a stretch of T's (Fig. 1). This structure could prevent entry of transcripts initiated outside the *his* operon into the auxotrophic *his* region.

RNA analysis. To determine whether transcription of the *his* operon of the auxotroph is similar to that of the prototroph, we prepared total RNA from the cells of IL1403 and NCDO2118 which were incubated with or without histidine. RNA was analyzed by Northern (RNA) hybridization, using as a probe a 700-bp segment that contains the putative *his* promoter and the beginning of the *hisC* gene. Two strong transcripts, of 10 and 3 kb, were detected in prototrophic cells incubated without histidine (Fig. 2, lane 2). The longer transcript has the length of the entire *his* operon, as deduced from the previous sequence analysis (5), and the shorter transcript might arise by processing of the longer one. Histidinol did not significantly affect this transcription, but histidine suppressed it almost completely (Fig. 2, lanes 1 to 3). In contrast, only a weak 10-kb transcript was present in the sample prepared from auxotrophic cells incubated without histidine, although the terminator structure which attenuates transcription might be weakened by a mismatch. This transcript was absent when histidine was added (Fig. 2, lanes 4 and 5). We conclude that histidine controls expression of the *L. lactis* subsp. *lactis* operon at the transcriptional level in the prototroph and that this control is still present, at least in part, in the auxotroph.

Reversion to histidine and histidinol independence. The analysis presented above shows that the histidine operon of strain IL1403 carries a number of mutations. To test whether other *L. lactis* subsp. *lactis* strains also contain a multiply mutated *his* operon, we studied reversion of auxotrophic strains to histidine independence (Table 1). None of the 10 His^- Hol^+ dairy strains tested was found to mutate to histidine independence at a rate above 10^{-9}. These strains may carry more than two mutations in the histidine operon. We also tested the capacity of several *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains to acquire the ability to grow on histidinol, which requires expression of a single biosynthetic gene, *hisD*. Eight of twenty-nine His^- Hol^- *L. lactis* subsp. *lactis* dairy strains and one of three *L. lactis* subsp. *cremoris* strains mutated to the Hol^+ phenotype, with rates between 6.10^{-8} and 5.10^{-10}. Six of the eight Hol^+ *L. lactis* subsp. *lactis* revertants were also His^+, suggesting that the *his* operon of these strains is inactivated by a single mutation in *hisD*. The two other strains contain at least one other lesion in addition to a point mutation inactivating *hisD*. As 22 strains did not yield Hol^+ revertants, it is probable that they carry multiple mutations in *hisD*. This observation supports the hypothesis that the histidine operon of dairy strains is most often inactivated by numerous lesions.

DISCUSSION

Sequence analysis of two operons, specifying the synthesis of histidine and BCAA, shows high sequence conservation (~99%) between two *L. lactis* subsp. *lactis* strains, IL1403, isolated from dairy material, and NCDO2118, isolated from frozen peas (this and the accompanying report [8]). Hybridization analysis of the two operons from a number of other dairy and nondairy strains, mostly from plants but also from the digestive tracts of plant-eating animals, also suggests a high degree of homology, which supports the notion that strains used in milk fermentations might derive from plant-associated strains (22). Interestingly, much less conservation was previously found between dairy strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* hybridization analysis (between 20 and 30%), which suggests that the two subspecies have diverged much earlier than the development of milk fermentation practice (9). Nevertheless, most *L. lactis* subsp. *lactis* and all *L. lactis* subsp. *cremoris* dairy strains are auxotrophs for histidine, whereas most nondairy *L. lactis* subsp. *lactis* strains are prototrophs (*L. lactis* subsp. *cremoris* nondairy strains were not tested, since they have never been isolated). As shown by the reversion test, the mutations which inactivate the *his* operon of different strains do not affect the same genes, indicating that these strains acquired independently the *his* auxotrophy. This observation suggests that dairy use might enrich the population in cells which have lost the ability to synthesize histidine. Similarly, BCAA synthesis is also often inactivated (8), whereas biosynthesis of other amino acids is only rarely impaired. The relevant selective

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				
hisC	16/1,079	3	4	9	12/4	6	10	1-bp deletion at positions 665 and 675; stop codon at position 671
ORF3	18/982	2	1	15	12/6	15	3	1-bp deletion at position 1808; stop codon at position 1964
hisG	4/624	2	0	2	2/2	2	2	1-bp deletion at position 3191; stop codon at position 3213
hisD	25/1,293	5	5	15	14/11	12	12	
ORF6	5/789	3	0	2	4/1	2	3	1-bp deletion at position 5095; stop codon at position 5181
hisB	8/600	2	2	4	4/4	2	4	
ORF8	2/792	0	1	1	2/0	1	1	
hisH	4/606	2	0	2	3/1	3	1	1-bp insertion at position 7216; stop codon at position 7223
hisA	16/717	4	4	8	9/7	5	9	
hisF	5/250	3	0	2	3/2	1	3	
Coding region	103/7,732	26	17	60	65/38	49	48	
Noncoding region	10/377				6/4			1-bp insertion at position 366
Total	113/8,109							

[a] NCDO2118 sequence is used; deletions are excluded; length is in nucleotides.
[b] Number refers to codon position.

pressures are not obvious at present, as histidine and BCAA are not particularly abundant in milk (19).

Sequence analysis and complementation of *E. coli* mutants have shown that all analyzed *his* genes (*hisCGDBH*) of *L. lactis* subsp. *lactis* IL1403 are inactive. Inactivation occurs predominantly by point mutations, as also observed for the *leu* genes of the same strain (8). Frameshift (this work) and nonsense (8) mutations were identified by sequence analysis, and the presence of missense mutations was deduced from complementation analysis. Only one small deletion, occurring between short direct repeats, was detected. Besides mutational gene inactivation, the histidine operon might be silenced also by lack of transcription, since much less *his* transcript was present in the auxotrophic strain than in the prototrophic strain. This could be due to mutations detected in the auxotroph in the region upstream of the biosynthetic genes, which could interfere with promoter activity. However, we cannot exclude the possibility that the promoter is equally active in the auxotroph and prototroph, but that transcript stability is lower in the auxotroph. Interestingly, initiation of transcription can be repressed by histidine in both strains, which suggests that this regulation of the auxotrophic operon is functional. The activity of the IL1403 *his* operon could therefore be impaired at three different levels in the presence of histidine, possibly involving repression, promoter inactivation, or transcript processing and structural gene inactivation. This might be an example of a far-advanced process of loss of a function. In contrast, inactivation of the *leu* genes is much less severe, since only two of the four biosynthetic genes are inactive, and the operon is efficiently transcribed and controlled (8).

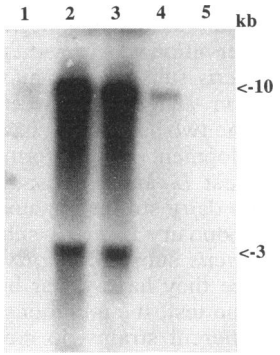

FIG. 2. Transcription of the histidine operon. Total RNA was hybridized with a 700-bp probe containing the putative promoter and the beginning of the *hisC* gene. Prototroph NCDO2118 cells were incubated with histidine (lane 1), with histidinol (lane 2), and without histidine and histidinol (lane 3). Auxotroph IL1403 cells were incubated without and with histidine (lanes 4 and 5, respectively).

Interestingly, the histidine and the BCAA operons are adjacent on the *L. lactis* subsp. *lactis* chromosome (unpublished results). One could thus speculate that the preferential loss of both pathways might be the result of their particular location on the chromosome. The rates of silent substitutions vary significantly among *E. coli* and *Salmonella typhimurium* chromosomes, being lower in the vicinity of the replication origin than in the vicinity of the terminus (23). *L. lactis* histidine and BCAA operons map in a location proposed to be far from the replication origin of the chromosome (12, 24). Obviously, large deletions do not play a role in gene inactivation in *L. lactis*, despite the relatively low size of its genome (12, 24).

The histidine biosynthetic genes in other *L. lactis* subsp. *lactis* dairy strains appear to be less severely impaired, since ~20% of strains are capable of growth on histidinol, which indicates that *hisD* is active. Of 29 strains unable to grow on histidinol, 8 (~27%) were found to become Hol$^+$ by spontaneous mutation. Furthermore, ~10% of dairy strains were prototrophs for histidine, and 6 of 39 (~15%) strains can be mutated to histidine prototrophy. This finding suggests that inactivation of *his* genes is a relatively recent and ongoing event. However, in contrast to the evolutionary pattern of cryptic genes in *E. coli* that are retained almost intact without known selective pressure, the lactococcal amino acid biosynthesis operons seem to accumulate mutations, as

predicted by the model of neutral evolution (11). This difference may reflect our incomplete knowledge of the role of *E. coli* genes which seem to have no function (10, 13, 20). Alternatively, enteric bacteria and dairy lactococci might evolve at different rates, possibly as the result of the recent change of the ecological niche undergone by the latter microorganisms.

ACKNOWLEDGMENTS

We thank C. Anagnostopoulos for critical reading of the manuscript and R. Raya for help with RNA analysis.

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

REFERENCES

1. **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.
2. **Chopin, A.** Personal communication.
3. **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.
4. **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.
5. **Delorme, C., D. S. Ehrlich, and P. Renault.** 1992. Histidine biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **174:**6571–6579.
6. **Garrick-Silversmith, L., and P. E. Hartman.** 1970. Histidine-requiring mutants of *Escherichia coli* K12. Genetics **66:**231–244.
7. **Gilson, T. J.** 1984. Ph.D. thesis. University of Cambridge, Cambridge, England.
8. **Godon, J. J., C. Delorme, J. Bardowski, M. C. Chopin, S. D. Ehrlich, and P. Renault.** 1993. Gene inactivation in *Lactococcus lactis*: branched-chain amino acid biosynthesis. J. Bacteriol. **175:**4383–4390.
9. **Godon, J. J., C. Delorme, S. D. Ehrlich, and P. Renault.** 1992. Divergence of genomic sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **58:**4045–4047.
10. **Hall, B. G., S. Yokoyama, and D. H. Calhoun.** 1983. Role of cryptic genes in microbial evolution. Mol. Biol. Evol. **1:**109–124.
11. **Kimura, M.** 1991. Recent development of the neutral theory viewed from the Wrightian tradition of theorical population genetics. Proc. Natl. Acad. Sci. USA **88:**5969–5973.
12. **Lebourgeois, P., M. Lautier, M. Matta, and P. Ritzenthaler.** 1992. Physical and genetic map of the chromosome of *Lactococcus lactis* subsp. *lactis* IL1403. J. Bacteriol. **174:**6752–6762.
13. **Lopilato, J., and A. Wright.** 1990. Mechanisms of activation of the cryptic *bgl* operon of *Escherichia coli* K-12, p. 435–444. *In* K. Drlica and M. Riley (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
14. **Loureiro Dos Santos, A. L., and A. Chopin.** 1987. Shotgun cloning in *Streptococcus lactis*. FEMS Microbiol. Lett. **42:**209–212.
15. **Low, B.** 1968. Formation of merodiploids in matings with a class of rec- recipient strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **60:**160–167.
16. **MacKay, L. L.** 1983. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek **49:**259–274.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Matney, T. S., E. P. Goldschmidt, N. S. Erwin, and R. A. Scroggs.** 1964. A preliminary map of genomic sites for F-attachment in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **17:**278–281.
19. **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.
20. **Parker, L. L., and B. G. Hall.** 1990. Characterization and nucleotide sequence of the cryptic *cel* operon of *Escherichia coli* K12. Genetics **124:**455–471.
21. **Reiter, B., and J. D. Oram.** 1962. Nutritional studies on cheese starters. J. Dairy Res. **29:**63–77.
22. **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.
23. **Sharp, P. M.** 1991. Determinants of DNA sequence divergence between *Escherichia coli* and *Salmonella typhimurium*: codon usage, map position, and concerted evolution. J. Mol. Evol. **33:**23–33.
24. **Tulloch, D. L., L. R. Finch, A. J. Hillier, and B. E. Davidson.** 1991. Physical map of the chromosome of *Lactococcus lactis* subsp. *lactis* DL11 and localization of six putative rRNA operons. J. Bacteriol. **173:**2768–2775.