The ldh Phylogeny for Environmental Isolates of Lactococcus lactis Is Consistent with rRNA Genotypes but Not with Phenotypes

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Lactate dehydrogenase (ldh) gene sequences, levels of 16S rRNA group-specific probe binding, and phenotypic characteristics were compared for 45 environmental isolates and four commercial starter strains of Lactococcus lactis to identify evolutionary groups best suited to cheddar cheese manufacture. ldh sequences from the environmental isolates showed high similarity to those from two groups of L. lactis used for industrial fermentations, L. lactis subsp. cremoris and subsp. lactis. Within each phylogenetically defined subspecies, ldh sequence similarities were greater than 99.1%. Strains with phenotypic traits formerly diagnostic for both subspecies were found in each ldh similarity group, but only strains belonging to L. lactis subsp. cremoris by both the newer, genetic and the older, superseded phenotypic criteria were judged potentially suitable for the commercial production of cheddar cheese. Identical evolutionary relationships were inferred from ldh sequences and from binding of subspecies-specific, 16S rRNA-directed oligonucleotide probes. However, groups defined according to these chromosomal traits bore no relationship to patterns of arginine deamination, carbon substrate utilization, or bacteriophage sensitivity, which may be encoded by cryptic genes or sexually transmissible genetic elements. Fourteen new L. lactis subsp. cremoris isolates were identified as suitable candidates for cheddar cheese manufacture, and 10 of these were completely resistant to three different batteries of commercial bacteriophages known to reduce starter activity.

Members of the gram-positive bacterial species Lactococcus lactis are important for commercial production of fermented milk products. During cheese production, lactic starter cultures are responsible for the curdling of milk via production of lactic acid and, by partial proteolysis of caseins, stabilizing fat micelles. Starters also contribute to cheese flavor by releasing peptides and carbonyl compounds from milk components. It is important that the growth characteristics of cheese starter bacteria result in rapid lactic acid production once the milk vat is inoculated. This inhibits growth of contaminating microorganisms which might compromise the product’s consistency or produce undesirable flavor compounds. During the production of cheddar cheese, the coagulation process is followed by “cooking” at an elevated temperature (38 to 40°C), which serves to anneal the developing curd, expel whey, and reduce populations of starter and adventitious bacteria. A cheddar starter culture must survive cooking to some extent, and these survivors, along with adventitious bacteria, continue producing acid and flavor compounds during cheddaring and ripening.

L. lactis subsp. cremoris is the preferred bacterium for use as a cheddar cheese starter. Strains of this subspecies have the proper growth response to the different stages of cheese production and typically yield cheese free of fruity, fermented, or bitter flavors (35, 36). This finding has been repeatedly confirmed in commercial cheddar cheese making, and as a result there is a great demand for new strains of this bacterium.

The taxonomy of L. lactis has undergone a number of revisions, with all strains once classified in the genus Streptococcus (7, 9, 21, 29). Until the most recent taxonomic revision, L. lactis subsp. cremoris was distinguished from L. lactis subsp. lactis (including the former Streptococcus lactis subsp. diacetylactis) according to four negative phenotypic criteria: L. lactis subsp. cremoris strains could not grow at 40°C, in 4% NaCl, or at pH 9.2 and could not deaminate arginine (21). L. lactis subsp. cremoris strains were also found to be sensitive to lithium chloride (1). More recently, it was discovered that L. lactis strains form two distinct phylogenetic groups differing in DNA sequence by 20 to 30%, as judged by Southern hybridization (9). It was therefore proposed that the subspecies diagnoses be redefined to reflect these natural relationships (9). The new taxonomic system required the transfer of several strains across subspecies lines, from L. lactis subsp. lactis to L. lactis subsp. cremoris and vice versa, with each new subspecies containing members of both superseded, phenotypic subspecies (9). Phylogenetic comparisons of 16S rRNA and lactate dehydrogenase (ldh) gene sequences have confirmed the new subspecies assignments for several of these strains (24, 31).

To date, only a few strains of L. lactis subsp. cremoris useful for cheddar manufacture have been classified according to the new taxonomic system. It is unclear whether strains carrying desirable cheddar traits fall exclusively into L. lactis subsp. cremoris as it is newly defined or whether strains belonging to both phylogenetic subspecies, but classified as L. lactis subsp. cremoris according to the old criteria, are likely to carry these traits.

Improvements in the manufacture of cheddar cheese have, until recently, been hampered by the lack of new L. lactis subsp. cremoris isolates. In particular, strains resistant to bacteriophage prevalent in the manufacturing environment were desired. While L. lactis subsp. cremoris bacteria can be found outside the cheese plant environment, their numbers are low relative to those of other lactose metabolizers. They are therefore extremely difficult to isolate with selective media alone. As a solution to this problem, we have described a genetic probe method that identifies L. lactis and L. lactis subsp. cremoris colonies on selective agar plates (24). With this technique, we have isolated several hundred environmental strains of L. lactis.

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from raw milk and cottage industry-fermented dairy products obtained in China, Morocco, Bosnia, and Ukraine and from vegetable and bovine sources in the United States (25, 26).

Some strains included in this study have been described previously (25, 26), and others are introduced here.

In order to confirm the new isolates' subspecies assignments according to the new taxonomy, to elucidate evolutionary relationships among these strains and strains derived from commercial starters, and to identify evolutionary groups best suited to cheddar manufacture, we compared ldh gene sequences for 45 different isolates. In addition, we evaluated the isolates for potential use as cheddar starters, assessed their carbon substrate utilization patterns, and screened candidate cheddar starter strains for bacteriophage resistance and bacteriocin production. The distribution of these characteristics was interpreted by comparison to the ldh phylogeny.

MATERIALS AND METHODS

Strain isolation and phenotypic characterization. The source materials, strain isolation method, and methods for taxonomic identification according to phenotypic criteria used in this study have been previously described (24–26). L. lactis isolates originated in vegetable matter, raw milk, and cottage industry-fermented milk products from China, Morocco (formerly Yugoslavia), China, Morocco, Ukraine, and the United States (Table 1).

Cloning and sequencing of ldh genes. Genomic DNAs were prepared from L. lactis cultures grown in M17 medium (32) by the guanidinium thiocyanate method (22). Genes encoding l-lactate dehydrogenase were PCR amplified with primers LDHF1 and LDHR1, complementary to conserved sequences at the extreme ends of the coding region (Table 2). Reaction mixtures contained 1× Taq buffer (Promega), 5% acetamide, 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.1 μg of genomic DNA. Thermal cycle parameters were 2 min 30 s at 94°C; 30 s at 94°C, 1 min at 52°C, 2 min at 72°C (35 cycles); and 7 min at 72°C. Taq DNA polymerase (2.5 U; Promega) was added during the first 52°C segment. PCR products were purified on QIAquick-spin columns (Qiagen) and cloned into vector pCRII (Invitrogen) according to the manufacturers' protocols.

Escherichia coli transformants producing white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates were screened for insert-containing plasmids by agarose gel electrophoresis, and plasmid DNAs were prepared with the Bio-Rad Miniprep kit. Sequencing reactions primed with the M13 forward primer, complementary to the vector sequence, were used to identify clones carrying ldh sequences. Five plasmids with ldh inserts were pooled for each L. lactis strain, and the pooled plasmids were sequenced with ldh-specific primers (Table 2). In a few instances in which all five chosen clones were in the same orientation, pooled plasmids were sequenced with the M13 reverse primer.

DYEdeoxy (Applied Biosystems) dye terminator sequencing reactions were analyzed with an ABI model 373A automated DNA sequencer. Completed sequences were assembled with Staden sequence assembly and editing software (3).

Phylogenetic analysis. ldh sequences were aligned according to their amino acid sequence, using the Genetic Data Environment sequence editor (provided by Steven Smith). The phylogenetic tree was inferred from DNA sequences by using the PHYLIP version 3.5c neighbor-joining program, with Kimura two-parameter genetic distances (6, 14, 23).

PCR amplification and probing of 16S rRNA genes. Binding of probes 212RLa and 68RCa (specific for L. lactis and L. lactis subsp. cremoris, respectively) to several environmental strains was double-checked by dot blot hybridization to PCR-amplified 16S rRNA genes. 16S rRNA genes were amplified from genomic DNAs by using domain Bacteria-specific primers 27F and 1492R (8, 15). Amplified DNAs were blotted and affixed to a Zetaprobe membrane as described elsewhere (10). The membrane was incubated for 20 min at 25°C with 15 ml of prehybridization buffer (6× SSPE [1.08 M NaCl, 60 mM NaPO4, 60 mM EDTA, pH 7.5], 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin], 0.1% sodium dodecyl sulfate [SDS]), after which the prehybridization buffer was replaced with 6 ml of the same buffer containing 50 ng of 32P-labeled oligonucleotide probe 212RLa or 68RCa and the membrane was incubated at 25°C for 5 h. After hybridization, the blot was washed three times for 15 to 20 min with 6× SSPE–0.1% SDS and then one time for 30 min at the stringent wash temperature 50°C. Hybridization intensities were measured with a PhosphorImager (Molecular Dynamics). Intensity of probe 68RCa binding was assessed as the ratio of 68RCa to 212RLa hybridization.

Carbon substrate utilization tests. The isolates' abilities to produce acid by using 49 compounds as sole carbon sources were evaluated with API 50 CH test kits (Bio-Merieux, Craponne, France). Liquid cultures inoculated from frozen stocks were spread over the following different agar II plates (28). Single colonies with typical appearance were picked, inoculated onto M17 broth, and processed according to the kit manufacturer's directions. API test results were evaluated at 48 h.

A dendrogram illustrating relationships among carbon substrate utilization phenotypes was constructed from the API data by using the PHYLIP version 3.5c neighbor-joining program (6) and genetic distances calculated according to the method of Jukes and Cantor (12).

Tests for characteristics useful to cheddar cheese manufacture. Isolates were subjected to preliminary tests for suitability as cheddar cheese starter strains, including tests for fast acid production and flavor development in milk. All strains were tested for rate of acid production by inoculating sterile 11% nonfat dry milk with 1.0% (vol/vol) overnight milk culture, incubating at 22°C, and scoring for clotting at 15 to 18 and 24 h (33). Selected strains positive for fast acid production were further tested for flavor development in milk (236-ml) cartons of 2.0% ultrahigh-temperature-pasteurized milk (Darigold Farms Inc.) with 1.0% overnight milk culture and incubating at 25°C for 7 days. After 7 days, each carton was sampled for flavor characteristics (32a). Some of these strains have been tested for flavor by a different method, with somewhat different results (25, 26).

Tests for bacteriophage sensitivity. Thirteen environmental isolates were tested for phage sensitivity against three industry phage banks (see Table 4). Tests were performed by the Bioproducts Group of Quest International, Rochester, Minn., the Culture Division of Waterford Foods, Inc., Millville, Utah, and Marschall Products of Rhone-Poulenc, Madison, Wis. Each company maintains independent phage banks isolated from whey samples obtained from commercial cheese factories.

Cultures transferred from milk to M17 lactose broth were grown overnight at 32°C and then challenged with phage. Individual L. lactis isolates were mixed with M17 lactose top agar containing 0.2% CaCl2 (Quest International and Waterford Foods, Inc.) or 0.08% CaCl2 (Marschall Products) and overlaid onto M17 lactose bottom agar plates. Phage bank preparations were spotted onto the top agar. Plates were incubated overnight (Quest International and Waterford Foods, Inc.) or 24 h (Marschall Products) and examined for clearing. Quest International tested five phage samples: a composite of phage-positive wheys collected at a midwestern cheddar cheese plant from 1990 to 1992, a composite of cheese plant phages from Quebec (20a), and three fresh buttermilk whey samples collected during 1993. Waterford Foods, Inc., used 32 phage preparations from their bank of phages. Marschall Products challenged environmental L. lactis with 102 whey samples from commercial cheese production: 72 whey samples, collected within a single month, which were positive for phage (whey samples 1 to 72), and 30 whey samples, collected within 2 weeks, which were negative (whey samples 73 to 102). A total of 38 single plaque isolates (SPI) from the Marschall Products phage bank were also tested (SPI 1 to 38). Clearing was assessed on a scale of 0 (no lysis) to 3 (total lysis), based on spot appearances.

GenBank accession numbers. The following nucleotide sequences were filed in GenBank under the assigned accession numbers: AMS, U78630; MSUA2, U78631; 1117M, U78632; AM4, U78633; CO3, U78634; CM1-3, U78635; BO34, U78636; 112, U78637; and BEN121, U78638. Identical ldh gene sequences were not filed.

RESULTS

Strain isolation and phenotypic characterization. The initial characterization of L. lactis environmental strains has been described previously (25, 26). A number of additional, undescribed strains from the Genetic Data Environment sequence editor (provided by Steven Smith) were identified to the subspecies level according to a reduced set of phenotypic criteria: growth at 40°C, in 4% NaCl, and at pH 9.2. The ability to deaminate arginine was not considered in the subspecies assignment, because this trait does not strictly correlate with the growth parameter criteria (2). While most strains fit into one or the other old subspecies category, according to these reduced criteria, a few strains exhibited some discordant characteristics (25). Both phenotypic subspecies were found among 68RCa-binding and nonbinding L. lactis (Table 1).

A total of 45 environmental strains were chosen for phylogenetic analysis, including strains which appeared promising for cheddar cheese production. Other strains were included in this analysis with genetic data for geographic isolate sites and to include strains belonging to both subspecies, defined by both phenotypic and L. lactis subsp. cremoris-specific probe binding criteria, and strains with discordant phenotypic characteristics. ldh sequences for four laboratory strains, ML1 (NCD0762), MG1363 (NCD0763), IL1403, and

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BU2-60, derived from commercial starter cultures, were included in the analysis (11, 18, 31).

Polymorphic patterns for L. lactis strains fell into two phylogenetic clusters exhibiting intercluster sequence similarities between 0.959 and 0.970. Each cluster included sequences from laboratory strains, which enabled subspecies identification. Within the L. lactis subsp. lactis group (the cluster including L. lactis subsp. lactis IL1403), 16S rRNA sequence similarities were ≥0.994. Within the L. lactis subsp. cremoris group (including L. lactis subsp. cremoris ML1 [NCDO762] and MG1363 [NCDO763]),

### TABLE 1. Characteristics of environmental isolates used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Growth at: 40°C</th>
<th>Growth at: 4% NaCl</th>
<th>Growth at: pH 9.2</th>
<th>Hydrolysis of arginine</th>
<th>Phenotypic L. lactis subspecies</th>
<th>Probe 68RCa</th>
<th>Coagulation of milk at 24 h</th>
<th>Flavor development in milk</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>112</td>
<td>Bosnia, cottage cheese sample 1</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>?</td>
<td>−</td>
<td>+/−</td>
<td>Bitter, off flavor</td>
<td>27</td>
</tr>
<tr>
<td>1117M</td>
<td>Bosnia, cottage cheese sample 1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Stomachy</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>AM4</td>
<td>Bosnia, cottage cheese sample 2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
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<td>AM5</td>
<td>Bosnia, cottage cheese sample 2</td>
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<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Stomachy</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>AM12</td>
<td>Bosnia, cottage cheese sample 2</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>BEN121</td>
<td>United States, <em>Phaseolus vulgaris</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>25, 27</td>
</tr>
<tr>
<td>BO32</td>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>BO34</td>
<td>Bosnia, raw milk</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>BO35</td>
<td>Bosnia, raw milk</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>BO36</td>
<td>Bosnia, raw milk</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>BO38</td>
<td>Bosnia, raw milk</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>CO3</td>
<td>United States, <em>Zea mays</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>CM1-3</td>
<td>China, raw milk sample 1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Stomachy</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>25, 26, 27</td>
</tr>
<tr>
<td>CM4-27</td>
<td>China, raw milk sample 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>25, 26, 27</td>
</tr>
<tr>
<td>CM5-6</td>
<td>China, raw milk sample 5</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>Acid, chalky</td>
<td>26, 27</td>
</tr>
<tr>
<td>FB1</td>
<td>United States, bovine Colostrum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>25, 27</td>
</tr>
<tr>
<td>FB2</td>
<td>United States, <em>Rubis discolor</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>25, 27</td>
</tr>
<tr>
<td>MS3</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS5</td>
<td>Morocco, raw milk</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>26, 27</td>
</tr>
<tr>
<td>MS7</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>Stomachy</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS9</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS11</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS13</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>25</td>
</tr>
<tr>
<td>MS16</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS17</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Slightly bitter</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS23</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS24</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS25</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS26</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS27</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS31</td>
<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
<td>MS33</td>
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<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
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<td>Morocco, raw milk</td>
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<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
</tr>
<tr>
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<td>Morocco, raw milk</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>27</td>
</tr>
<tr>
<td>MS45</td>
<td>Morocco, raw milk</td>
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<td>−</td>
<td>−</td>
<td>+/−</td>
<td>Clean, acid</td>
<td>−</td>
<td>+/−</td>
<td>Bitter</td>
<td>27</td>
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*+, −, and +/−, positive, negative, and intermediate reaction, respectively, for characteristic indicated; ?, indeterminate.

Hybridization to L. lactis subsp. cremoris 16S rRNA-specific oligonucleotide probe 68RCa (24).

Reference(s): 25, 26, 27.
sequence similarities were \( \geq 0.991 \). A number of strains in each group had identical \( ldh \) sequences (Fig. 1).

In initial colony hybridization tests, one strain belonging to the \( L. lactis \) subsp. \( cremoris \) group did not bind 16S rRNA probe 68RCa (strain AM5), and several strains belonging to the \( L. lactis \) subsp. \( cremoris \) group did (strains 112, MS3, MS7, and MS11). In order to investigate this apparent discrepancy between the two subspecies identification methods, we tested the binding of probe 68RCa, using PCR-amplified 16S rRNA genes as targets. This technique increases the amount of 16S rRNA sequence target bound to the hybridization membrane and reduces background due to cell debris and non-16S rRNA nucleic acid sequences. Using this higher-resolution protocol, all strains belonging to the \( ldh \) \( L. lactis \) subsp. \( cremoris \) group bound the \( L. lactis \) subsp. \( cremoris \)-specific 16S rRNA probe, and strains in the \( L. lactis \) subsp. \( cremoris \) group did (data not shown). The results of the 16S rRNA probe and \( ldh \) sequence analyses were thus congruent, yielding the same grouping of strains into \( L. lactis \) subsp. \( cremoris \) and \( L. lactis \) subsp. \( lactis \).

Each phylogenetic subspecies cluster included representatives of both phenotypic subspecies, as well as isolates from most geographic sites (the three Chinese milk isolates and three Bosnian cottage cheese sample 2 isolates were exceptions; these were found only in the \( L. lactis \) subsp. \( cremoris \) group). Arginine hydrolysis did not distinguish members of the two phylogenetic groups (Table 1).

In carbon substrate utilization tests, all strains were capable of using galactose, \( \alpha \)-glucose, \( \alpha \)-fructose, \( \alpha \)-mannose, \( N \)-acetyl glucosamine, and lactose (except strains MG1363 and FB1) as sole carbon sources. However, for other carbon sources the patterns of substrate utilization showed no strict phylogenetic pattern (Table 3; Fig. 2). In fact, strains with identical \( ldh \) sequences frequently had different profiles. Groups defined according to the old, phenotypic subspecies corresponded more closely to carbon substrate utilization patterns, with phenotypically \( L. lactis \) subsp. \( cremoris \) strains generally using fewer substrates than phenotypically \( L. lactis \) subsp. \( lactis \) strains. Ribose was closest to being a diagnostic substrate: all of 10 phenotypically \( L. lactis \) subsp. \( lactis \) isolates produced acid from ribose, compared to 3 of 24 phenotypically \( L. lactis \) subsp. \( cremoris \) isolates.

Tests to identify potential cheddar starter strains were performed on all environmental isolates (Table 1). All phenotypically identified \( L. lactis \) subsp. \( lactis \) strains were rejected as potential starters because they failed to coagulate milk after 15 to 18 h (BEN121, BO32, BO34, BO35, BO36, BO38, BO39, FB1, and FB62) and/or developed undesirable flavors in milk (BEN121, BO32, BO34, BO35, BO36, BO38, BO39, and CO3). This is consistent with the findings of Vedamuthu et al. (35, 36), who identified phenotypically \( L. lactis \) subsp. \( lactis \) strains as the frequent cause of fruity, fermented, or bitter flavors in cheeses made with uncharacterized, mixed-strain commercial starters. In addition, phenotypically \( L. lactis \) subsp. \( lactis \) strains’ ability to grow at 40°C would likely result in growth during cooking (19). All strains with indeterminate phenotypic subspecies identities also were rejected due to slow milk coagulation (CM1-3, MS3, MS13, MSUA2, and MSUA10) and/or undesirable flavors (112, AM4, AM12, CM1-3, CM4-27, CM5-6, MS3, MS70, MSUA2, and MSUA10). The two strains phenotypically \( L. lactis \) subsp. \( cremoris \) but phylogenetically \( L. lactis \) subsp. \( lactis \) were rejected due to slow acid production.
### TABLE 3. Carbon substrate utilization profiles for *L. lactis* strains

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<th>Strain</th>
<th>Acid production from:</th>
<th>Ribose</th>
<th>Galactose</th>
<th>l-Glucose</th>
<th>d-Galacto</th>
<th>d-ribo</th>
<th>d-xylose</th>
<th>d-Mannose</th>
<th>Maltitol</th>
<th>N-acetyl glucosamine</th>
<th>Amygdalin</th>
<th>Arabin</th>
<th>Escalin</th>
<th>Sialin</th>
<th>Cellobiose</th>
<th>Cellobiose</th>
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a All strains were negative for acid production from glycerol, erythritol, d-arabinose, l-arabinose, d-xylose, aldonitol, β-methyl-xyllose, side, sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-d-mannoside, α-methyl-d-glucoside, melibiose, trehalose, melezitose, d-raffinose, glycogen, xylitol, d-turanose, d-lyxose, d-tagatose, d-fucose, d-arabitol, l-arabitol, 2-ceto-gluconate, and 5-ceto-gluconate. Strains are listed in the order in which they appear in Fig. 1.

b Laboratory strain derived from commercial starter culture.
either by the milk coagulation assay (MS7) or by temperature effects on ΔpH during growth (MS11) (1a). Several strains which were phenotypically and phylogenetically members of \textit{L. lactis} subsp. \textit{cremoris} were eliminated due to slow milk coagulation (AM5, MS34, MS55, and 1117M) and/or flat, bitter, or malty flavors (AM5, MS5, MS16, MS34, and MS58). The remaining 14 strains belonging to \textit{L. lactis} subsp. \textit{cremoris} by both phenotypic and phylogenetic criteria were judged to be good candidates for new cheddar starter strains (strains MS9, MS17, MS23, MS24, MS25, MS26, MS27, MS31, MS33, MS44, MS45, MS51, MS52, and MS53). Although these strains had identical \textit{ldh} sequences and were isolated from the same Moroccan raw milk sample, they exhibited a variety of different carbon substrate utilization profiles (Table 3) and are therefore unlikely to have arisen from a single parent cell. Several of these strains are now being evaluated as cheddar starters in pilot- and commercial-scale cheese production at the Tillamook County Creamery Association, Tillamook, Oreg.

Sixteen strains were tested for bacteriophage sensitivity by three commercial starter companies (\textit{L. lactis} subsp. \textit{cremoris} AM4, CM1-3, CM4-27, CM5-6, CO3, MS5, and MS17 and \textit{L. lactis} subsp. \textit{lactis} BEN121, FB1, FB62, MS11, and MS70). All were resistant to most of the 177 phage preparations tested, including one of two composite whey samples (Quest International composite collected from 1990 to 1992), all of three buttermilk whey samples (Quest International), 40 of 72 phage-positive commercial cheese whey samples (lethal to commercial starter strains; Marschall Products), 30 phage-negative commercial cheese whey samples (Marschall Products), and 44 of 70 plaque-purified phages (12 of 32 and 32 of 38; Waterford Foods, Inc., and Marschall Products, respectively). Ten strains were completely resistant to all phage preparations; the remaining six exhibited a variety of phage sensitivity profiles (Table 4).

CO3 was the most sensitive environmental isolate, with 44 of 177 phage preparations producing either turbid or clear plaques. Strain MS70 was sensitive to 15 preparations, all of which also produced plaques on CO3. Strain CM4-27 was the only isolate belonging to \textit{L. lactis} subsp. \textit{cremoris} by both phenotypic and genotypic criteria which also showed sensitivity to phages, producing turbid plaques when challenged with one plaque-purified phage and five cheese whey preparations. Strains FB1, FB62, and BEN121, were sensitive to 4, 12, and 6 phage preparations, respectively (Table 4).

**DISCUSSION**

Our most important finding is that environmental isolates of \textit{L. lactis} which show potential as cheddar cheese starters are restricted to strains that are \textit{L. lactis} subsp. \textit{cremoris} by both new and old taxonomic criteria.

According to \textit{ldh} sequence phylogenies, the 45 newly identified isolates of \textit{L. lactis} are very closely related to laboratory strains isolated from industrial starters that have been in use since the early to mid-1900s. Eighty new identified strains isolated from bovine colostrum, raw milk, and cottage cheese from the United States, Morocco, and Bosnia have \textit{ldh} sequences identical to that of \textit{L. lactis} subsp. \textit{lactis} IL1403; four

**FIG. 2.** Dendrogram constructed by the neighbor-joining method from carbon substrate utilization data shown in Table 3. Strains able to use the fewest carbon sources appear at the left, with catabolic diversity increasing towards the right. Relationships shown here correlated neither with relationships among \textit{ldh} genes nor with phenotypic subspecies designations. Boldface type, phenotypically \textit{L. lactis} subsp. \textit{cremoris} strain; lightface, roman type, phenotypically \textit{L. lactis} subsp. \textit{lactis} strain; italic type, subspecies phenotypically indeterminate; asterisk, laboratory strain derived from commercial starter culture; dagger, strain belonging to the \textit{L. lactis} subsp. \textit{cremoris} phylogenetic group shown in Fig. 1.
strains from maize and raw milk collected in the United States, Bosnia, and China are identical to *L. lactis* subsp. *cremoris* MG1363; and 26 strains from raw milk and home-style cottage cheese collected in Morocco, Bosnia, China, and Ukraine have only 1-nucleotide differences from *ldh* of *L. lactis* subsp. *cremoris* ML1 (Table 1; Fig. 1).

According to the *ldh* phylogeny, *L. lactis* strains form two very tight phylogenetic clusters in which *L. lactis* subsp. *cremoris* strains vary by a maximum of 8 nucleotides (similarity, $0.991$) and *L. lactis* subsp. *lactis* strains vary by a maximum of 6 nucleotides (similarity, $0.994$) (Fig. 1). This tight clustering is consistent with relationships inferred from DNA hybridization data for laboratory and commercial strains, including strains MG1363, IL1403, and ML1 (9). It therefore appears that strains from environmental sources belong to the same evolutionary groups as commercial strains and do not fall into older, more diverse clades as might have been hypothesized. It should be noted, however, that our isolation method would fail to identify *L. lactis* strains which do not bind the species-specific 212RLa probe (24). These results are also consistent with the recent, independent diversification of the two *L. lactis* subspecies, perhaps coincident with the origin of dairying among human populations (9).

Despite the limited evolutionary breadth recorded in the *ldh* sequence, the new *L. lactis* isolates exhibit a high degree of phenotypic variation, even among strains identical in *ldh* sequence. For instance, among the 23 *L. lactis* subsp. *cremoris* isolates with *ldh* sequences identical to that of strain AMS5 (Fig. 1) there are eight different carbon substrate utilization profiles, both Arg$^+$ and Arg$^-$ phenotypes, and at least two different phenotypic sensitivity profiles (phage insensitivity in MS5 and MS17, the CM4-27 profile, and profiles for untested members of the group [Table 4]). Most significantly, strains indistinguishable by *ldh* sequence may vary in their suitability as cheddar cheese starters (Table 1).

This high phenotypic variability, despite low sequence divergence, is not surprising given the current knowledge of genetic instability among low-G+C-content gram-positive bacteria. The low-G+C-content gram-positive bacteria have extraordinarily fluid genomes, in both their chromosomal and plasmid components (4, 17, 20, 37). Their phenotypic diversity can

| TABLE 4—Continued |
|-------------------|-------------------|
| Infected phase preparation | Spot appearance$^b$ on sensitive isolate: |
| CM4-27 | CO3 | FB1 | FB62 | MS70 | BEN121 |
| 57 | 2 |
| 60 | 1 |
| 63 | 1 |
| 64 | 3 |
| 68 | 3 |

$^a$Ten isolates were insensitive to all phase preparations, and 118 phase preparations were ineffective against all *L. lactis* strains.

$^b$Spot appearance key: 1, slightly reduced cell density; 2, turbid; 3, clear.

### TABLE 4. Phage sensitivity profiles for environmental isolates of *L. lactis*

<table>
<thead>
<tr>
<th>Infective phage preparation</th>
<th>Spot appearance$^b$ on sensitive isolate:</th>
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<tr>
<td>Quest International composite whey: Quebec</td>
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<tr>
<td>Waterford Foods, Inc., plaque-purified phage</td>
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| Marschall Products plaque-purified whey | |
| 1 | 1 |
| 4 | 1 |
| 6 | 3 |
| 7 | 1 |
| 10 | 3 |
| 11 | 2 |
| 12 | 2 |
| 13 | 1 |
| 14 | 1 |
| 18 | 2 |
| 21 | 1 |
| 26 | 2 |
| 27 | 3 | 2 |
| 28 | 2 |
| 29 | 2 |
| 31 | 1 | 1 |
| 39 | 1 | 3 | 1 | 1 |
| 40 | 3 | 1 |
| 41 | 1 |
| 44 | 2 |
| 45 | 2 |
| 46 | 2 |
| 47 | 3 |
| 49 | 2 |
| 51 | 1 |
| 52 | 1 |
| 55 | 1 |

Continued
often be attributed to the acquisition or loss of sexual plasmids and transposons, to gene inactivation or reactivation of cryptic genes, and to DNA rearrangements.

A striking feature of the low-G + C-content group is that many genes subject to strong selective pressures are carried on sexually transmissible plasmids and conjugative transposons. In *L. lactis*, plasmid genes encode enzymes for the metabolism of citrate, proteins, and sugars (including lactose, sucrose, galactose, mannose, xylose, and glucose), as well as bacteriocin production and phage resistance (reviewed by von Wright and Sibakov [37]). Some of these plasmids can reversibly integrate into the chromosome, leading to strains which differ in heritability and levels of gene expression (5, 20). Wide dissemination of sexual plasmids is demonstrated by the finding that plasmid-encoded citrate permease genes in a *Leuconostoc* strain are nearly identical to *citP* in an *L. lactis* strain (34). In addition to demonstrating gene acquisition and loss associated with the frequently unstable sexual elements, *L. lactis* strains harbor a number of cryptic genes that can be reactivated by spontaneously or chemically induced mutagenesis (4, 13, 30). It is therefore not surprising that closely related *L. lactis* strains exhibit markedly different phenotypes, especially for traits important to the dairy industry.

Among strains of *L. lactis*, chromosomal rearrangements are so common that restriction fragment length polymorphisms are more frequently due to these events than to point mutations (16). While restriction fragment length polymorphisms are frequently attributed to the insertion or deletion of plasmids, mobile genetic elements, or temperature bacteriophages, large chromosomal inversions can be a significant cause of fragment length variation as well (16, 17). The discovery that stretches of *L. lactis* chromosomal DNA appear to have been transferred from outside the low-G + C-content gram-positive cluster suggests another potential source of chromosomal variation (4).

Our data indicate that relationships among *L. lactis* strains inferred from *ldh* sequences and binding of 16S rRNA-specific oligonucleotide probes are congruent, despite the disorganizing influences of mobile sexual elements, gene transfer, and DNA rearrangement. This suggests that the set of *L. lactis* chromosomal sequences has remained in overall linkage disequilibrium (intact) since the subspecies diverged. 16S rRNA, *ldh*, and presumably other chromosomal sequences can therefore be used to discriminate *L. lactis* subspecies with some confidence. However, it seems advisable to evaluate sequence similarities at more than one locus in order to make definitive subspecies assignments.

We have discovered that environmental isolates of *L. lactis* showing promise as cheddar cheese starter strains fail exclusively into *L. lactis* subsp. *cremoris*, as defined by both phylogenetic and phenotypic criteria. Although this finding considerably narrows the field of new strains to be screened for manufacturing uses, there remains a large amount of phenotypic diversity among the closely related candidate strains, many of which are unsuitable for industrial cheesemaking. More-efficient molecular screens for cheddar starter potential will depend upon the identification of genes and regulatory elements controlling desirable traits, most likely encoded on transposons or plasmids or as reversibly encrypted chromosomal genes.

**ACKNOWLEDGMENTS**

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**REFERENCES**


2. Daniels, B. Unpublished data.


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