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EXPERIMENTAL ARTICLES

The Lactic Acid Enterococci *Enterococcus faecium* and *Enterococcus durans*: Nucleotide Sequence Diversity in 16S rRNA Genes

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Abstract—Among the strains used as starters for making sour milk products on the territory of the CIS, the bacteria *Enterococcus faecium* and *Enterococcus durans* are frequently found. In this work, we studied a new collection of lactic acid enterococci and also obtained more complete data on the nucleotide sequences of 16S rRNA genes in some strains studied earlier and found that most strains had certain distinctions in their 16S rRNA genes as compared with the *E. durans* and *E. faecium* genes available in the NCBI database. Based on these data, it is suggested that the strains of lactic acid enterococci represent new, earlier unknown taxa of enterococci that use milk as an ecological niche.

Key words: thermophilic lactic acid bacteria, enterococci, sequencing of 16S rRNA genes, DNA–DNA hybridization, speciation in bacteria.

We showed in our earlier work [1] that enterococci of the species *E. faecium* and *E. durans* are widely used as starters on the territory of the CIS, especially for domestic production of sour milk products. In that paper, based on the results of sequencing of the proximal region (500 nucleotides) of the 16S rRNA genes, seven out of ten enterococcal strains used as starters and erroneously considered earlier as thermophilic streptococci were assigned to *E. durans*, and three strains were assigned to *E. faecium.* The latter three strains were ascribed earlier [2, 3] to different genomovars (II, III, IV) based on the results of DNA hybridization. The strains assigned to *E. durans* also represented three genomovars (I, V, VI).

The level of DNA hybridization between the enterococcal strains of one genomovar was usually at least 80%, and the one for the strains of different genomovars did not usually exceed 30–40% [2, 3]. These data ran counter to the common opinion that the DNA–DNA hybridization level between strains of the same species cannot be below 60–70% [4, 5]. However, it should be taken into account that bacteria reproducing themselves vegetatively, in contrast to higher organisms existing in the regime of regular genetic exchange, have almost no limitations for the divergence in the DNA primary structure, especially in the case of geographically remote populations.

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In this work, we investigated a number of strains from the new collection of thermophilic lactic acid bacteria collected in Stavropol' krai and the Caucasus and obtained data showing that the level of DNA hybridization between strains assigned to one species of enterococci varies within 40–60%. We also obtained more complete data on the nucleotide sequences of the 16S rRNA of the strains studied earlier and new isolates of lactic acid enterococci. As a result, it appeared that the *E. durans* strains used as starters or isolated from sour milk products of different origin exhibit, in most cases, certain distinctions in the 16S rRNA nucleotide sequences from the *E. durans* strains that are represented in the National Center for Biotechnology Information (NCBI) database. Moreover, we discovered, as a result of sequencing the distal region of the 16S rRNA genes, that in strains of different origin, the nucleotide sequence in the proximal region of the 16S rRNA genes was the same as in *E. durans*, and in the distal region, the same as in *E. faecium.* These data may give evidence of the existence of specialized taxa of enterococci that use milk as an ecological niche.

MATERIALS AND METHODS

Bacterial strains. Six strains from the new collection of thermophilic lactic acid bacteria isolated from sour milk products (cheese, matsoni (yogurt), sour milk) in Armenia (CK1101) and Stavropol' krai (CK1106, CK1107, CK1109, CK1114, and CK1116)

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were the subjects of this study. These six strains were chosen for study from the initial collection of 15 strains as exhibiting different *Sma*I restriction patterns revealed by pulsed-field electrophoresis (data not shown). In addition, in this work, we used eight strains of lactic acid enterococci identified earlier [1], including six strains assigned to the species *E. durans*: 6kb (B8249), B3371, B2095, CK1025, CK1026, and B3166 and two strains assigned to *E. faecium*: 5 (B8251) and CK1013. These strains were used as starters for making sour milk products in different CIS regions under the wrong species name *S. thermophilus.* The type strain *S. faecium* ATCC19434 was provided by Dr. G. Giraffa (Italy).

The 525-nucleotide sequences in the proximal region of the 16S rRNA genes in strains CK1013, 5, 6kb, B3371, and CK1025 were earlier submitted to the GenBank; the accession numbers are AY683831, AY683833, AY683834, AY683835, and AY683836, respectively. When comparing the 16S rRNA genes in different species of enterococci and streptococci, we used the nucleotide sequences of these genes from the NCBI database for the strains of *E. durans* (AJ276454 and AJ420801), *E. faecium* (AJ420800 and AY172570), and *Streptococcus macedonicus* (AF088900 and SMZ94012). The designations of all the other strains (we used the NCBI accession numbers for the corresponding 16S rDNA sequences) are given hereinafter in the text of the article.

Cultivation. The bacteria were grown at 42°C on agarized medium M21 containing (g/l) peptone, 2.5; yeast extract, 2.5; KH_2PO_4, 2; Na_2HPO_4, 8.5; $MgSO_4$, 0.12; agar–agar, 15; glucose, 5; milk hydrolysate, 200 ml; and distilled water, 800 ml (pH 7.0).

DNA–DNA hybridization was carried out according to the De Ley method as described earlier [6].

Determination of the 16S rDNA nucleotide sequence and their analysis were performed as described earlier [1].

RESULTS AND DISCUSSION

The DNA G+C content of strains of the new collection of thermophilic lactic acid bacteria varied between 37.7 and 39.2 mol % (data not shown), which may be considered typical both of thermophilic lactic acid streptococci and enterococci. All six strains, except CK1101, were capable of fermenting esculin, and strains CK1107 and CK1114 were tolerant of polymyxin, which could be evidence of their phenotypic similarity to enterococci. All the strains fermented lactose at 44°C.

To identify the new strains, we determined the nucleotide sequences of their 16S rRNA genes in a 500-nucleotide-long proximal region that approximately corresponded to positions 50–550 in *Escherichia coli* 16S rRNA. Based on the comparison with the sequences determined earlier [1] or retrieved from the NCBI database (see MATERIALS AND METHODS), strains CK1106, CK1107, CK1114, and CK1116 were identified as *E. durans.* These strains hardly differed from one another in the proximal gene region, as well as from the 16S rRNA gene of strain 6kb, sequenced by us earlier, except for a single nucleotide substitution (C for T) in CK1107. Strain CK1101, originating from Armenia, was identified in the same way as *E. faecium* based on complete similarity of its nucleotide sequence to that of strain CK1013 identified earlier [1]. However, it should be taken into account that, in the proximal region of the 16S rRNA gene, both of these strains have one distinction in common (substitution of A for G) from typical strains of *E. faecium* (see below).

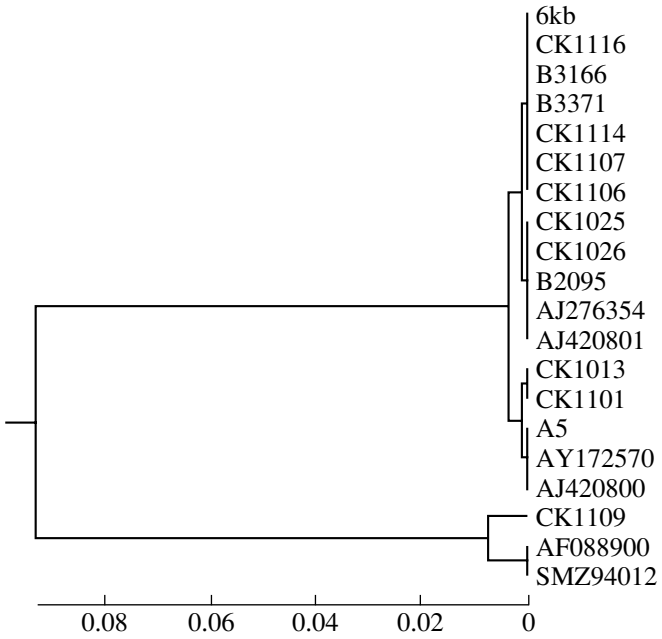

Fig. 1. Dendrogram of the relationships between strains of enterococci and streptococci constructed based on the homologies of 16S rRNA sequences in the proximal region (500 nucleotides).

Strain CK1109 was identified as *Streptococcus macedonicus.* In this case, the closest 16S rDNA nucleotide sequences for the species *S. macedonicus* were in the strains whose accession numbers to the GenBank were AF088900 and Z94012.

Figure 1 shows the phylogenetic tree constructed with the use of the 16S rRNA gene sequences determined by us in this work and earlier [1] and sequences retrieved from GenBank. The genetic distances (scaled at the bottom of the figure) reflect the numbers of nucleotide substitutions per 100 nucleotide sequences in a 500-nucleotide-long portion of the 1542-nucleotide-long 16S rRNA gene. According to these data, the strains assigned to *E. durans* form two branches characterized by 99.8% homology. The strains assigned to *E. faecium* also form two branches with the same high homology level. The degree of homology between

The extents of DNA reassociation (%) among enterococcal and streptococcal strains of different origin

Strain	CK1106	CK1107	CK1116	CK1114	CK1101	CK1109
CK1106						
CK1107	91					
CK1116	42	57				
CK1114	50	55	56			
CK1101	36	32	37	35		
CK1109	34	33	30	33	30	
E. faecium ATCC19434	45	48	41	62	51	31

these species is also sufficiently high, varying between 99.2 and 99.6%, whereas the degree of homology between the genera *Enterococcus* and *Streptococcus* is at a level of 81.2–81.5%.

The data on the DNA hybridization level may be of interest for the general characterization of the genome properties in the strains from the new collection. As follows from the results shown in the table, four strains assigned to *E. durans* (CK1106, CK1107, CK1114, and CK1116) are characterized by relatively high (from 42 to 91%) levels of DNA hybridization between one another and lower hybridization levels (30–37%) with strains CK1101 and CK1109 assigned to other species. The high level (91%) of DNA hybridization between CK1106 and CK1107 indicates that they belong to one genomovar. The very low hybridization level (42%) between strains CK1106 and CK1116 was quite unexpected, given that all the other DNA–DNA hybridization values in the group of the four *E. durans* strains (including the combination CK1107 × CK1116) are relatively high (50–57%). When the DNA hybridization experiments were repeated using independently prepared DNA preparations, virtually the same values of the degree of DNA reassociation were obtained (the difference was no more than 1–2%). Thus, the four strains identified as *E. durans* should represent three different genomovars with DNA hybridization levels between them of approximately 40 to 60%.

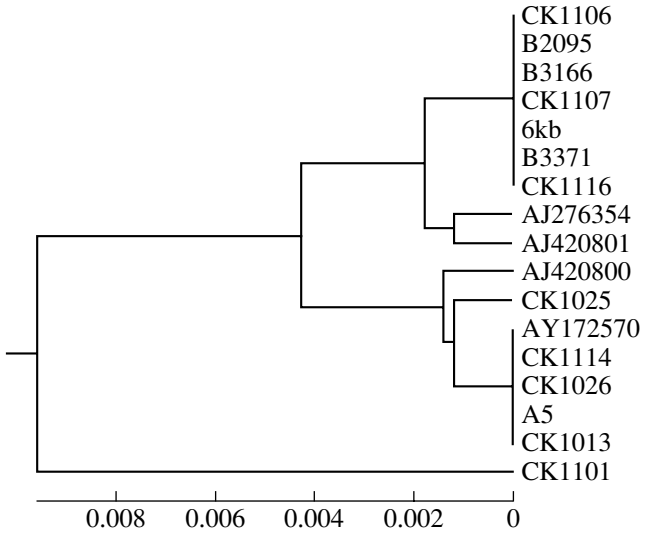

Fig. 2. Dendrogram of the relationships between strains of enterococci and streptococci constructed based on the homologies of 16S rRNA sequences in the distal region (440 nucleotides).

It follows from the data presented in the table that, with the *E. faecium* type strain ATCC19434, all the strains of *E. durans* had higher DNA hybridization values than with strain CK1101, also assigned to *E. faecium.* This result may give evidence of a significant divergence of strain CK1101 from Armenia in relation to the type strain of the same species. In addition, the high (62%) value of DNA reassociation between *E. faecium* ATCC19434 and CK1114 is noteworthy. Indeed, strain CK1114, as it appeared as a result of sequencing the distal region of 16S rRNA genes (see below), does not belong to *E. durans* but rather represents a separate species close to *E. faecium.*

The comparison of the known nucleotide sequences of the 16S rRNA genes of *E. durans* and *E. faecium* [7] made it possible to conclude that the most variable 16S rRNA domains in these species correspond to the proximal and distal regions of the gene. That was the reason why in the five strains from the new collection identified as *E. durans* and *E. faecium*, as well as in the eight strains assigned to these species earlier, we sequenced the distal region of the 16S rRNA genes using the primer 1R (5'-cgc acc ttc cga tac ggg cta cct-3'). The most interesting conclusion from this sequencing consisted in that strain CK1114, as well as strains CK1025 and CK1026, is much closer to *E. faecium* than to *E. durans*. Figure 2 shows in the form of a phylogenetic tree the values of the degree of homology of the distal regions of 16S rRNA genes of the strains studied. According to these data, the degree of homology of the strains within the species *E. durans* varied between 99.5 and 99.8%. The same degree of homology exists within the species *E. faecium* if strain CK1101 is not taken into account. The latter strain reveals more significant deviation from the other strains of *E. faecium* (98.3–98.6%) and an even greater deviation from *E. durans* (97.3–97.8%). This result confirms the conclusion on a considerable divergence of strain CK1101 that was made based on the data of DNA–DNA hybridization (table).

Figure 3 shows data demonstrating the differences in the nucleotide sequences in different strains in the most important positions of the 16S rRNA gene as revealed by the results of sequencing the proximal and distal regions of this gene. It is these distinctions that allow the species affiliation (either with *E. durans* or *E. faecium*) to be determined. Figure 3 shows such

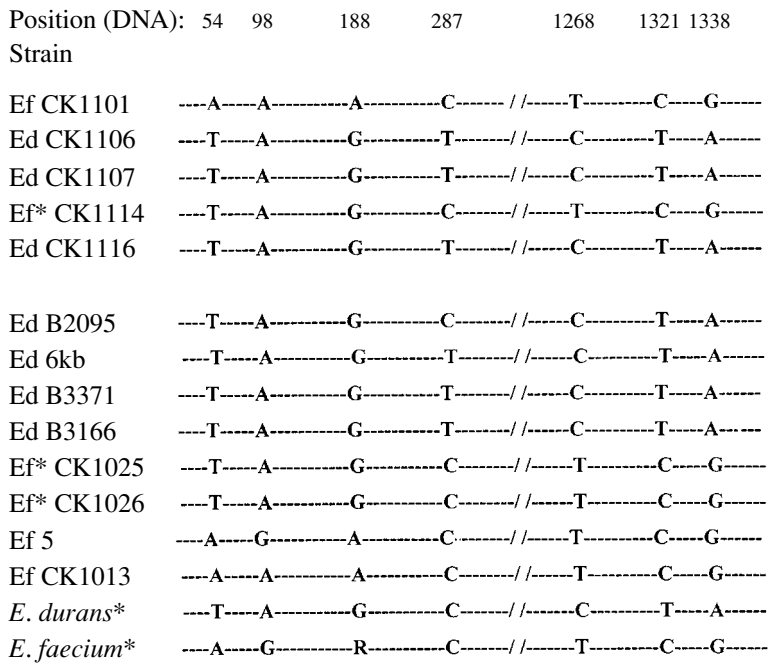

Fig. 3. Positions of the common variable nucleotides in the 16S rRNA genes of different *E. durans* and *E. faecium* strains. Ed, *E. durans*; Ef, *E. faecium*; Ef*, a separate taxon close to *E. faecium* (see text). *E. durans** represents sequences common for the strains from the NCBI database whose accession numbers are AJ420801, AJ276354, Y18359, and AF061000. *E. faecium** represents the NCBI strains under the accession numbers AJ420800, Y172570, AJ291732, AJ276355, AY057055, AY665974, AF145258, and AF070223. R means A or G.

positions in the 16S rRNA genes of different species of enterococci (the sequences were taken from the paper by Patel *et al.* [7]). Position 99 in these sequences corresponds to position 100 of the *E. coli* 16S rRNA.

The lower part of Fig. 3 shows the combinations of variable nucleotides characteristic of *E. durans, E. faecium*, and *E. hirae* strains represented in the NCBI database. In most *E. faecium* strains, position 188 in occupied by A (as in strains 5 and CK1013), but some of the strains contain G. It follows from Fig. 3 that in positions 54, 98, 1268, 1321, and 1338, the strains of *E. durans* and *E. faecium* have nucleotide combinations characteristic of these species. However, as follows from the data obtained by us, in strain CK1114, as well as in CK1025 and CK1026, the proximal region of the 16S rRNA gene exhibits, in positions 54, 98, and 188, a combination of nucleotides characteristic of *E. durans*, whereas the distal region of this gene displays, in positions 1268, 1321, 1338, a combination of nucleotides typical of *E. faecium.* Thus, strain CK1114, as well as CK1025 and CK1026, as judged from the combination of variable nucleotides in the distal portion of the 16S rRNA genes, must be close to *E. faecium.* This is also evidenced by the aforementioned high DNA hybridization level (62%) between the *E. faecium* strains ATCC19434 and CK1114.

According to our data on the ability of the strains studied to utilize different carbohydrates (not shown), strains CK1114, CK1025, and CK1026, as distinct from the strains of *E. durans*, are capable of utilizing mannitol, which serves as an important differentiating characteristic in identification of *E. durans* and *E. faecium* [8]. These strains may represent an emerging or even already sufficiently widespread new species, *Enterococcus lactis*, which uses milk as a biotope. At least it is evident that these strains represent a separate taxon that is phenotypically close to *E. faecium* but has a special 16S rRNA nucleotide sequence. Interestingly, there is an *E. faecium* strain in the NCBI database (accession number AB018210) isolated from the carp stomach in Japan whose 16S rRNA sequence is the same as that in CK1114, CK1025, and CK1026 (i.e., it is similar to *E. durans* in the proximal portion and to *E. faecium* in the distal portion). This fact may be considered evidence in favor of the new species being already sufficiently widespread.

According to our data shown in Fig. 3, the strains assigned by us to *E. durans* reveal one distinction in the 16S rDNA nucleotide sequence compared to the strains from the NCBI database. This distinction consists in the substitution of T for C in position 287 in all the strains assigned to *E. durans*, except strain B2095. In this connection, attention should be given to the fact that enterococci are usual inhabitants of the human and animal gastrointestinal tract and that, in most cases, the enterococcal strains in the NCBI database are clinical isolates. Taking into consideration this circumstance, it can be suggested that most of the strains isolated from

sour milk products or used as starters also comprise a separate isolated taxon (within the framework of the species *E. durans*) that often uses milk as an ecological niche.

The nucleotide sequences of 16S the rRNA genes were deposited in the GenBank database under the accession numbers AY902456 (CK1106), AY902457 (CK1109), AY902458 (CK1107), AY902459 (CK1114), AY902460 (CK1101), and AY902461 (CK1116).

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