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Characterization of the *Lactobacillus casei* group based on the profiling of ribosomal proteins coded in *S10-spc-alpha* operons as observed by MALDI-TOF MS

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ABSTRACT

The taxonomy of the members of the *Lactobacillus casei* group is complicated because of their phylogenetic similarity and controversial nomenclatural status. In this study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of ribosomal proteins coded in the *S10-spc-alpha* operon, termed *S10*-GERMS, was applied in order to classify 33 sample strains belonging to the *L. casei* group. A total of 14 types of ribosomal protein genes coded in the operon were first sequenced from four type strains of the *L. casei* group (*L. casei* JCM 1134^T, *L. paracasei* subsp. *paracasei* JCM 8130^T, *L. paracasei* subsp. *tolerans* JCM 1171^T, and *L. rhamnosus* JCM 1136^T) together with *L. casei* JCM 11302, which is the former type strain of '*L. zeae*'. The theoretical masses of the 14 types of ribosomal proteins used as biomarkers were classified into five types and compiled into a ribosomal protein database. The observed ribosomal proteins of each strain, identified by MALDI-TOF MS, were categorized into types based on their masses, summarized as ribosomal protein profiles, and they were used to construct a phylogenetic tree. The 33 sample strains, together with seven genome-sequenced strains, could be classified into four major clusters, which coincided precisely with the taxa of the (sub)species within the *L. casei* group. Three "ancient" strains, identified as *L. acidophilus* and *L. casei*, were correctly re-identified as *L. paracasei* subsp. *paracasei* by *S10*-GERMS. *S10*-GERMS would thus appear to be a powerful tool for phylogenetic characterization, with considerable potential for management of culture collections.

Introduction

The use of lactic acid bacteria (LAB) in the fermentation of a variety of fermented foods and drinks has a long history. There is a growing body of scientific evidence for their beneficial effects on health and, nowadays, LAB are widely used as probiotics in foods and pharmaceuticals. *Lactobacillus casei* and related organisms (the *L. casei* group) are typical LAB widely used in dairy products and lactic beverages. However, the taxonomic position and nomenclature of the *L. casei* group remains controversial [20,28].

The former *L. casei* consisted of five subspecies, with ATCC 393 being the type strain [29]. In 1989, Collins et al. [6] reclassified *L. casei* into three species: *L. casei* (containing ATCC 393 as the type strain), *L. paracasei* with two subspecies (subsp. *paracasei* and subsp. *tolerans*), and *L. rhamnosus*. Dellaglio et al. [7] requested an opinion on this proposal from the International Committee on Systematic Bacteriology (ICSB), specifically concerning a plan to reject the name *L. paracasei* and to designate strain ATCC 334 as a neotype of *L. casei*. Although this request was initially turned down [39], Dicks et al. [10] then again proposed: (1) the reclassification of *L. casei* subsp. *casei* ATCC 393 and *L. rhamnosus* ATCC 15820 as '*L. zeae*' (with ATCC 15820 as the type strain); (2) the designation of ATCC 334 as the neotype strain of *L. casei*; and (3) the rejection of the name *L. paracasei*. This proposal was considered reasonable [5,7,11,19,25] and a Request for an Opinion was subsequently made in 2002 [8]. However, the decision of the Judicial Commission of the ICSB in 2008 was to reject the Request again [35]. The current taxonomy of the *L. casei* group is thus still based on the 1989 proposal by Collins et al. [6].

Due to the controversy concerning the nomenclatural status that has persisted since 1989, the classification of the *L. casei* group has wavered between *L. casei* and *L. paracasei*. This is because the former *L. casei* subsp. *casei* strains remaining in the new *L. casei* are limited to ATCC 393 and its related strains. There is a view that most of the

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other former *L. casei* subsp. *casei* strains, which are similar to ATCC 334 and widely used in the dairy food industry, should be transferred to *L. paracasei*. Huys et al. [16] reported that more than 28% of commercial LAB cultures involving the *L. casei* group intended for probiotic use had been misidentified at the genus or species level. Therefore, gaining a correct understanding of the status of the strains in the *L. casei* group is important for culture collection management in the industry and research institutes, as well as in microbial resource centers. Moreover, a key question is whether or not the strains previously thought to be *L. casei* truly have a close phylogenetic relationship to type strain ATCC 393.

The discrimination and classification of the *L. casei* group strains is, however, not an easy task [28]. Since the 16S rRNA gene sequences may not be suitable for discriminating closely related *Lactobacillus* taxa, other housekeeping gene sequences have been used to discriminate within the *L. casei* group [3,11,15,22]. Nevertheless, the use of much more genotypic information would assist in achieving a higher resolution. Automated ribotyping has been used successfully to segregate the *L. casei* group into five genotypic clusters (see Table 1) [25]. Multilocus sequence typing (MLST) has also proved to be a powerful tool for studying the strain diversity of the *L. casei* group [9,24]. These DNA-based methods, however, require time-consuming procedures, such as DNA extraction and amplification, gel electrophoresis, or DNA sequencing.

The characterization of bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is attracting increasing attention as a research tool [27,40]. The experimental procedure of MALDI-TOF is significantly simpler and faster than those of other DNA-based methods. In some cases, even intact cells can be subjected directly to mass spectrometric measurements [38]. We have proposed a bioinformatics-based phylogenetic classification method on the basis of ribosomal protein profiling, as observed by MALDI-TOF MS [26,31,33,34]. Unlike previous MALDI-TOF approaches based on mass spectral fingerprinting, our proposed method is based on the sequence variations of ribosomal subunit proteins, which are the most representative house-keeping proteins.

In our initial studies [26,30,32–34], the reference mass list for the ribosomal protein biomarkers (ribosomal protein database) relied on translated amino acid sequences of genome-sequenced strains registered in public databases. Although the taxonomic position of bacterial strains should be determined by comparison with type strains, in many cases the genome-sequenced strains differ from the type strains. In spite of aggressive genome sequencing of the *L. casei* group, there are no type strains of the group with fully finished status. *L. paracasei* ATCC 25302^T is the only fully genome-sequenced type strain at this time, but the sequencing status has been announced as a permanent draft. As a result of a homology search, the registered amino acid sequences of the ribosomal proteins of ATCC 25302^T appear to be incomplete. Furthermore, the taxa names of several genome-sequenced strains of the *L. casei* group appear to be incorrect.

To overcome the problem of mismatching between genome-sequenced strains and type strains, we have promoted the gene sequencing of ribosomal proteins coded in the *S10-spc-alpha* operon of type strains [12–14]. A set of theoretical masses for the operon coding ribosomal proteins is listed as the ribosomal protein database. This approach, termed *S10*-GERMS (the *S10-spc-alpha* operon gene encoded ribosomal protein mass spectrum), has been successfully applied to the classification of several bacterial taxa at the species level without using whole-genome information [12–14]. In this study, *S10*-GERMS was applied to the taxonomic classification of the *L. casei* group. The ability of *S10*-GERMS was evaluated in comparison with results previously reported using DNA-based methods.

Table 1
Strains used in this study.

Species name[a]	Strains[b,c]	Ribotype[d]
L. casei	ATCC 334	C1
	JCM 1134^T (=ATCC 393^T = DSM 2011^T = NBRC 15883^T)	C5
	JCM 8129	C5
	JCM 11302[e] (=ATCC 15820 = DSM 20178 = KCTC 3804)	C4
	JCM 20024 (=IAM 1045)	–
	JCM 20304 (=IAM 10062)	–
(*L. acidophilus*)[f]	JCM 20315 (=IAM 10074)	–
L. paracasei subsp. *paracasei*	JCM 1053	C1
	JCM 1109	C1
	JCM 1111	C1
	JCM 1133 (=ATCC 27216 = DSM 20020)	C1
	JCM 1161 (=DSM 20207)	C1
	JCM 1163 (=DSM 20006)	C1
	JCM 1172 (=DSM 20012)	C3
	JCM 1181 (=ATCC 25598 = DSM 20008)	C1
	JCM 1556 (=ATCC 335)	C1
	JCM 2123 (=DSM 20356)	–
	JCM 2769	C1
	JCM 8130^T (=ATCC 25302^T = DSM 5622^T)	C1
	JCM 8131	C1
	JCM 8132 (=ATCC 11974)	C1
	JCM 8133	C1
L. paracasei subsp. *tolerans*	JCM 1171^T (=ATCC 25599^T = DSM 20258^T)	C3
L. rhamnosus	JCM 1136^T (=ATCC 7469^T = DSM 20021^T)	C2
	JCM 1165 (=ATCC 7469a = DSM 20022)	C2
	JCM 1553 (=ATCC 11443)	C2
	JCM 1561 (=ATCC 9595 = DSM 20245)	C2
	JCM 1563 (=ATCC 27773)	C2
	JCM 2771	C2
	JCM 2772	C2
	JCM 8134 (=ATCC 11981)	C2
	JCM 8135 (=ATCC 11982)	C2
	JCM 8136	C2

[a] Taxon names follow the proposal by Collins et al. [6].
[b] *Abbreviations*: JCM, Japan Collection of Microorganisms; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; IAM, IAM Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, The University of Tokyo; NBRC, National Institute of Technology and Evaluation (NITE) – Biological Resource Center; KCTC, Korean Collection for Type Cultures; ^{T}, type strain.
[c] Underlined strain names indicate genome-sequenced strains.
[d] Ribotypes reported in Ref. [25].
[e] Former proposed type strain of '*L. zeae*'.
[f] Proposed to be re-identified as *L. casei* based on the results of reactions with species-specific primers [37].

Materials and methods

Strains and growth conditions

Thirty-two strains of the *L. casei* group, listed in Table 1, were obtained from the Japan Collection of Microorganisms at RIKEN (JCM, Wako, Saitama, Japan) and the American Type Culture Collection (ATCC, Rockville, MD). Since most of the sample strains were the same as those used in a previous report, obtained by ribotyping [25], the ribotype of each strain is also indicated in Table 1. Each bacterial strain was grown aerobically in the medium and at the temperature recommended by its suppliers. In this paper, the taxon name of the *L. casei* group refers to the proposal by Collins