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Diversity of *Lactobacillus sakei* strains investigated by phenotypic and genotypic methods

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Abstract

The diversity of 10 strains of *Lactobacillus sakei*, a commercially important species of lactobacilli, was characterized by studying food isolates. Growth characteristics varied among the strains when examined after growth in a complex medium and a defined medium with either glucose or ribose. A commercial starter culture strain showed the fastest growth rates and high biomass formation on all media, while two of the strains hardly grew on ribose. Based on acidification properties in a meat model, some of the strains had the ability to compete with the indigenous microbiota of the meat batter in addition to being fast acid producers. Carbohydrate-fermentation abilities revealed a relatively wide variation, clustering the strains into two phenotypic groups. The isolates were analyzed using different genetic fingerprinting techniques, demonstrating a distinction between two genetic groups, a grouping consistent with previous studies dealing with *L. sakei* strains. Comparative genome hybridization (CGH) was introduced for clustering the strains and the same division into two genetic groups was observed. Chromosomal sizes of the strains were estimated by pulsed field gel electrophoresis (PFGE) and were found to vary from 1884 to 2175 kb. The genetic groups did not correlate with the clustering obtained with carbohydrate-fermenting abilities or with chromosomal sizes.

Keywords: *Lactobacillus sakei*; Meat; Fermentation; Diversity; AFLP; RAPD; CGH; PFGE

Introduction

Lactobacillus sakei is a member of the lactic acid bacteria (LAB) group. It differs from many LAB in being tolerant to various adverse conditions such as low temperature, high salt concentrations and varying oxygen levels [12,13]. Although the organism can be isolated from diverse habitats, such as plant fermentations [52] and fermented fish products [32,36], its major habitat is meat [24]. The bacterium is one of the commercially important species of lactobacilli often used in starter cultures for industrial production of fermented sausage [22,23], and it has a great potential as a biopreservative culture in meat and fish products [10,11,26,51]. The primary metabolism, essentially glycolysis, resulting in production of lactic acid is an important property both in a starter and a protective culture. The acidification caused by lactic acid contributes to food preservation and affects the quality of

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the product [3,22,31]. Many strains also produce different antibacterial substances including bacteriocins, which contribute to biopreservation [5,17,38]. Recently, the complete genome sequence of the plasmid-cured sausage isolate *L. sakei* 23K [9] was published, and the 1.88-Mb chromosome encoding 1883 predicted genes was explored, revealing a specialised metabolic repertoire that reflect the adaptation to meat products [12].

Early classification of species of *Lactobacillus* relied on phenotypic properties, and for *L. sakei*, phenotypic differentiation between strains and other species was mostly based on the type of lactic acid isomer produced, the type of sugar fermentation pattern and whether ammonia was produced from arginine. In particular, strains of *L. curvatus* and *L. sakei* might be difficult to differentiate [8,13,29,48]. Studies based on biochemical and physiological features have reported a wide phenotypic heterogeneity within strains of *L. sakei*. Phenotypic techniques such as the API 50 CH assay, where fermentation of 49 carbohydrates is tested, has often been used as a tool for discriminating between species of *Lactobacillus*, though the use of genetic methods has been shown to be more reliable [4,39,40]. High-resolution genotypic techniques such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are often used to generate species-specific electrophoretic profiles when investigating genetic diversity between *Lactobacillus* species [7,8,40,48,49]. On the basis of phenotypic and genetic properties, *L. sakei* has been divided into two sub-groups based mainly on results from numerical analyses of whole-cell protein and RAPD patterns [8,29,48]. The sub-groups are in several publications described as sub-species: *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus* [13,30,48]. Analyses of *Eco*RI and *Hind*III ribotypes and 16S rRNA genes, on the other hand, did not allow a clear separation between sub-groups of *L. sakei* [30]. Identification of *Lactobacillus* species is an important step in the development of new and interesting cultures, but also the identification at an intraspecies level may be important. Although more accurate than other typing techniques, the RAPD and AFLP provide limited information about the complete genome. Previous DNA–DNA hybridization experiments have revealed close relatedness between *L. sakei* strains at the genomic level ($\geqslant$75% identity) [29,48], but only after the completion of a genome sequence is it possible to perform genome-wide comparisons of strains within the species. Microarray-based comparative genome hybridization (CGH), also named genomotyping, is a powerful tool for estimating whole-genomic diversity [15,18]. By allowing hybridization of genomic DNA from a test strain to a microarray representing the 23K genome, a measurement of the similarity and divergence between two closely related *L. sakei* strains can be obtained.

The purpose of this work was to study the diversity of *L. sakei*, analyzing 10 strains of different origins from meat and fermented meat products, sake and fermented fish. Some of the strains are commercially used starter or protective cultures, and some are bacteriocin producers. Based on growth characteristics, pH- and fermentation profiles, we investigated phenotypic variation between the strains. We compared different genetic fingerprint analyses and determined the sizes of the chromosomes by pulsed field gel electrophoresis (PFGE), CGH using an open-reading frame (ORF)-specific *L. sakei* microarray based on the sequenced strain 23K was introduced for clustering the strains, and the clustering from the molecular techniques was compared. This work provides new insights into the diversity among isolates of the *L. sakei* species, and we introduce the application of AFLP and microarray-based CGH for the separation of the species into two genetic groups.

Materials and methods

Bacterial strains, media and growth

The bacterial strains used in this study are listed in Table 1. Strains were maintained at −80 °C in MRS broth (Oxoid, Hampshire, UK) supplemented with 20% glycerol. Cells were cultivated in Honeycomb microplates (Labsystems, Helsinki, Finland) in wells with 400 μl medium at 30 °C in a Bioscreen C (Labsystems). Growth was monitored by optical density at 600 nm (OD_{600}) every 30 min, after 10 s agitation, for 70 h. Cells from overnight cultures grown in the complex medium MRS were washed twice with sterile physiological saline (0.9% sodium chloride) before inoculation to OD_{600} 0.05 in MRS or in the completely defined medium DML [37], supplemented with either 0.5% glucose (DMLG) or 0.5% ribose (DMLR). All conditions were examined in triplicate and performed twice. The average maxOD_{600} from the two independent experiments was calculated. The average specific growth rate (μ) from the two experiments was calculated for the interval OD_{600} 0.2–0.4.

Acidification properties in a meat model

A recipe for traditional Norwegian salami was used to prepare a meat batter containing (% w/w) beef (37.85), pork (37.85), pork back fat (20.00), sodium chloride (3.19), sodium nitrite (0.0096), dextrose (0.70), spices (0.30) and ascorbic acid (0.05). The *L. sakei* strains were added to 150 g aliquots of this batter at a concentration of 10^6 cfu g^{-1}, and pH was recorded as described by Hagen et al. [21]. Duplicate samples were incubated at 24 °C for 72 h. Mean and standard deviations were