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Evaluation of $(GTG)_5$-PCR for identification of *Enterococcus* spp.

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Abstract

A set of reference strains and a group of previously unidentified enterococci were analysed by rep-PCR with the $(GTG)_5$ primer to evaluate the discriminatory power and suitability of this method for typing and identification of enterococcal species. A total of 49 strains representing all validly described species were obtained from bacterial collections. For more extensive evaluation of this identification approach 112 well-defined and identified enterococci isolated from bryndza cheese were tested. The $(GTG)_5$-PCR fingerprinting assigned all strains into well-differentiated clusters representing individual species. Subsequently, a group including 44 unidentified enterococci isolated from surface waters was analysed to evaluate this method for identification of unknown isolates. Obtained band patterns allowed us to identify all the strains clearly to the species level. This study proved that rep-PCR with $(GTG)_5$ primer is a reliable and fast method for species identification of enterococci.

Keywords: *Enterococcus*; rep-PCR; $(GTG)_5$-PCR; Identification; Bryndza; Water

1. Introduction

Enterococci are commonly isolated from intestines of man and animals, from food and from the environment. They became a serious problem in human medicine because of their increasing antibiotic resistance and frequent association with human infections including nosocomial infections. On the other hand, they are a natural part of many dairy products and certain enterococcal strains are used as probiotics for their beneficial effect in the human digestive tract [1]. Nowadays there are 28 validly described species of the genus *Enterococcus*. Their identification in a routine laboratory using classical biochemical tests became difficult because of many variable tests revealed by individual species and even because of different biochemical profiles revealed by strains of the same species isolated from different sources [2].

Many phenotypic and molecular methods have been described for identification of enterococci [3], nevertheless, they are often laborious, time consuming and/or they require special equipment. The rep-PCR fingerprinting represents easy to perform method based on primers complementary to certain repetitive sequences dispersed in bacterial genomes [4]. They are frequently used in bacterial taxonomy and they have been successfully applied for reliable and fast identification of different bacterial groups e.g. lactobacilli [5], staphylococci [6], mycobacteria [7] or streptomycetes [8].

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Only a handful studies describing application of rep-PCR for characterization of enterococci have been published. Dunne and Wang [9] used rep-PCR with ERIC primers ERIC1R and ERIC2 to analyse relatedness of vancomycin-resistant *E. faecium* strains isolated from human clinical material in different hospitals. Similarly, Bedendo and Pignatari [10] used REP-PCR with REP1R and REP2 primers for typing of *E. faecium* clinical strains. The BOX-PCR with the BOXA1R primer was applied for typing of *E. faecium* VanA phenotype strains to study horizontal transmission from poultry to humans via the food chain [11]. Malathum et al. [12] used the BOX-PCR and REP-PCR for typing of *E. faecalis* strains.

Our study focused on evaluation of rep-PCR fingerprinting for identification of enterococci at the species level and its application using the $(GTG)_5$ primer as a fast identification method for screening of unidentified enterococcal strains.

2. Materials and methods

2.1. Bacterial strains

Three sets of enterococcal strains were included into this study. The first group contained 49 type and reference strains obtained from the BCCM/LMG bacteria collection (http://www.belspo.be/bccm/) and from the Czech Collection of Microorganisms (http://www.sci.muni.cz/ccm/) representing all hitherto validly described enterococcal species. The second group consisted of 112 enterococcal strains isolated from a traditional Slovakian soft ripened cheese bryndza produced from ewe milk. These strains representing 76 *E. faecium* strains, 25 *E. faecalis*, 10 *E. durans* and one *E. hirae* strain were previously well characterized and identified by biochemical tests, ITS-PCR as well as by $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ PCR assays as described by Drahovská et al. [13]. The third group was formed by 44 enterococcal strains with unclear taxonomic position isolated from surface waters during a routine analysis of water quality in the Czech Republic in the region of North Moravia as described previously [14]. For strain numbers included in this study see Fig. 1. The strains isolated from bryndza cheese were marked by the letter "B" and the water isolates by the letter "W". Representative strains were deposited in the Czech Collection of Microorganisms (CCM) and in the BCCM/LMG bacteria collection (LMG).

2.2. Repetitive sequence-based PCR (rep-PCR) fingerprinting

Isolation of the total genomic DNA, rep-PCR, horizontal agarose gel electrophoresis of the PCR products as well as analysis of the rep-PCR fingerprint profiles by BioNumerics v. 4.0 software and dendrogram construction was performed as described by Gevers et al. [5]. The REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′ -IIICGNCGNCATCNGGC-3′); ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′); BOX-A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) oligonucleotide primers were evaluated in this study. Optimal PCR program for each of the primer set used was described by Versalovic et al. [15].

2.3. Analysis of whole-cell protein profiles (SDS–PAGE)

Preparation of whole cell-protein extracts from the cells grown on MRS agar (Oxoid) at 37% in a 6% CO_2 atmosphere and polyacrylamide gel electrophoresis (SDS–PAGE) was done as described by Pot et al. [16]. Protein profiles obtained were compared to a database of the BCCM/LMG collection comprising representative strains from different environments covering all described enterococcal species and identified by GelComparII v. 4.0 software package (Applied Maths, Belgium).

3. Results and discussion

The initial screening with the REP, ERIC, BOX and $(GTG)_5$ primer sets was performed on a subset of 24 well-characterized type and reference enterococcal strains obtained from the LMG/BCCM bacteria collection to evaluate discriminatory power of each individual primer set. All four primers generated DNA fragments of size ranging from 200 to 5000 bp. ERIC-PCR yielded the lowest number of bands ranging from 0 to maximally 13, but most of the strains analysed showed less than five bands. REP-PCR and BOX-PCR fingerprinting revealed comparable fingerprints with average band numbers 8 and 13, respectively. The rep-PCR with the $(GTG)_5$ primer provided the most discriminative and complex fingerprints with band numbers ranging approximately from 15 to 20. Therefore, the $(GTG)_5$ primer was used for further evaluation.

Thereafter, these reference strains were retested to verify reproducibility of the $(GTG)_5$-PCR fingerprinting (data not shown). All strains gave repeatedly the same band patterns without qualitative differences due to missing bands although differences in band intensity of some fingerprints occurred. Identical results were obtained by Gevers et al. [5] who tested reproducibility of the $(GTG)_5$-PCR fingerprinting in their study dealing with identification of lactobacilli. Although they occasionally found minor quantitative variations in band intensity none of the strains tested in their study showed qualitative differences in the fingerprint patterns.

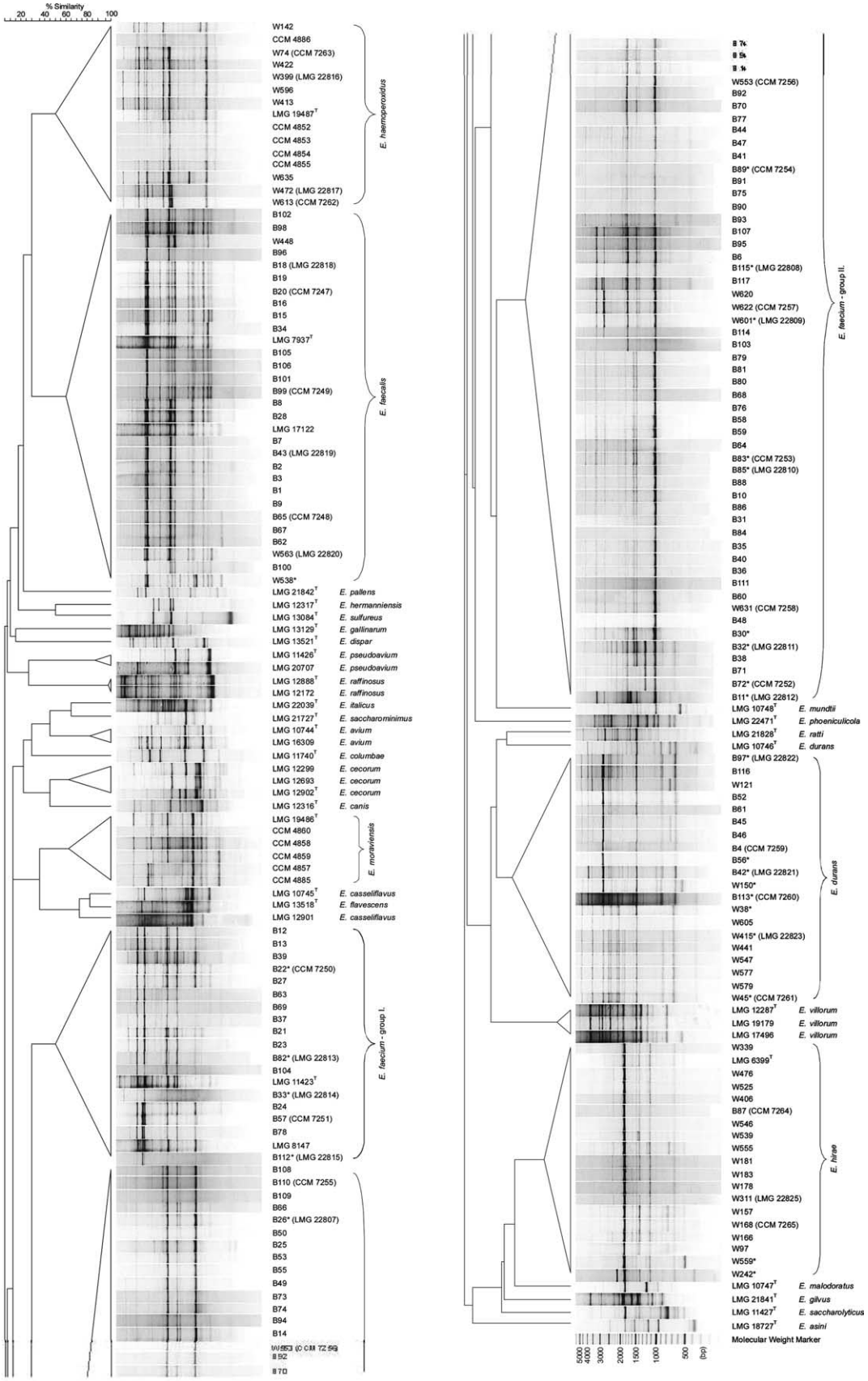

Fig. 1. Dendrogram based on cluster analysis of the $(GTG)_5$-PCR fingerprints. The letter "B" indicates strains isolated from bryndza cheese and the letter "W" the water isolates. The strains marked by asterisk were identified by whole-cell protein profile analysis (SDS–PAGE).

Similarly, a good reproducibility of rep-PCR fingerprinting with ERIC and RW3A primers was proved by Kang and Dunne [17]. They demonstrated high stability of fingerprints obtained from DNA isolated from 24, 48 and 72 h old bacterial cultures and from 5-, 10- and 15-times subcultured strains.

Subsequently, further 25 strains obtained from bacterial collections and a total of 112 previously well-identified strains isolated from bryndza cheese were characterized by $(GTG)_5$-PCR fingerprinting. All strains were grouped clearly into well-separated clusters representing single species (Fig. 1). The only minor difference was noted for *E. durans* strains. The *E. durans* type strain LMG 10746^T was slightly separated from the other reference *E. durans* strains. Visual examination of their band patterns showed typical basic band-pattern revealed by all *E. durans* strains including the type strain, but the type strain lacked (repeatedly) a strong 2600 bp long fragment affecting the cluster analysis. *E. faecium* strains were split into two groups in confirmation with previously published data. Similarly, Vancanneyt et al. [18] found two genomic *E. faecium* groups by AFLP and RAPD analyses. Although these two groups revealed in their study were clearly separated by both methods used further studies by DNA hybridisation analysis showed high DNA-DNA homology values confirming both groups as *E. faecium* species. The $(GTG)_5$-PCR fingerprints revealed by *E. casseliflavus* and *E. flavescens* representatives were nearly identical and confirmed generally accepted synonymy of these two species documented previously in numerous studies [e.g. 19,20].

A group of 44 unidentified enterococci isolated from water were included in this study in order to evaluate this method for identification of unknown isolates. Seventeen strains were separated into a cluster representing *E. hirae*, 10 strains matched *E. durans*; nine strains *E. haemoperoxidus*, five clustered with *E. faecium* and three showed fingerprints similar to *E. faecalis* reference strains (see Fig. 1). Band patterns revealed by these environmental strains generally matched well with the fingerprints obtained from reference as well as from dairy strains and allowed us to assign them clearly at the species level.

Whole-cell protein profile analysis (SDS–PAGE) was performed on selected strains (see the strains marked by asterisk in the Fig. 1) representing different sub-clusters or revealing slightly aberrant band patterns to verify their taxonomic position. Identification obtained by SDS–PAGE was in all cases in full agreement with $(GTG)_5$-PCR results.

Application of rep-PCRs for typing and characterization of single enterococcal species at the subspecies level has been described in a few studies as discussed above. Nevertheless, its using for identification purposes has been described meagrely in the literature. Usefulness of rep-PCR with RW3A primer for identification of *E. faecalis* and *E. faecium* and for their differentiation from other enterococci was shown by Namdari and DelVecchio [21]. Callon et al. [22] used rep-PCR with Rep-1R-Dt and REP2-D for typing of lactic acid bacteria from Salers cheese. They identified *E. durans*, *E. faecalis* and *E. faecium* using this method and clearly differentiated these enterococcal species from other LAB species isolated from cheese samples. Although these two studies showed rep-PCR as a suitable method for identification of enterococci they did not cover all validly described species of the genus *Enterococcus*.

This study, based on an extended and diverse selection of enterococcal reference strains and field isolates covering all currently described enterococcal species, showed that $(GTG)_5$-PCR fingerprinting is a suitable, reliable and fast identification tool for enterococci. All species constitute separate entities. Additional information is provided at the intra-species level as some species constitute different clusters. In general, the taxonomic resolution at the strain-level was limited as strains from different ecological niches and geographic locations revealed analogous $(GTG)_5$-PCR fingerprint patterns.

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