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Short communication

Differentiation of mixed lactic acid bacteria communities in beverage fermentations using targeted terminal restriction fragment length polymorphism

Nicholas A. Bokulich [a,b], David A. Mills [a,b,*]

[a] Department of Viticulture & Enology, University of California, One Shields Ave., Davis, CA 95616-8749, USA
[b] Department Food Science & Technology, University of California, One Shields Ave., Davis, CA 95616-8749, USA

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ABSTRACT

Lactic acid bacteria (LAB) are an important group of bacteria in beer and wine fermentations both as beneficial organisms and as spoilage agents. However, sensitive, rapid, culture-independent methods for identification and community analyses of LAB in mixed-culture fermentations are limited. We developed a terminal restriction fragment length polymorphism (TRFLP)-based assay for the detection and identification of lactic acid bacteria and *Bacilli* during wine, beer, and food fermentations. This technique can sensitively discriminate most species of *Lactobacillales*, and most genera of *Bacillales*, in mixed culture, as indicated by both bioinformatic predictions and empirical observations. This method was tested on a range of beer and wine fermentations containing mixed LAB communities, demonstrating the efficacy of this technique for discriminating LAB in mixed culture.

1. Introduction

Lactic acid bacteria (LAB) play a dual role in wine and some beer fermentations, both as beneficial microbiota and as nefarious spoilage agents (Boulton et al., 1996). Sensitive methods are required to detect and differentiate species of LAB, given the multiple points of entry and diverse cast of species associated with wine/beer fermentations. This is to detect species with spoilage potential before spoilage occurs, differentiating them from beneficial or benign species, and to track fermentation progress, ensuring that it is being conducted by desired (or inoculated) species. While traditional, culture-based methods remain the norm for identifying LAB, these methods are prone to select for the most dominant and/or competitive organisms present, presenting an incomplete picture of community structure (Heard and Fleet, 1986). In addition, the development of viable-but-not-culturable (VBNC) cells, which remain alive and metabolically active but incapable of growth on detection media, can occur under the low-pH, high-ethanol, low-nutrient conditions of wine fermentation (Millet and Lonvaud-Funel, 2000), further hampering culture-dependent detection of potentially detrimental bacteria. Thus, culture-independent, molecular methods have been developed for the detection of LAB in wine, including real-time PCR (qPCR; Neeley et al., 2005), fluorescent in situ hybridization (FISH; Blasco et al., 2003), and denaturing gradient gel electrophoresis (DGGE; Lopez et al., 2003). While qPCR enables sensitive enumeration of wine bacteria, and FISH direct visualization of specific bacterial taxa in wine using targeted fluorescent probes, both methods are targeted toward either broad taxonomic groups (e.g., lactobacilli) or specific species (e.g., *Lactobacillus brevis*) and thus lack the ability to be both comprehensive and simultaneously resolve the community present. DGGE, on the other hand, is a community profiling technique that meets both of these goals, but is technically challenging and poorly adapted for high-throughput and routine analyses.

Terminal restriction fragment length polymorphism (TRFLP) is a high-throughput, culture-independent method for community profiling originally developed for characterizing highly diverse bacterial communities (Liu et al., 1997). This method almost always employs universal primers targeting different regions of the 16S small subunit ribosomal RNA (rRNA) of prokaryotes (Liu et al., 1997) or rRNA internal transcribed spacer (ITS) in fungi (Bokulich et al.,

Abbreviations: LAB, Lactic acid bacteria; TRFLP, Terminal restriction fragment length polymorphism; TRF, Terminal restriction fragment; OTU, Operational taxonomic unit; LAB-TRFLP, TRFLP using LAB-specific primers described herein; 16S-TRFLP, TRFLP using universal bacterial 16S primers.

* Corresponding author. Department of Viticulture & Enology, University of California, One Shields Ave., Davis, CA 95616-8749, USA. Tel.: +1 530 754 7821; fax: +1 530 752 0382.
E-mail address: damills@ucdavis.edu (D.A. Mills).

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2012). TRFLP is a useful technique for profiling fermentation microbiota as it is adaptable, high-throughput, sensitive, and easy, enabling comparison of large, time- and treatment-based sample sets but is also inexpensive and rapid enough to be applied to diagnostic testing scenarios. However, as taxonomic assignment is inferred from restriction fragmentation and comparison to a database—as opposed to actual sequence data—observed populations are typically traced to operational taxonomic units (OTUs) representing the common phylogenetic category shared by all potential matches in the database. This limits the depth of taxonomic assignment achievable when using TRFLP with universal 16S primers, given the growing size of public sequence databases. The use of targeted primers, on the other hand, limits the number of potential hits in the database, increasing resolution within the targeted population without resorting to assumptions to eliminate "unlikely" hits.

Here we describe the development of a TRFLP assay (LAB-TRFLP) for the detection and differentiation of species of LAB and *Bacilli* in wine and beer using targeted primers. We designed a primer set with broad specificity for the *Lactobacillales* family and the closely related *Bacillales* targeting the 16S rRNA gene, and compared empirical TRFLP data to *in silico* predictions of LAB and *Bacilli* terminal restriction fragments (TRFs), demonstrating the discriminatory power afforded by this technique. This technique was tested on a range of wine and beer fermentations to demonstrate the level of resolution achieved using this technique.

2. Methods

2.1. Strains/sample collection

All strains used in this study were obtained from the UC Davis Viticulture and Enology Culture Collection and are presented in Table 1. Samples of wine (spoiled, bottled samples), beer (finished fermentations in barrels), and malt extract were sampled aseptically, transported refrigerated, and processed immediately.

2.2. DNA extraction

Samples were processed according to the modified protocol of Martinez et al. (2010) for using the QIAamp DNA stool kit (Qiagen, Valencia, CA). Briefly, samples were centrifuged at 4000 × g for 10 min and decanted. Of the resulting cell pellet, 100 μL were removed and washed 3 times by suspension in 1 mL ice-cold PBS, centrifugation at 8000 × *g* (5 min), and the supernatant discarded. The pellet was then suspended in 200 μL DNeasy lysis buffer (20 mM Tris-Cl [pH 8.0], 2 mM Sodium EDTA, 1.2% Triton X-100) supplemented with 40 mg/mL lysozyme and incubated at 37 °C for 30 min. From this point, the extraction proceeded following the protocol of the QIAamp DNA stool mini kit protocol (Qiagen), with the addition of a bead beater cell lysis step of 2 min at maximum speed following addition of "buffer ASL" using a FastPrep-24 bead beater (MP Bio, Solon, OH). DNA extracts were stored at −20 °C until further analysis.

2.3. Primer design

The forward primer, NLAB2F (5′-[HEX] GGCGGCGTGCCTAATACATGCAAGT), was designed targeting a consensus region (positions 502899-502925 in *Lactobacillus plantarum* WCFS1, GenBank AL935263.2) of the 16S rRNA gene from 45 *Lactobacillales*, *Bacillales,* and *Acetobacteriaceae* (as a non-target alignment) chosen from the Ribosomal Database Project (RDP) Release 10 (Cole et al., 2007, 2009) and aligned using ClustalX 2.0 (Larkin et al., 2007). The reverse primer (WLAB1R) chosen was the reverse homolog of WLAB1 (5′-TCGCTTTACGCCCAATAAATCCGGA-3′), previously designed for detecting LAB using DGGE (Lopez et al., 2003). These primers were chosen to target the V1-V3 domains of the 16S rDNA. Primer specificity and taxonomic coverage were predicted using PrimerProspector (Walters et al., 2011) checking against a representative subset of the Greengenes 16S rRNA database filtered at 97% identity (DeSantis et al., 2006) and the ARB-SILVA Ref 106 filtered 18S database (Pruesse et al., 2007).

Table 1
TRFLP Reference Table for Strains Used in This Study.

Species[a]	#	Source	MseI		Hpy118I		Hpy118III		HhaI	
			P[b]	O	P	O	P	O	P	O
Oenococcus oeni PSU-1	21	Wine	563	563	448	445	528	525	563	561
Lactobacillus plantarum B-10	1	Unknown	429	426	180	176	59	54	565	564
Lactobacillus sakei NJ2	126	Stool, breast-fed infant	571	568	380	376	61	58	571	567
Lactobacillus kunkeei (T) YH-15	26	Stuck wine fermentation	436	433	79	74	572	546	232	229
Lactobacillus hilgardii G45-2	10	Wine, California	63	59	382	378	473	470	573	572
Lactobacillus delbrueckii subsp. b*ulgaricus*	246	Mash, sour grain	561	558	370	364	561	533	224	221
Lactobacillus casei MGC11-6	4	Wine	117	112	375	370	566	538	566	562
Lactobacillus brevis I23	276	Wine must, California	64	59	374	369	211	201	565	558
Pediococcus damnosus Bpe238	13	Wine, California	197	192	386	381	223	219	577	573
Pediococcus ethanolidurans IMAU80017	244	Wine	40	192	386	382	223	220	577	573
Pediococcus pentosaceus ATCC 25745	222	Plants	40	35	384	380	221	217	575	571
Pediococcus parvulus Bpe301	253	Wine	197	192	386	382	223	221	577	574
Leuconostoc mesenteroides (T) ATCC 8293	14	Olives	170	165	548	545	513	508	179	174
Lactococcus lactis 04	172	Unknown	150	148	357	354	517	511	548	547
Streptococcus thermophilus G49-5	240	Unknown	548	548	392	390	416	414	548	546
Weissella confusa	238	Sugar cane	197	192	65	59	576	551	576	573
Bacillus cereus	683	Wine	38	33	358	353	550	523	210	209
Bacillus megaterium	30	Wine, red, Bulgaria	549	548	358	354	118	113	553	548
Bacillus simplex	346	Wine	547	543	356	351	116	111	212	207
Bacillus acidicola	349	Soil	550	547	358	354	550	523	550	548
Bacillus ginsengihumi	309	Wine	550	546	358	355	550	523	249	209
Lysinibacillus sphaericus	334	Wine	549	545	170	167	157	154	208	209
Paenibacillus barcinonensis	335	Wine	551	545	56	49	551	548	150	155
Paenibacillus humicus	361	Wine, Chile	543	539	352	348	44	39	210	203
Staphylococcus pasteuri	339	Wine	548	543	393	388	118	113	210	205

[a] Also tested with no amplification: *Gluconobacter oxydans* (UCD 131), *Acetobacter aceti* (UCD 114), *A. pasteurianus* (UCD 255), *A. malorum* (UCD 212), *Gluconoacetobacter hansenii* (UCD 113), *Saccharomyces cerevisiae* (UCD 522), *Pichia membranifaciens* (UCD 7), *Candida zemplinina* (UCD 2097).
[b] P = predicted, O = observed.