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Phenotypic and genotypic diversity of *Lactobacillus buchneri* strains isolated from spoiled, fermented cucumber☆

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ABSTRACT

Lactobacillus buchneri is a Gram-positive, obligate heterofermentative, facultative anaerobe commonly affiliated with spoilage of food products. Notably, *L. buchneri* is able to metabolize lactic acid into acetic acid and 1,2-propanediol. Although beneficial to the silage industry, this metabolic capability is detrimental to preservation of cucumbers by fermentation. The objective of this study was to characterize isolates of *L. buchneri* purified from both industrial and experimental fermented cucumber after the onset of secondary fermentation. Genotypic and phenotypic characterization included 16S rRNA sequencing, DiversiLab® rep-PCR, colony morphology, API 50 CH carbohydrate analysis, and ability to degrade lactic acid in modified MRS and fermented cucumber media. Distinct groups of isolates were identified with differing colony morphologies that varied in color (translucent white to opaque yellow), diameter (1 mm–11 mm), and shape (umbonate, flat, circular or irregular). Growth rates in MRS revealed strain differences, and a wide spectrum of carbon source utilization was observed. Some strains were able to ferment as many as 21 of 49 tested carbon sources, including inulin, fucose, gentiobiose, lactose, mannitol, potassium ketogluconate, saccharose, raffinose, galactose, and xylose, while others metabolized as few as eight carbohydrates as the sole source of carbon. All isolates degraded lactic acid in both fermented cucumber medium and modified MRS, but exhibited differences in the rate and extent of lactate degradation. Isolates clustered into eight distinct groups based on rep-PCR fingerprints with 20 of 36 of the isolates exhibiting > 97% similarity. Although isolated from similar environmental niches, significant phenotypic and genotypic diversity was found among the *L. buchneri* cultures. A collection of unique *L. buchneri* strains was identified and characterized, providing the basis for further analysis of metabolic and genomic capabilities of this species to enable control of lactic acid degradation in fermented plant materials.

1. Introduction

Lactobacillus buchneri is a Gram-positive, facultative anaerobe that has been isolated from a wide range of food, feed, and bioprocessing environments due to its broad range of metabolic capabilities. Notably, *L. buchneri*'s ability to anaerobically metabolize lactic acid into acetic acid and 1,2-propanediol (Oude Elferink et al., 2001) makes this organism both detrimental to the cucumber pickling industry and suitable as an adjunct starter culture for the silage industry. In addition to fermented cucumber (Franco et al., 2012; Johanningsmeier et al., 2012) and silage (Cooke, 1995; Muck, 1996), *L. buchneri* has also been isolated from human intestines (Zeng et al., 2010), fermented sorghum product (Yousif et al., 2010), wine (de Orduña et al., 2001), beer wort (Sakamoto and Konings, 2003), tomato pulp (Hammes and Hertel, 2015) and spoiled Swiss cheese (Sumner et al., 1985). Considering the various ecological niches that *L. buchneri* strains occupy, diversity within the species is likely.

Pickling cucumbers are fermented in a sodium chloride (NaCl) solution in open-top, 10–40 kL vats where naturally occurring lactic acid bacteria, principally homolactic *Lactobacillus* species, convert sugars to lactic acid to preserve the fruits (Franco et al., 2016). Prior to processing, it is common for fermented cucumbers to be held in bulk storage for up to 1 year. During bulk storage, secondary fermentation may ensue, particularly when the salt concentration is low (Fleming et al., 1989; Johanningsmeier et al., 2012; Kim and Breidt, 2007). Spoilage-associated secondary cucumber fermentation occurs during bulk

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storage after residual carbohydrates have been depleted (Fleming et al., 1989; Franco et al., 2012, 2016). This particular spoilage is characterized by an increase in pH and decrease in lactic acid with a concomitant increase in acetic and propionic acids (Fleming et al., 1989; Franco et al., 2012; Johanningsmeier et al., 2012). In numerous studies, *L. buchneri* has been shown to initiate spoilage in fermented cucumber by degrading lactate to acetate and 1,2-propanediol under aerobic or anaerobic conditions, causing a rise in pH (Johanningsmeier et al., 2012; Johanningsmeier and McFeeters, 2013). As lactate is depleted and pH rises, spoilage occurs through a succession of microbial metabolic activity of less acid-tolerant species. *Propionibacterium* species, *Pectinatus sotaceto*, *Enterobacter cloacae*, *Clostridium tertium*, and *Clostridium bifermentans* produce additional propionic acid and butyric acid, creating undesirable off-odors and compromising food safety (Breidt et al., 2013; Caldwell et al., 2013; Fleming et al., 1989; Franco et al., 2012; Franco and Pérez-Díaz, 2012). Ultimately, this renders the final product unfit for consumption and leads to significant economic loss (Franco et al., 2012).

The unique metabolic activities of *L. buchneri* that make it a robust spoilage organism in foods also hold potential for industrial applications in food and feed technology. Strains of *L. buchneri* sourced from a multitude of environments have been proposed for a variety of industrial applications. One particular strain isolated from pickle juice was shown to have potential probiotic effects, such as the reduction of serum cholesterol levels (Zeng et al., 2010). This strain of *L. buchneri* produces a bile salt hydrolase, which catalyzes the hydrolysis of conjugated bile salts into amino acid residues and bile acids. A strain of *L. buchneri* isolated from sauerkraut produced bacteriocins targeting several Gram-positive bacteria, including species from *Listeria*, *Bacillus*, *Micrococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*, and *Pediococcus* (Yildirim and Yildirim, 2001; Yildirim et al., 2002). *L. buchneri* strain NRRL B-30929, originally isolated as a contaminant in a commercial ethanol facility, has since been exploited for bioethanol production and was the first of this species to have its genome fully sequenced and annotated (Liu et al., 2011). Anaerobic lactic acid utilization by *L. buchneri* in silage results in the production of acetic and propionic acids, which act as natural antimicrobials for the inhibition of aerobic spoilage yeasts and fungi that are often responsible for heating and degradation of dry matter in silage upon exposure to air. This has been demonstrated in various silage fodder, including grass (Driehuis et al., 2001), corn (Kleinschmit et al., 2005; Muck, 1996; Ranjit and Kung, 2000), barley (Kung and Ranjit, 2001; Taylor et al., 2002) and alfalfa (Kung et al., 2003). Relying on this same metabolic pathway, *L. buchneri* was used in concert with *Lactobacillus diolivorans* to naturally produce propionate in sourdough bread, extending product shelf life without added preservatives (Zhang et al., 2010). Despite common features observed in several cases, it is not yet clear whether the ability to metabolize lactic acid is strain-dependent.

To date, diversity within the *L. buchneri* species has not been fully explored. Understanding the genotypic and phenotypic differences among *L. buchneri* strains will facilitate the development of strategies to eliminate the onset of secondary cucumber fermentation by this species. Furthermore, this knowledge may also be exploited to develop improved starter culture adjuncts for the silage industry. The objective of this study was to characterize *L. buchneri* cultures isolated from spoiled, fermented cucumber to determine the phenotypic and genotypic diversity of this species from a single environmental niche.

2. Materials and methods

2.1. Bacterial strain cultivation

L. buchneri cultures isolated from spoiled, fermented cucumber (Franco and Pérez-Díaz, 2012; Johanningsmeier et al., 2012) and the type strain for *L. buchneri* (ATCC® 4005™, American Type Culture Collection, Manassas, VA, USA) were obtained from the USDA-ARS Food Science Research Unit Culture Collection (Raleigh, NC, USA). The sources of the isolates are described in Table 1. Species determination was confirmed using 16S rRNA sequencing. Frozen stocks were prepared in de Man-Rogosa-Sharpe (MRS) broth and 30% vol/vol glycerol, and stored at −80 °C. Prior to experiments, isolates were streaked onto MRS agar and incubated at 30 °C under anoxic conditions for 4–5 days followed by anaerobic propagation in MRS broth for 2–3 days at 30 °C.

2.2. 16S rRNA sequencing

All isolates were subjected to 16S rRNA sequencing for species verification prior to characterization studies. Genomic DNA was isolated from broth cultures of each of the 36 isolates using a DNA purification kit (Ultra-Clean Microbial DNA Isolation Kit, MoBio laboratories, Carlsbad, CA, USA). Polymerase Chain Reaction (PCR) was conducted to amplify a 500 bp hypervariable region with 16S rRNA primers. The primers used in this study were plb16 5′ AGAGTTTGAT CCTGGCTCAG 3′ and mlb16 5′ GGCTGCTGGCACGTAGTTAG 3′ (Kullen et al., 2000). DNA amplification was performed in a BioRad MyCycler Thermocycler programmed for 5 min at 94 °C (initial denaturation), and 30 cycles of 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing), 40 s at 72 °C (extension) and 7 min 72°C (final extension). Amplicons were visualized with ethidium bromide in a 1% agarose gel and sequenced using Eton Bioscience Inc. (Durham, NC, USA). The NCBI Basic Local Alignment Search Tool was used to identify the bacterial cultures. All isolates were confirmed to be *L. buchneri* based on alignment matches with ≥99.7% identity.

2.3. Colony and cellular morphology

Each of the isolates was streaked onto MRS agar, and incubated anaerobically for 4 days at 30 °C. Colony morphology was evaluated based on diameter, pigmentation, form, elevation, margin, texture and opacity of each colony for all isolates of *L. buchneri* using the method found in the Manual of Microbiological Methods (Pelczar Jr., 1957). Isolates were further propagated anaerobically in MRS broth for 36 h at 30 °C prior to cellular morphology observation. Cellular morphology was visualized using a Nikon Eclipse E600 phase contrast microscope with a Q-Imaging Micropublisher Camera attachment (Nikon, Japan). Isolates that represented a range of distinct colony morphologies and differed in DiversiLab® genotype fingerprints were selected for detailed photographs (Fig. 1).

2.4. Growth curves in De Man Rogosa and Sharpe (MRS) broth

Growth curves were generated by measuring optical density (OD_{600nm}) of cultures grown in standard MRS medium for 64 h using a microtiter plate reader (Spiral Biotech QCount; Advanced Instruments, Inc.; MA, USA). A 5 µL aliquot of a 10^8 CFU/mL culture was inoculated into a single well of 245 µL of sterile MRS broth for each of the 36 isolates and incubated at 30 °C under modified anaerobic conditions using a mineral oil overlay. Readings were taken every 30 min after a 5-s vibrational shake of the microtiter plate. This experiment was independently conducted in duplicate. Lag times, growth rates, and max OD were calculated using a published algorithm (Breidt et al., 1994). Isolates that varied in DiversiLab® genotype and colony morphology were selected for presentation.

2.5. Carbohydrate fermentation profiles

The ability to ferment a variety of carbon sources was determined by the API 50 CHL method per the manufacturer's guidelines (bioMerieux, Marcy l'Etoile, France). Each of the 36 cultures of *L. buchneri* was cultivated in standard MRS broth under anaerobic conditions at 30 °C for 4 days prior to inoculation.