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Characterization of *Bacillus megaterium*, *Bacillus pumilus*, and *Paenibacillus polymyxa* isolated from a Pinot noir wine from Western Washington State

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ABSTRACT

This report provides the first confirmed evidence of *Bacillus*-like bacteria present in a wine from Washington State. These bacteria were isolated from a 2013 Pinot noir wine whose aroma was sensorially described as being 'dirty' or 'pond scum.' Based on physiological traits and genetic sequencing, three bacterial isolates were identified as *Bacillus megaterium* (strain NHO-1), *Bacillus pumilus* (strain NHO-2), and *Paenibacillus polymyxa* (strain NHO-3). These bacteria grew in synthetic media of low pH (pH 3.5) while some survived ethanol concentrations up to 15% v/v. However, none tolerated molecular SO_2 concentrations $\geq$0.4 mg/l. Growth of strains NHO-1 and NHO-3 in a Merlot grape juice resulted in increases of titratable and volatile acidities while decreases in titratable acidity were noted for NHO-2.

1. Introduction

Bacillus spp. represent a wide array of bacteria which have been isolated from a variety of sources. Though commonly present in soils or added as biological fertilizers (Ling et al., 2011; Ahmad et al., 2012; Hu et al., 2013), a few reports have described isolation from grape vines, juice, wine, and or packaging materials (Gini and Vaughn, 1962; Murrell and Rankine, 1979; Alvarez-Rodriguez et al., 2003; Bae et al., 2004; Salunkhe et al., 2013). Given the scarcity of isolations, specific impacts on wine quality are largely unknown. In fact, Boulton et al. (1996) described isolating unidentified *Bacillus* spp. from bottled wines where aroma/flavor characteristics were not apparently affected.

Bacterial resistance to intrinsic antimicrobial factors of wine such as low pH, high ethanol, and sulfites may allow *Bacillus* spp. to survive in wine (Vaughn, 1955; Gini and Vaughn, 1962; Murrell and Rankine, 1979). To date, research has not evaluated the extent that these factors affect *Bacillus* spp. under vinification conditions. Thus, the objectives of this study were to identify and characterize bacterial isolates recently obtained from a spoiled red wine.

2. Materials and methods

2.1. Isolation and identification

Bottles of a 2013 Pinot noir wine (pH 3.46, 12.3% v/v ethanol, and 0.56 g/l titratable acidity) made from three grape clones (Precoce, 667, and 777) were obtained from Cloud Mountain Farm Center (Everson, WA). Harvested grapes exhibited bunch rot (*Botrytis*) and damage due to yellow jacket insects. At crush, 60–100 mg/l total SO_2 was added to all musts. Each grape clone was separately fermented, with Precoce and 667 fermented by *Saccharomyces cerevisiae* strain Bourgovin RC 212 and 777 by strain Assmanshausen (Danstar Ferment AG, Fredericia, Denmark). Following malolactic fermentation, the wines were blended with the concentration of free SO_2 adjusted to 25 mg/L prior to bottling without filtration.

As bottle spoilage became evident during storage, infected wines were first filtered through 0.45 μm membranes (Fisher Scientific, Waltham, MA) to collect representative microbes. Membranes were transferred onto WL agar (Fisher Scientific) for

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incubation at 25 °C for 48 h. Representative colonies were then streaked for isolation using the same medium before being characterized by microscopic evaluation of cells as well as Gram, capsule, and endospore staining (Anthony, 1931, Burdon and Williams, 1968; Schaeffer and Fulton, 1993). Based on preliminary evaluation, biochemical analyses were conducted using the Microgen™ *Bacillus* ID kit (Microbiology International, Frederick, MD) while identities were confirmed by 16S rRNA sequencing (MIDI Labs, Newark, NJ).

2.2. Culture maintenance

Bacterial strains were maintained by streaking onto fresh WL agar once a week and stored long-term in 30% w/w glycerol at −80 °C.

When needed, starter cultures were prepared by aseptically transferring strains to the nutrient medium described by Kurtzman et al. (2011). All tubes were incubated at 25 °C with turbidity assessed using a Klett-Summerson photoelectric colorimeter equipped with a green filter (Klett Manufacturing Co., New York, NY). Upon reaching a turbidity associated with approximately 10^7 cfu/ml, cultures were harvested by centrifugation at 3000 × *g* for 20 min. Pellets were washed 3× with 10 ml 0.2 M phosphate buffer (pH 7.0) made up of 9.36 g/l potassium monobasic, anhydrous and 32.73 g/l sodium phosphate dibasic heptahydrate (Fisher Scientific). After the final wash, cells were resuspended in 0.2 M phosphate buffer (pH 7.0) and used as starter cultures to inoculate media or grape juice.

2.3. Tolerances to pH, ethanol, and SO_2

The medium described by Kurtzman et al. (2011) was prepared using chemicals obtained from Sigma-Aldrich (St. Louis, MO). Here, the base medium consisted of ammonium sulfate (5 g/l), dextrose (10 g/l), L-histidine monohydrochloride (10 mg/l), DL-methionine (20 mg/l), DL-tryptophan (20 mg/l), H_3BO_3 (500 μg/l), $CuSO_4$ (40 μg/l), KI (100 μg/l), $FeCl_3$ (200 μg/l), $MnSO_4$ (400 μg/l), Na_2MoO_4 (200 μg/l), $ZnSO_4$ (400 μg/l), KH_2PO_4 (0.85 g/l), K_2HPO_4 (0.15 g/l), $MgSO_4$ (0.5 g/l), NaCl (0.1 g/l), and $CaCl_2$ (0.1 g/l). A vitamin solution containing biotin (2 μg/l), calcium pantothenate (400 μg/l), folic acid (2 μg/l), *myo*-inositol (2 mg/l), nicotinic acid (400 μg/l), *p*-aminobenzoic acid (200 μg/l), pyridoxine hydrochloride (400 μg/l), riboflavin (200 μg/l), and thiamin (1 mg/l) was sterile-filtered (0.22 μm) before aseptic addition to the base medium.

The base medium was adjusted to different pH values using 1 N HCl for characterization to pH (pH 3.5, 4.0, 4.5 and 5.0), ethanol (pH 5.0) or SO_2 (pH 3.7). For pH and ethanol studies, media were transferred into 15 × 150 mm Klett tubes (4.2 ml), fitted with Identi-Plug foam inserts (Jaece Industries, North Tonawanda, NY), a plastic dilution blank cap, and autoclaved. Upon cooling, the vitamin solution (0.8 ml) was added to all tubes. A variable mixture of ethanol:water (1 ml) was aseptically added to yield ethanol concentrations of 0, 5, 10 and 15% v/v. For SO_2 studies, enough potassium metabisulfite was added to a liter of autoclaved media to yield 0, 0.2, 0.4, 0.6, or 0.8 mg/l molecular SO_2 with concentrations of free SO_2 verified by aeration-oxidation (Buechsenstein and Ough, 1978). After addition of SO_2 and the vitamin solution, media were sterile-filtered (0.45 μm) and distributed, in triplicate, into autoclaved 15 × 150 mm Klett tubes (5 ml) as described previously. All tubes were incubated at 25 °C while viability was evaluated by the spread plate method using WL agar (Fugelsang and Edwards, 2007).

2.4. Growth in grape juice

Grape juice was reconstituted using Merlot grape juice concentrate (California Concentrate Co., Acampo, CA) according to manufacturer's instructions. Equal ratios of glucose:fructose (80 g/l) and malic:tartaric (2 g/l) were added to yield a juice of 19 °Brix and 6.0 g/L titratable acidity (final pH = 3.5). Yeast extract (0.5 g/l) was also added while sulfites were removed by addition of 3% w/v H_2O_2 as confirmed by aeration-oxidation method. Grape juice was sterile-filtered (0.45 μm) into autoclaved one liter bottles fitted with fermentation locks prior to inoculation (10^5 cfu/ml) and incubation at 25 °C. All fermentations were replicated in triplicate with populations periodically determined by the spread plate method using WL agar (Fugelsang and Edwards, 2007).

2.5. Volatile analysis of 2013 Pinot noir wine

Bottled wines were analyzed using a modification of the methods of Lu et al. (2003). For the headspace solid phase microextraction, 2 ml of standards or wine were added to a 4.0 ml vial (Supelco, Bellefonte, PA) containing 30% w/v NaCl. A fused silica fiber coated with 65 μm polydimethylsiloxane/divinylbenzene (Supelco) was exposed to the sample for an hour with continuous sample mixing. A 6890/5973 GC/MSD (Agilent, Wilmington, DE) equipped with Chemstation C1024-A02 was used for analysis. Splitless injection was utilized for 2 min at 200 °C with a DB-1MS 0.32 mm × 60 m column (1.0 μm thickness; Phenomenex, Torrance, CA) under chromatographic conditions described by Mattheis et al. (1991) with the exception that the transfer line and ion source temperatures were held at 250 °C and 150 °C, respectively. The GC inlet contained a 0.75 mm SPME injection sleeve which insured sharpness for early eluting peaks (Yang and Peppard, 1994). Identifications were made using the Wiley/NIST library (Wiley 125 K) and later confirmed using commercial standards including 1-octen-3-one, 1-octen-3-ol, 2-isopropyl-3-methoxypyrazine, 4-ethylphenol,4-ethylguaiacol, 2,4,6-trichloroanisole, and geosmin (Sigma-Aldrich, St. Louis, MO).

3. Results and discussion

3.1. Identification

The three bacterial strains, NHO-1, NHO-2, and NHO-3, microscopically appeared as Gram (+) rods, straight or slightly curved, and occurred either as single cells or in chains. Colonies on WL agar appeared filamentous, pulvinate, and white-blue (NHO-1), irregular, flat, wrinkled, and gray-blue-green (NHO-2), or circular, umbonate, mucosal, and white-blue (NHO-3). All of the strains were catalase (+), formed endospores and capsules, did not utilize arginine, citrate, or indole, and reacted positively to Voges-Proskauer (Table 1). While some carbohydrates were not metabolized (arabinose, methyl-α-D-mannoside, or rhamnose), only xylose was metabolized by all three isolates. Utilization of other carbohydrates tested (cellobiose, galactose, inulin, mannitol, melizitose, raffinose, salicin, sorbitol, sucrose or trehalose) depended on the isolate.

Based on subsequent sequencing, the three isolates were identified as *Bacillus megaterium* (NHO-1), *Bacillus pumilus* (NHO-2), and *Paenibacillus polymyxa* (NHO-3) with genomic agreements of 99.9%, 100%, and 99.5%, respectively. While the highly conserved 16S rRNA sequence can reveal some variation between strains (Turenne et al., 2001), biochemical and physiological characterizations yielded matches of 97.0% (*B. megaterium*), 99.1% (*B. pumilus*), and 98.8% (*P. polymyxa*).

Although infrequently isolated from wines, this report represents the first observation of *Bacillus* or related species present in a wine from Washington State. One of the first to describe *Bacillus* spp. isolated from wine was Gini and Vaughn (1962) who described