Characterization of Cucumber Fermentation Spoilage Bacteria by Enrichment Culture and 16S rDNA Cloning

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Abstract: Commercial cucumber fermentations are typically carried out in 40000 L fermentation tanks. A secondary fermentation can occur after sugars are consumed that results in the formation of acetic, propionic, and butyric acids, concomitantly with the loss of lactic acid and an increase in pH. Spoilage fermentations can result in significant economic loss for industrial producers. The microbiota that result in spoilage remain incompletely defined. Previous studies have implicated yeasts, lactic acid bacteria, enterobacteriaceae, and *Clostridia* as having a role in spoilage fermentations. We report that *Propionibacterium* and *Pectinatus* isolates from cucumber fermentation spoilage converted lactic acid to propionic acid, increasing pH. The analysis of 16S rDNA cloning libraries confirmed and expanded the knowledge gained from previous studies using classical microbiological methods. Our data show that Gram-negative anaerobic bacteria supersede Gram-positive *Fermincutes* species after the pH rises from around 3.2 to pH 5, and propionic and butyric acids are produced. Characterization of the spoilage microbiota is an important first step in efforts to prevent cucumber fermentation spoilage.

Keywords: pickled vegetables, *Pectinatus*, *Propionibacteria*, secondary cucumber fermentation, spoilage

Practical Application: An understanding of the microorganisms that cause commercial cucumber fermentation spoilage may aid in developing methods to prevent the spoilage from occurring.

Introduction

Commercial cucumber fermentations are typically carried out in large 40000 L outdoor tanks (reviewed by Breidt and others 2007). The cucumbers are held under the surface of the brine by wooden headboards, and the brine surface is exposed to UV light from the sun, which helps to prevent yeast or mold growth. Cucumber fermentations are typically done in a 6% NaCl brine, and preservation is achieved by sugar utilization, pH reduction, and accumulation of lactic acid (typically, pH < 3.3 and approximately 1% lactic acid). However, spoilage-associated secondary fermentations have been observed in fermented cucumbers (Fleming and others 1989; Kim and Breidt 2007; Johanningsmeier and McFeeters 2011; Franco and others 2012).

The first research report of spoilage in fermented cucumbers was from an anaerobic cucumber fermentation tank (4500 L), with cucumbers fermented at 2.3% NaCl (Fleming and others 1989). In this fermentation tank, the initial lactic acid fermentation was completed within 2 wk, with 1.2% lactic acid formed (pH 3.6) and no detectable sugar remaining in the brine. The cucumbers in the fermentation brine were microbiologically stable for more than 8 mo, with no change in pH or organic acid concentrations. However, after 9 mo, a secondary fermentation occurred. Lactic acid was completely consumed, and acetic, propionic, and butyric acids were produced. During this spoilage fermentation, the pH rose above 4.6, resulting in a potential health hazard due to the possibility of *Clostridium botulinum* spore germination and growth. *C. butyricum* was isolated from spoilage samples and the ability to convert lactic acid to butyric acid was demonstrated (Fleming and others 1989). However, the organisms responsible for production of acetic and propionic acids were not identified.

Spoilage fermentations have also been documented in conventional 6% NaCl cucumber fermentations (Franco and others 2012). These fermentations were aerated during the primary fermentation to prevent the buildup of CO_2, which can cause destructive gas pockets (bloating) that form within the fruit during the primary fermentation. To prevent bloating, commercial fermentation tanks are typically purged with compressed air using a sparging system that circulates the brine (Humphries and Fleming 1988). The microbiota responsible for these spoilage fermentations were identified by classical microbiological methods. Isolates from spoilage fermentations included: *Pichia* and *Issatchenkia* yeast species, as well as 3 Gram-positive bacteria: *Lactobacillus buchneri, Clostridium* sp., and *Pediococcus ethanolidurans*; and 1 Gram-negative bacterium *Enterobacter cloacae* (Franco and others 2012; Franco and Perez-Diaz 2012). Previously, it has been shown that rumen and

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silage lactic acid bacteria can degrade lactic acid. Rumen microbiota have been shown to convert lactic acid to propionic acid (Baldwin and others 1962). Similarly, *Lactobacillus buchneri* has been shown to convert lactic acid to acetic acid and 1,2-propanediol under acidic conditions in silage (Driehuis and others 1999; Holzer and others 2003), fermented cucumbers (Johanningsmeier and others 2012), and microbiological media (Oude-Elferink and others 2001). Lindgren and others (1990) have shown that *Lactobacillus plantarum*, in the absence of fermentable sugar, can metabolize L-lactic acid to acetic and formic acids. Other microbiota have been implicated in the anaerobic conversion of lactic acid to other organic acids. In olive fermentations, *Propionibacterium* species and *Clostridium* species have been implicated as causative agents of Zapatera spoilage, converting lactic acid to propionic and butyric acids (Vaughn and Levin 1966; Fernandez and others 1995).

Classical methods for microbial ecology may often identify only a minority of the microbiota present in a given environment (Amann and others 1995; Hugenholtz and others 1998), indicating the need for culture-independent methods. One potential difficulty with nucleic-acid-based methods for the investigation of the spoilage microbiota in fermented cucumbers is interference in sequencing or cloning due to the presence of cell-free nucleic acids of *Lactobacilli* from the primary fermentation. Following fermentation, viable *Lactobacilli* decline in numbers by several orders of magnitude (Breidt and others 2007). It has been shown that propidium monoazide (PMA) pretreatment of samples prior to DNA isolation can be used to selectively amplify DNA from living cells (Nocker and others 2006; Pan and Breidt 2007). The objective of this study was to investigate the bacteria present during the spoilage fermentations, using both enrichment-culture- and nonculture-based methods to supplement the knowledge gained from existing-culture-based studies (Kim and Breidt 2007; Franco and others 2012; Franco and Pérez-Díaz 2012; Johanningsmeier and others 2012). We report here the microbial characterization of laboratory and commercial spoilage fermentation samples using a combination of enrichment and PMA cloning techniques.

Materials and Methods

Cucumber fermentation spoilage samples

Commercial cucumber fermentation spoilage samples for cloning experiments were obtained from 40000 L commercial cucumber fermentations (4 total) that exhibited spoilage characteristics at varying stages, as described below. In addition, laboratory spoilage samples were prepared by inoculating commercially brined cucumbers (6% NaCl, approximately 100 mM lactic acid, pH adjusted to pH 5) with brine from a commercial spoilage fermentation. Cucumbers and brine (50% cucumbers, 50% brine) were packed into 1.36 L pickle jars containing rubber septa (approximately 15 mm in diameter) inserted into the lids. Aliquots (1.0 mL) of commercial spoilage samples (as described below) were then inoculated through the septa and the jars stored at room temperature for up to 4 mo, along with uninoculated controls. Samples (1 mL) were removed through the septa using a 1 mL syringe for biochemical and microbiological analysis.

Culture handling and media

To prepare fermented cucumber slurry medium, size 2B cucumbers (3.5 to 3.8 cm in diameter) obtained from commercial sources were fermented in a 19 L (5 gallon) plastic pail with a 50/50 ratio of cucumbers and brine. The initial cover brine contained 60 mM calcium acetate (prior to equilibration with the cucumbers). *L. plantarum* MU45 (malolactic decarbolylase mutant, USDA Food Fermentation Culture collection, Raleigh, N.C., U.S.A.) was grown for 15 h at 30 °C in De Man, Rogosa and Sharp (MRS) broth (BD Diagnostic Systems, Sparks, Md., U.S.A.). The cucumber fermentation was inoculated immediately after brining with 20 mL of the overnight culture, to give an approximate initial inoculum of 1×10^6 CFU/mL in the brine. After 10 d of fermentation in the pail sealed with a lid containing a gasket, equal amounts of brine and cucumber pickles were removed and blended into slurry and frozen in 350 mL (12 oz) jars at −20 °C. Prior to use, the slurry was thawed, NaCl was added to 2%, the pH was adjusted to 5.0 as indicated, and fermented slurry medium (FSM) was autoclaved followed by refrigeration prior to use. A fermented slurry broth (FSB) prepared by centrifugation at 10000 × *g* in a Sorvall GSA rotor (Thermo Fisher Scientific Inc., Waltham, Mass., U.S.A.) for 10 min to remove particulate matter. The supernatant was then filtered through a 0.45 μm filter. Tryptic soy agar (TSA) and MRS agar (BD Diagnostic) were used for isolating spoilage organisms. Plates were incubated anaerobically at 30 °C for up to 4 d in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich., U.S.A.).

Biochemical analysis

Spoilage samples were centrifuged at 13000 × *g* in a microcentrifuge to remove any residual particulate matter. The pH was determined with an IQ240 pH meter (IQ Scientific Instruments, San Diego, Calif., U.S.A.). High-performance liquid chromatography (HPLC) analysis was used to determine organic acid and sugar concentrations using a modification of the method of McFeeters and Barish (2003). Organic acid concentrations were measured with a Thermo Separation Products HPLC (ThermoQuest, Inc., San Jose, Calif.) system consisting of a P2000 pump, an SCM100 solvent degasser, an AS3000 autosampler, and a UV6000 diode array detector (ThermoQuest). A Bio-Rad HPX-87H column, 300 × 7.8 mm (Bio-Rad Laboratories, Hercules, Calif.), was used to resolve malic, lactic, acetic, propionic, and butyric acids, as well as ethanol. The operating conditions of the system included a column temperature of 37 °C, and 0.03 N H_2SO_4 eluent at a 0.6 mL/min flow rate. The UV6000 detector was set to 210 nm at a rate of 1 Hz for data collection. ChromQuest version 4.1 chromatography software was used to control the system and analyze the data, utilizing the peak heights for quantitative integration. Sugars, glucose and fructose, were determined on the same system with a refractive index detector (Waters 410, Millipore, Milford, Mass.).

Isolation and characterization of spoilage bacteria

Enrichment of spoilage bacteria was carried out by serial passage of laboratory spoilage fermentation samples. One milliliter of brine from laboratory spoilage was inoculated into 9 mL of FSM. Samples were incubated at 30 °C for 2 to 3 wk. Once complete utilization of lactic acid was observed by HPLC analysis, 1 mL was transferred to 9 mL of fresh FSM medium. Samples were sequentially transferred by this method 5 times, plated on TSA and MRS, and bacterial colonies were randomly selected and streaked for isolation. Pure culture isolates were stored in tryptic soy broth (TSB, BD Diagnostic) containing 15% glycerol at −80 °C. Reproduction of spoilage fermentations with pure culture of the isolates

Table 1–Chemical characterization of 6 commercial spoilage fermentations.

Sample ID[a]	pH	Glucose[b]	Fructose	Lactic	Acetic	Propionic	Butyric	Ethanol
A	5.2	nd	nd	2	23	62	42	nd
B	4.1	nd	nd	56	33	50	nd	nd
C	4.9	nd	nd	nd	39	46	51	nd
D	4.9	nd	nd	nd	37	34	16	12
E	4.3	nd	nd	39	22	6	nd	18
F	3.2	nd	nd	91	32	13	nd	nd

[a]Brine samples from 6 commercial cucumber fermentations, following completion of the lactic acid fermentation.
[b]The concentration of all compounds is expressed in mM amounts, nd = not detected.

was carried out by inoculation of 50 mL FSB to an initial cell concentration of approximately 10^7 CFU/mL from a 2 d anaerobic TSB broth culture grown at 30 °C. Samples were incubated for up to 5 d at 30 °C in the anaerobic chamber prior to biochemical and microbiological analysis.

Molecular methods

For the 16S cloning experiments, commercial spoilage brine aliquots of 10 mL were centrifuged (Sorvall, Thermo Fisher Scientific Inc., Waltham) at 2000 × *g* for 10 min at 4 °C, washed in saline, and centrifuged as before. Pellets were resuspended in 490 μL sterile saline and treated with PMA to eliminate dead bacterial and extracellular DNA (Pan and Breidt 2007). Ten microliters of 2.5 mM PMA (Biotium, Inc., Hayward, Calif.) stock solution (1.3 mg/mL PMA in 20% DMSO) were added to 490 μL of the spoilage sample in saline (final concentration 57 μM PMA) in a microcentrifuge tube. Suspensions were vortexed briefly and placed in the dark for 5 min at room temperature. Sample tubes were then placed on ice with the tops open and exposed to a 650-W halogen lamp 20 cm above the tubes for 5 min. After light exposure to allow cross-linking of the dye to DNA, the cell suspensions were centrifuged at 6000 × rpm for 5 min at room temperature. Supernatant was removed and pellet was treated with PMA again as described. PMA-treated samples were then stored as cell pellets at −20 °C until DNA extraction.

DNA isolation was done using 1 of 3 different methods as indicated: DNeasy kit (Qiagen, Valencia, Calif.) with the addition of mutanolysin (10 units/μL) and/or lysozyme (20 mg/mL), or using the Power Soil DNA (MoBio, Carlsbad, Calif.) extraction kit (including mechanical disruption with a bead beater apparatus (Eppendorf, Thermomixer). Polymerase chain reaction (PCR) was done using 2 to 50 ng of DNA per PCR reaction. For PCR, primers 8f/fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r/rP1 (5-GGTTACCTT GTTACGACT T-3′) (Weisburg and others 1991; Turner and others 1999) were used to amplify the bacterial 16S ribosomal DNA gene. Primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used for yeast 26S rDNA gene amplification (Kurtzman and Robnett 1997). For rDNA clone analysis, Petri plates with the rDNA clones (in *Escherichia coli*) were sent to either GeneWiz (Genewiz, Research Triangle Park, N.C.) or Eton Bioscience (Eaton Bioscience Inc., Research Triangle Park). Sequence data were formatted and analyzed using custom Biopython (http://biopython.org/wiki/Biopython) scripts to facilitate web-based BLAST analysis, and/or BioEdit software processing (www.mbio.ncsu.edu/bioedit). Sequence similarities were determined using the NCBI BLASTN algorithm (www.ncbi.nlm.nih.gov) with the 16S ribosomal DNA (Bacteria and Archaea) database. All sequences were submitted to the National Center for Biotechnology Information Genbank database (NCBI accession numbers JX950891, JX951173, and JF28008).

Table 2–Isolates from laboratory spoilage with and without enrichment culture.

Strain ID	Strain Name[a]	Enrichment	%ID[b]
BI0237	*Clostridium diolis*	no	96
BI0238	*Clostridium spp.*	no	75
BI0239	*Bacillus spp.*	no	86
LA0599	*Lactobacillus plantarum/pentosus*	no	95
LA0600	*Lactobacillus brevis*	no	92
LA0601	*Lactobacillus casei/paracasei*	no	96
LA0602	*Lactobacillus paracasei*	no	99
B506	*Propionibacterium acidipropionici*	yes	97
LA1170	*Lactobacillus paracasei*	yes	100
LA1171	*Lactobacillus plantarum*	yes	100
LA1172	*Lactobacillus acidipiscis*	yes	99

[a]The tentative identification of the species based on the BLASTN alignment of the NCBI Bacterial and Archaeal database.
[b]The percent sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database.

Pectinatus spp. isolation

Pectinatus strain B405 was isolated by serial dilution of spoilage brine using peptone yeast lactate (PYL) broth. PYL broth contained per liter: 5 g peptone, 5 g tryptone, 10 g yeast extract, 5 g sodium lactate, 2 g sodium phosphate dibasic, 1 mL Tween 80, 5 g cysteine HCl, and 5 μg/mL vancomycin (R. Juvonen, personal communication). Cultures were incubated for up to 5 d at 30 °C, followed by plating on PYL agar. Identification was confirmed by phase-contrast microscopy, Gram staining, and 16S rDNA PCR. Cultures were stored under liquid nitrogen. To determine fermentation characteristics, biochemical analysis (as described above) was carried out after 96 h anaerobic incubation in PYL broth at 30 °C. An electron micrograph of the culture was obtained from the Center for Electron Microscopy, at NC State Univ., Raleigh.

Results and Discussion

The spoilage of fermented cucumbers in a commercial environment was first characterized by Fleming and others (1989), with an experimental low-salt (2.3%) anaerobic fermentation. Recently, an outbreak of fermentation spoilage has been reported in air-purged commercial cucumber fermentations. The air purging technology used by the cucumber pickling industry may have contributed to this outbreak (Franco and others 2012). Although the frequency of occurrence of spoilage in commercial cucumber fermentations is typically low, perhaps 1% of fermentation tanks per year, commercial brining operations may have up to 1000 tanks of 40000 L capacity each. Because of the scale of brining operations, spoiled tanks can be very costly. The onset of commercial fermentation spoilage can be detected as an increase in pH, resulting from the conversion of lactic acid to propionic, acetic, and butyric acids, and

Table 3–Biochemistry of the enrichment cultures after incubation during 6 sequential passages.

Passage	1	2	3	4	5	6	FSM[b]
Days	20	8	16	11	13	13	–
Lactic*	31.3 (7.4)	40.1 (4.8)	6.1 (3.9)	12.6 (7.2)	1.3 (0.9)	11.2 (14.4)	85.6
Acetic	96.7 (2.8)	96.5 (1.7)	105.9 (4.9)	99.9 (3.0)	102.3 (2.0)	97.9 (3.2)	63.7
Propionic	120.2 (7.2)	54.9 (1.5)	65.5 (4.0)	58.0 (2.9)	67.5 (2.9)	60.3 (7.2)	1.7
pH	5.22 (0.02)	5.17 (0.03)	5.19 (0.05)	5.35 (0.06)	5.31 (0.13)	5.36 (0.10)	4.97

[a]All acid data for each passage from independent replications in mM units (standard deviation in parenthesis).
[b]FSM, fermentation slurry medium, no glucose or fructose detected.

the generation of other unknown compounds. The biochemistry of these fermentations has been characterized elsewhere (Fleming and others 1989; Johanningsmeier and McFeeters 2011; Franco and others 2012). As pH rises above 4.6, the brine can become potentially hazardous due to the risk of *C. botulinum* spore outgrowth (Ito and others 1976).

Bacterial and vegetative cells of viable but not actively growing microorganisms may be present after the primary fermentation of cucumbers has ceased due to consumption of available sugar and accumulation of lactic acid. Surviving organisms may include lactobacilli present during the primary fermentation. These organisms may not contribute to spoilage but would be identified by classical or molecular ecology methods. Some lactic acid bacteria such as *L. buchneri* isolated from laboratory cucumber spoilage fermentations has been found to convert lactic acid to acetic acid and 1,2-propanediol, which is co-metabolized to propionic acid and propanol in a process that may involve multiple bacterial species (Johanningsmeier 2011). We initially conducted experiments with culture-based methods. Laboratory spoilage was prepared by inoculation of FSB with a commercial spoilage sample exhibiting propionic and butyric acids (commercial spoilage sample A, Table 1) production. Plating spoilage brine from laboratory reproduction of spoilage fermentations under anaerobic conditions resulted in the isolation of spore-forming bacteria and *Lactobacilli* (Table 2). None of the isolates obtained without enrichment could utilize lactic acid at pH 5.0 or produce the typical spoilage fermentation acids, propionic, butyric, and acetic acids in FSB.

In this study, direct plating did not yield cultures capable of metabolizing lactic acid under anaerobic conditions; thus, an enrichment method was used. To enrich for microorganisms that convert lactic acid to propionic or butyric acids during cucumber fermentation spoilage, a fermented cucumber medium lacking sugar (<0.1 mM glucose or fructose) and containing lactic acid and acetic acid as the primary carbon sources was prepared (FSM, Table 3). The initial pH of FSM was raised to 5.0 to facilitate growth of spoilage bacteria, as lower pH values resulted in no obvious growth or biochemical changes in the medium for periods exceeding 1 mo. Enrichment cultures were initially inoculated from spoilage sample B (Table 1) and serially passaged after 3 wk incubations, during which time the pH rose by 1 to 2 pH units (Table 3). Several species of bacteria were isolated from the 5th successive enrichment culture: *Propionibacterium acidipropionici, L. plantarum, Lactobacillus paracasei*, and *Lactobacillus acidipiscis* (Table 2). Of the bacterial isolates from the enrichment experiments, only *P. acidipropionici* was found to consume lactic acid and produce propionic acid under anaerobic conditions with pH 5.0 in FSM (Figure 1). At lower pH values (pH 3.2 and 3.8), little or no conversion of lactic acid to propionic acid was observed by *P. acidipropionici* (F. Breidt and D. Wafa, unpublished), indicating that this organism may participate in the later stages of fermented cucumber spoilage after the pH has risen. Since this organism does not utilize lactic acid at low pH, it would not be capable of initiating the spoilage process.

Culture-based methods may not fully identify the complexity of the microbial ecology in cucumber spoilage fermentations. To further identify spoilage organisms that may be responsible for the spoilage fermentation at pH values < 5.0, nonculture-based methods were applied using 4 commercial fermentation samples (C–F) with pH values of 3.2 to 4.9 (Table 1). All brines were typical of commercial fermentations having between 4% and 6% NaCl, and the spoilage samples were all found to contain propionic acid and 2 (C and D) had butyric acid (Table 3). Because cell free DNA or DNA from dead cells may be present in the brine after the completion of the primary lactic fermentation, we used a PMA treatment prior to DNA isolation (Nocker and others 2006; Pan

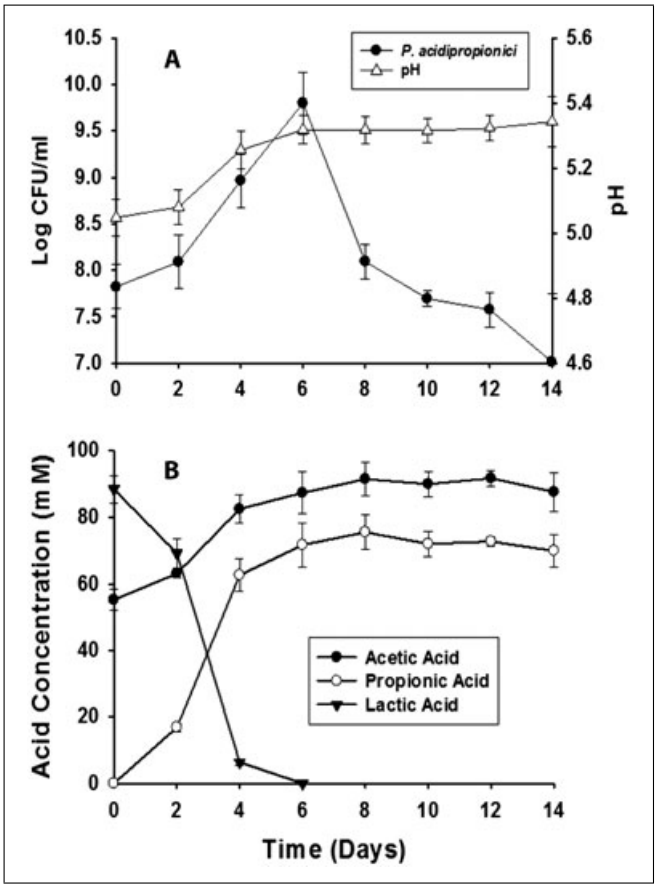

Figure 1–Lactic acid utilization by *P. acidipropionici*. (A) Cells (circles) and pH (triangles). (B) Biochemical analysis, acetic acid (filled circles), propionic acid (open circles), and lactic acid (triangles). The error bars indicate the standard deviation of 3 or more samples. Because pH replicates were within 0.1 units, log numbers were used for the mean values.

Table 4–The 16S rDNA clone sequence BLASTN results from commercial brine sample C, pH 4.9.

Tentative ID[a]	No.[b]	% Similarity[c]	Isolation method[d]			Phylum; Class; Order
			M	ML	BB	
Lactobacillus buchneri	5	99		1	4	Firmicutes; Bacilli; Lactobacillales
Lactobacillus sunkii	6	99 to 95		2	4	Firmicutes; Bacilli; Lactobacillales
Pediococcus cellicola	3	99			3	Firmicutes; Bacilli; Lactobacillales
Clostridium sporogenes	9	97 to 96	1	6	2	Firmicutes; Clostridia; Clostridiales
Butyricicoccus pullicaecorum	1	94		1		Firmicutes; Clostridia; Clostridiales
Pectinatus haikarae	41	97 to 93	19	13	9	Firmicutes; Negativicutes; Selenomonadales
Acidaminococcus fermentans	2	94	2			Firmicutes; Negativicutes; Selenomonadales
Acidaminococcus intestini	1	92		1		Firmicutes; Negativicutes; Selenomonadales
Pseudomonas fluorescens	2	99		2		Proteobacteria; Gammaproteobacteria; Pseudomonadales
Janthinobacterium lividum	1	99		1		Proteobacteria; Gammaproteobacteria; Pseudomonadales
Bacteroides intestinalis	2	94	2			Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Bacteroides eggerthii	1	91	1			Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Alistipes onderdonkii	1	88		1		Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Odoribacter splanchnicus	1	88	1			Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Prolixibacter bellariivorans	1	88		1		Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Paludibacter propionicigenes	2	87			2	Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Proteiniphilum acetatigenes	41	89 to 86	20	9	12	Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Cytophaga fermentans	19	83 to 81	2	9	8	Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia
Totals	139		48	47	44	

[a]The tentative identification of the species based on the BLASTN alignment using the NCBI Bacterial and Archaeal database.
[b]The number of clone sequences.
[c]The percentage sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database.
[d]For each method, the number of clones is shown: M = mutanolysin, ML = mutanolysin plus lysozyme, BB = mechanical lysis method.

Table 5–The 16S rDNA clone sequence BLASTN results from commercial brine sample D, pH 4.9.

Tentative ID[a]	No.[b]	% Similarity[c]	Phylum; Class; Order
Pseudoflavonifractor capillosus	1	91	*Firmicutes; Clostridia; Clostridiales*
Acidaminococcus fermentans	5	92 to 93	*Firmicutes; Negativicutes; Selenomonadales*
Suttonella spp.	13	95 to 96	*Proteobacteria; Gammaproteobacteria; Cardiobacteriales*
Rikenella microfusus	2	82	*Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia*
Proteiniphilum acetatigenes	8	88	*Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia*
Prevotella amnii	1	88	*Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia*
Odoribacter splanchnicus	2	91	*Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia*
Total	26		

[a]The tentative identification of the species based on the BLASTN alignment using the NCBI Bacterial and Archaeal database.
[b]The number of cloned sequences.
[c]The percent sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database.

Table 6–The 16S rDNA clone sequence BLASTN results from commercial brine sample E, pH 4.3

Tentative ID[a]	No.[b]	% Similarity[c]	Phylum; Class; Order
Lactobacillus acetotolerans	1	99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus amylovorus	5	95	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus gallinarum	4	97	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus namurensis	16	99 to 100	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus parabrevis	1	99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus paracasei	1	99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus plantarum[d]	7	99 to 100	*Firmicutes; Bacilli; Lactobacillales*
Pediococcus cellicola	2	99	*Firmicutes; Bacilli; Lactobacillales*
Vibrio spp.	4	94 to 98	*Proteobacteria; Gammaproteobacteria; Vibrionales*
	41		

Mixture of hetero and homofermentative strains, mostly homoferm.
*includes plantarum, paraplantarum, and pentosis

[a]The tentative identification of the species based on the BLASTN alignment using the NCBI Bacterial and Archaeal database.
[b]The number of cloned sequences.
[c]The percent sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database.
[d]Includes *plantarum*, *paraplantarum*, and *pentosus* species.

and Breidt 2007) from fermentation brine spoilage samples. This allowed us to selectively amplify DNA from cells with an intact membrane. Because the method of cell lysis is known to introduce bias in the DNA isolation from mixed culture environments, we used 3 different methods for cell lysis with mutanolysin (M), mutanolysin plus lysozyme (ML), and a mechanical lysis method (using a "bead beater" apparatus, BB), generating about 45 clones for each method. For primer selection, we used the 8f/1492R primer pair (Weisburg and others 1991; Turner and others 1999) that is widely used in microbial ecology studies. While it has been

Table 7–The 16S rDNA clone sequence BLASTN results from commercial brine sample F, pH 3.2.

Tentative ID[a]	No.[b]	% Similarity[c]	Phylum; Class; Order
Pediococcus cellicola	1	99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus sunkii/buchneri	27	98 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus parafarraginis	2	99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus namurensis	3	95 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus acetotolerans	5	98 to 99	*Firmicutes; Bacilli; Lactobacillales*
Total	38		

[a]The tentative identification of the species based on the BLASTN alignment using the NCBI Bacterial and Archaeal database.
[b]The number of cloned sequences, and the total number of clones.
[c]The percent sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database.

Table 8–Metabolites of *Pectinatus* B405 in culture media.

Medium[a]	Lactic[b]	Acetic	Propionic	glucose	pH
PYL-Van[c]	183	nd	nd	7.8	6.3
PYL-Van + culture	4.9	98	188	7.7	6.8

[a]PYL-Van with and without the added culture, after incubation at 30 °C for 72 h.
[b]All concentrations are in mM, nd = not detected.
[c]PYL-Van = PYL medium with vancomycin at 5 μg/mL.

reported that amplification of eukaryotic DNA may occur with this primer set (Galkiewicz and Kellogg 2008), we did not observe this.

The different DNA isolation methods (M, BB, and ML) each resulted in identification of 8 to 12 different species. A larger sample size would be needed to confirm whether there is a significant difference in the diversity of clones generated by each method. Because we considered mechanical lysis to be the least subject to interference by the sample contaminants, we chose the BB methods for continued studies. With the combined methods, a total of 139 16S rDNA clones were generated from this spoilage sample, including 49 M, 46 ML, and 44 BB clones (Table 4). The 139 sequences had a mean of 826 bp of contiguous sequence with <0.04% unidentified bases per sequence. A custom BioPython script (F. Breidt, unpublished) was used to remove ambiguous bases prior to taxonomic identification and only the first 600 bp of the selected 139 clones were used for sequence alignments. Approximately 52% of the clones had sequence identities with microorganisms in the rDNA database of >93% similarity, including 41 with an average of 96% similarity to *Pectinatus haikarae* (strain DSM 16980). Other clones tentatively identified using the 16S rDNA database included Gram-positive *Lactobacilli, Pediococcus*, and *Clostridium* species (Table 4).

Of the remaining sequence data from spoilage sample C, 68 clones were identified as members of the "*Cytophaga–Flavobacterium–Bacteroides*" superphylum of anaerobic Gram-negative rod-shaped bacteria, although most of the sequences had low sequence identity (<90%) to known species. Two groups represented the majority of clones; 1 group of 41 clones had sequence similarity (87%) to *Proteiniphilum acetatigenes*, and a 2nd group of 19 clones had similarity to *Cytophaga fermentans* (82%). For each of these groups, the cloned sequences were approximately 99% similar to each other, possibly representing 2 distinct species (Table 4). Because yeasts have previously been identified in fermentation spoilage (Franco and others 2012), we used yeast 26S primers with the same DNA sample (spoilage sample C), 24 clones were obtained, which were identified as *Pichia* species, including *Pichia occidentalis* and *Pichia manshurica* all with 99% or 100% sequence identity. Of the 3 additional commercial spoilage tanks examined using BB method for DNA isolation (samples D, E, and F), one had a majority of Gram-negative anaerobic *Gammaproteobacteria* and *Bacteroidetes* species (sample D, Table 5). The Gram-negative anaerobes were predominantly represented in spoilage fermentations C and D, which both had pH values of 4.9, and contained butyric acid. The remaining 2 spoilage samples (E, F) had the majority of sequences indicative of Gram-positive *Fermicutes* bacteria (Table 6 and 7, respectively) and no evidence of butyric acid. These spoilage samples had pH values of 4.3 and 3.2, respectively.

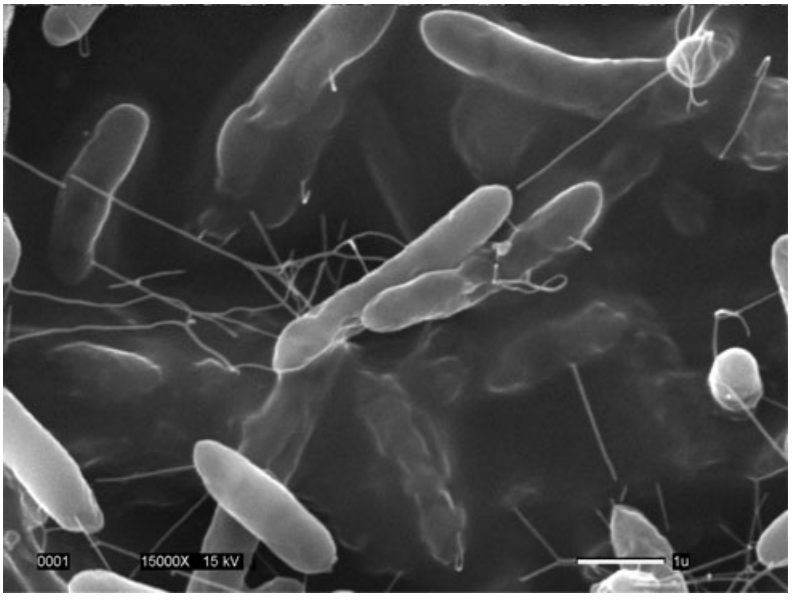

Figure 2–Electron micrograph of *Pectinatus* species. The electron micrographs were taken using 150000-fold magnification, which show the rod-shaped cells with fimbriae. The white bar represents 1 μm.

Pectinatus haikerae has recently been isolated from beer spoilage (Juvonen and Suihko 2006). However, no isolates from our direct plating or enrichment culture were previously identified as *Pectinatus. Pectinatus* is an anaerobic rod that stains Gram negative, and is a member of the *Fermicutes* phylum that has been implicated in beer spoilage. These organisms are strict anaerobes, lacking catalyze, and oxidase enzymes, and are difficult to cultivate on agar media. In an attempt to isolate the *Pectinatus* species identified in fermentation C, an antibiotic containing selective medium was used (PYL medium, R. Juvonen and others, personal communication). To select for *Pectinatus,* vancomycin was added to FSM, and a pure culture was obtained following plating on PYL agar containing vancomycin. The culture, *Pectinatus* strain B405, exhibited the characteristic X-shaped motion of *Pectinatus* species under phase contrast microscopy, which is due to the single centrally located flagellum of *Pectinatus* (Figure 2), and was capable of converting lactic acid to propionic acid (Table 8). The organism was strictly anaerobic and the pure culture obtained was stored under liquid nitrogen. Interestingly, the complete 16S sequence of the *Pectinatus* B405 was identical (100% match) to 11 of the cloned *Pectinatus* sequences from spoilage fermentation D. Further characterization of this organism will be published elsewhere (J. Caldwell, J. Brown, R. Juvonen, and F. Breidt, unpublished data).

Conclusions

Bacterial isolates identified from the laboratory or commercial spoilage samples using culture-based methods may not be comprehensively representative of the microbial populations present in secondary cucumber (spoilage) fermentations. A combination of culture-based and molecular ecology methods, resulted in the isolation of 2 species that may contributed to the spoilage fermentation, *P. acidipropionici,* identified by enrichment culture from a laboratory spoilage fermentation and *Pectinatus* B405, first identified by PCR cloning and then subsequently isolated by selective enrichment. Both organisms could convert lactic acid to propionic acid in pure culture. However, neither organism was capable of converting lactic acid to propionic acid below pH 5. A unique aspect of this work is the isolation of Pectinatus B405, which had an identical 16S rDNA sequence as found by 16S cloning. This shows the value of combining the molecular and classical microbiology approaches. Research conducted previously in our group suggest that lactic acid utilization at low pH (around pH 3.3) is initiated by lactic acid bacteria and yeasts in anaerobic and air-purged fermentations, respectively (Franco and Pérez-Díaz 2012; Johanningsmeier and other 2012). Our data support the hypothesis that Gram-positive *Firmicutes* dominate the early stages of the spoilage fermentation, and as pH values approach 5, Gram-negative anaerobes mostly representative of the *Bacteriodetes/Chloribi* group dominate, with concurrent production of butyric acid. Future research will include high-throughput next-generation sequencing of 16S rDNA from different stages of individual spoilage fermentations, to help identify the sequential changes in microbiota, as well as determining the conditions that prevent the growth of organisms responsible for the initiation of the spoilage fermentation.

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