SHORT COMMUNICATIONS

The Characterization of Microorganisms in Dairy Wastewater Storage Ponds

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Dairy wastewaters from storage ponds are commonly land applied to irrigate forage crops. Given that diverse microbial populations are associated with cattle feces, the objective of this study was to use a culture-independent approach to characterize bacteria and archaea in dairy wastewaters. Using domain-specific primers, a region of the 16S rRNA gene was amplified from pooled DNA extracts from 30 dairy wastewaters and subsequently used to create a clone library. A total of 152 bacterial clones were examined and sequence matches were affiliated with the following groups: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes. Firmicutes was identified as the largest phylum, representing up to 69% of the clone sequences. Of 167 clones representing Archaea, seven genera were found to be closely related (91–100% sequence similarity) to isolates obtained from sediments and feces. Most of the putative sequence matches (98%) represented members from the class Methanomicrobia. With respect to the archaeal clones, only one of the putative sequence matches was affiliated with a methanogenic bacterium known to inhabit the rumen.

In Idaho, there are 536,000 milk cows (National Agricultural Statistics Service, 2009), each producing approximately 50 to 70 kg d^{-1} of wet manure. To deal with the vast quantity of manure generated, the solids are removed and the liquid fraction is sent to ponds for storage (Ham and DeSutter, 2000). The solids are often land applied before the crop growing season because they are an effective soil conditioner and fertilizer. Similarly, the wastewater is blended with irrigation water and pumped through pressurized irrigation systems to distribute it to dairy forage crops such as silage corn (*Zea mays* L.) and alfalfa (*Medicago sativa* L.).

Because dairy cattle harbor a diverse microbial population, there is significant opportunity for zoonotic pathogens in land-applied manures to be transported in runoff and potentially as aerosolized particles (Thurston-Enriquez et al., 2005; Dungan, 2010). In untreated liquid manures, pathogens may persist for long periods of time depending on storage conditions and temperature (Arrus et al., 2006; Grewal et al., 2006). Furthermore, the survival period for pathogens is known to be longer when manures are immediately incorporated into the soils than when left on the surface (Hutchison et al., 2004). Because it is common practice among dairymen to land apply untreated manures, an examination of the microbial communities in such materials is warranted, especially given the fact that exposure to manure-borne microorganisms is a well-recognized public issue.

While the topic of manure pathogens generally receives the most attention (Klein et al., 2010b), microorganisms within livestock manures and wastewaters are responsible for a variety of actions including nutrient transformations and breakdown of organics leading to the formation of CH_4 or NH_3 and other offensive odorous compounds (Merrill and Halverson, 2002; Todd et al., 2011; Trabue et al., 2011). Despite the importance of microorganisms, very few studies to date have been conducted to characterize the microbial communities within dairy wastewaters (McGarvey et al., 2005). With the advent of molecular or culture-independent techniques, increased resolution of microbial communities is possible in complex environmental materials (e.g., feces) compared with culture-dependent techniques (Leung and Topp, 2001; Ibekwe et al., 2003; Shanks et al., 2011).

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Abbreviations: LB, Luria-Bertani; PCR, polymerase chain reaction; PSB, purple sulfur bacteria.

In this study, we used random cloning of 16S rRNA gene sequences amplified from community DNA to characterize bacteria and archaea in samples obtained from dairy wastewater ponds in south-central Idaho. Our objective was to generate data to improve the general understanding of microbial populations present in dairy wastewaters.

Materials and Methods

Dairy Operations and Wastewaters

Thirty wastewater storage ponds at open-lot and free-stall dairies in south-central Idaho were targeted for this study (Table 1). Surface samples were obtained from the storage ponds on three separate dates in June, August, and October of 2011. To ensure anonymity of the dairies, the samples were collected on a single-blind basis by trained individuals. Eight 500-mL samples were collected from the perimeter of each pond, then composited in a sterile 4-L container. The composited samples (a total of 90 for the study) were transferred to the laboratory in coolers, then stored under refrigeration at 5°C and processed within 24 h of collection. Chemical properties of the dairy wastewaters are presented in Table 2.

Table 1. Characteristics of the 30 dairies in south-central Idaho targeted for this study.

Dairy type	Lactating cows	Manure handling system
	no.	
Open lot	1,000–5,000	scrape
Free stall	10,000 +	flush
Open lot	1,000–5,000	scrape
Open lot	5,000–10,000	scrape
Open lot	>10,000	vacuum
Open lot	5,000–10,000	scrape
Open lot	5,000–10,000	scrape
Free stall	1,000–5,000	vacuum
Open lot	<1,000	scrape
Free stall	5,000–10,000	vacuum
Open lot	<1,000	scrape
Open lot	1,000–5,000	scrape
Free stall and open lot	1,000–5,000	flush
Free stall and open lot	1,000–5,000	flush
Open lot	1,000–5,000	scrape
Open lot	1,000–5,000	scrape
Open lot	1,000–5,000	scrape
Open lot	1,000–5,000	scrape
Free stall	1,000–5,000	flush
Open lot	5,000–10,000	flush
Open lot	1,000–5,000	scrape
Free stall	5,000–10,000	flush
Open lot	1,000–5,000	scrape
Open lot	1,000–5,000	scrape
Open lot	>10,000	vacuum
Free stall	1,000–5,000	flush
Free stall	1,000–5,000	scrape
Open lot	<1,000	scrape
Free stall	>10,000	flush
Free stall	>10,000	flush

Isolation of Microbial DNA

Community DNA was extracted from each composite sample as described by Dungan et al. (2012). In brief, the wastewater samples were washed twice with cold phosphate-buffered saline, with centrifugation at $10,000 \times g$ for 10 min between steps. The final pellet was then transferred to a bead beating tube from a FastDNA SPIN Kit for feces (MP Biomedicals) and processed using a FastPrep FP120 instrument at a speed setting of 6 m s^{-1} for 45 s. The DNA was eluted with 100 μL of TES buffer and stored at −20°C until amplified by polymerase chain reaction (PCR) as described below.

Amplification of 16S Ribosomal RNA

Before PCR amplification, the DNA extracts were thawed on ice at room temperature, then an equal volume of each extract was combined in a clean tube and gently mixed. A total of three pooled DNA samples were prepared, each one representing the 30 wastewater ponds on the three separate dates.

The V1 to V3 region of the bacterial 16S rRNA gene was amplified using the primer pair 63F–BA518R (Muyzer et al., 1993; Marchesi et al., 1998). The PCR reaction mixtures were prepared with 1 μL of DNA template, 0.3 μmol L^{-1} of each primer, 15 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems), and molecular-grade water to a final volume of 30 μL. The PCR was performed at 95°C for 5 min, then 30 cycles of 92°C for 1 min, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

The primer pair AR340F–AR1100R was used to amplify the V3 to V6 region of the archaeal 16S rRNA gene (Zhu et al., 2003). Thirty microliters of PCR reaction mixtures were prepared as described above. The thermocycler conditions for PCR were 95°C for 5 min, then 30 cycles of 94°C for 45 s, 46°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

Clone Library Construction

The 63F–BA518R and AR340F–AR1100R PCR products were cloned using pGEM-T Easy Vector (Promega Corp.). After transformation of the ligated plasmids, the clones were plated onto Luria-Bertani (LB) agar plates containing ampicillin (0.1 g L^{-1}), isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mmol L^{-1}), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)(80 μg L^{-1}) and incubated overnight at 37°C. Approximately 20 white colonies were randomly selected from each plate, which were then grown overnight in 10 mL of LB medium containing 0.1 g ampicillin L^{-1} at 37°C with shaking. Plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen).

Table 2. Chemical properties of the 30 wastewater ponds.

Variable	Min.	Max.	Mean	Median
Total NH$_3$ + NH$_4$–N, mg L^{-1}	1.4	1,512	378	240
Total Kjeldahl N, mg L^{-1}	3.4	2,286	674	468
Electrical conductivity, mS cm^{-1}	0.35	26	8.8	6.9
Total solids, mg L^{-1}	276	57,253	13,742	8,198
Volatile solids, mg L^{-1}	41	32,939	6,903	4,099
Chemical O$_2$ demand, mg L^{-1}	549	41,273	12,872	9,899
pH	6.6	8.8	7.7	7.7

Sequencing and Phylogenetic Analysis

Plasmid inserts were amplified with the 63F or AR340F primer and sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems). Raw sequences were handled using BioEdit Sequence Alignment Editor (Hall, 1999). The clone sequences were checked for putative chimeric sequences and aligned using tools available on the Greengenes web site (DeSantis et al., 2006). While >500 clones were generated and processed, only 152 bacterial and 167 archaeal sequences passed the quality control measures. Sequence identification was performed using the Basic Local Alignment Search Tool (BLAST) in GenBank.

Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987), and evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). To evaluate the stability of the phylogenetic relationships, bootstrap analyses of the neighbor-joining data were conducted based on 1000 iterations.
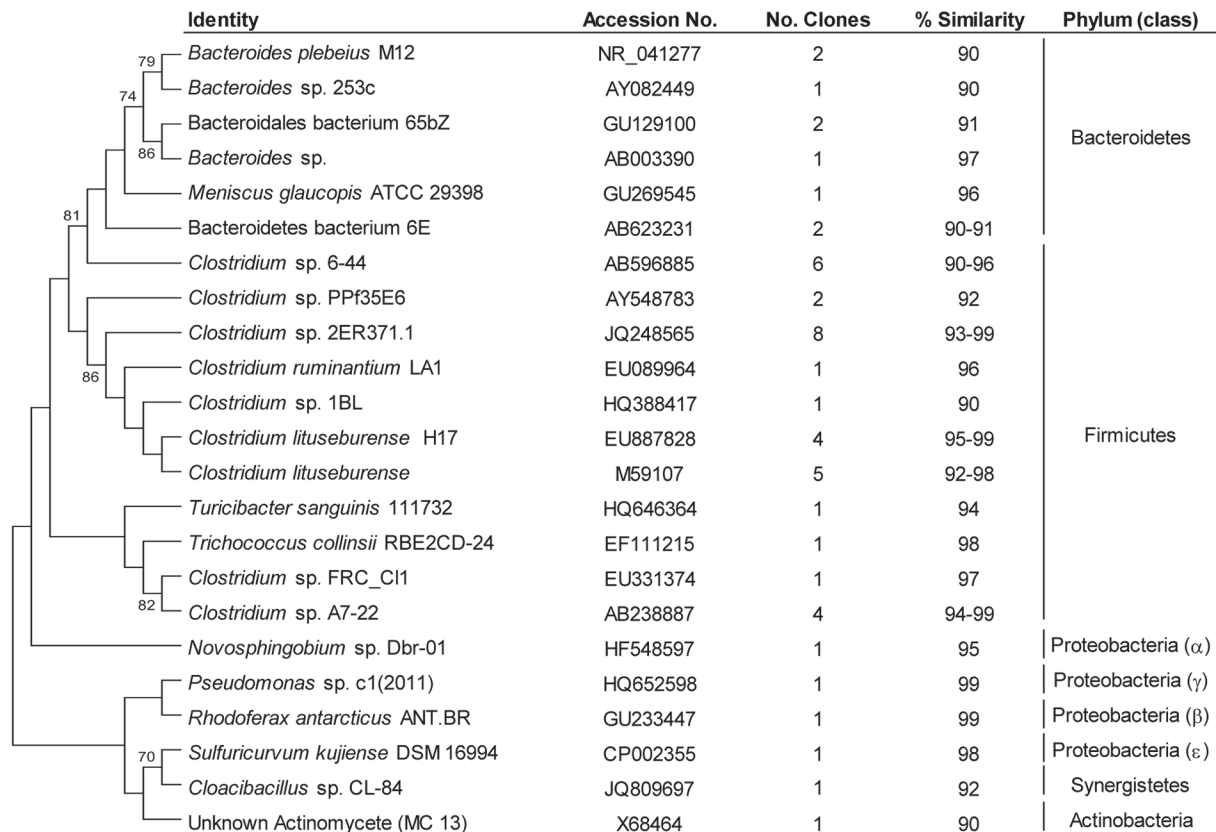
Results and Discussion

The 152 clone sequences representing bacteria in the wastewater ponds were found to be affiliated with five phyla: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes (Table 3). While all five phyla were present in June, just four and three phyla were present in the August and October samples, respectively. Regardless of month, Firmicutes was identified as the largest phylum, representing ≥46% of the clone sequences, while Actinobacteria and Synergistetes represented ≤4% of the sequences. Although only 8% of our sequences were affiliated with Proteobacteria in June, an

Table 3. Percentage of clones affiliated with the bacterial phyla in the dairy wastewaters.

Phylum	June	Aug.	Oct.
Actinobacteria	2	4	0
Bacteroidetes	18	9	10
Firmicutes	69	60	46
Proteobacteria	8	26	44
Synergistetes	2	0	0

increase to 44% occurred by October. Conversely, the number of Bacteroidetes and Firmicutes were found to decrease during the same time period. These observed differences may be related to seasonal changes within the cattle or ponds; however, it should be noted that a limited number of sequences were obtained and that random cloning results are not necessarily an accurate quantitative measure. A few studies, however, have found that levels of bacterial pathogens in fresh cattle manures were affected in a season-dependent manner (Chapman et al., 1997; Stanley et al., 1998).

Figure 1 presents the phylogenetic relationship among of the bacterial clone sequences that were derived from the June wastewater pond samples. In June, only four sequences with 95 to 99% similarity to the GenBank database were related to the α, β, ε, and γ subdivisions of Proteobacteria. Firmicutes was the predominant phylum, and 32 of 34 sequences were 90 to 99% similar to *Clostridium* spp., with the remaining two sequences being 94 and 98% similar to *Turicibacter sanguinis* and *Trichococcus collinsii*, respectively. The lack of detection of *C. perfringens* and *Enterococcus* spp. in the dairy wastewaters was

Identity	Accession No.	No. Clones	% Similarity	Phylum (class)
Bacteroides plebeius M12	NR_041277	2	90	Bacteroidetes
Bacteroides sp. 253c	AY082449	1	90	
Bacteroidales bacterium 65bZ	GU129100	2	91	
Bacteroides sp.	AB003390	1	97	
Meniscus glaucopis ATCC 29398	GU269545	1	96	
Bacteroidetes bacterium 6E	AB623231	2	90-91	
Clostridium sp. 6-44	AB596885	6	90-96	Firmicutes
Clostridium sp. PPf35E6	AY548783	2	92	
Clostridium sp. 2ER371.1	JQ248565	8	93-99	
Clostridium ruminantium LA1	EU089964	1	96	
Clostridium sp. 1BL	HQ388417	1	90	
Clostridium lituseburense H17	EU887828	4	95-99	
Clostridium lituseburense	M59107	5	92-98	
Turicibacter sanguinis 111732	HQ646364	1	94	
Trichococcus collinsii RBE2CD-24	EF111215	1	98	
Clostridium sp. FRC_Cl1	EU331374	1	97	
Clostridium sp. A7-22	AB238887	4	94-99	
Novosphingobium sp. Dbr-01	HF548597	1	95	Proteobacteria (α)
Pseudomonas sp. c1(2011)	HQ652598	1	99	Proteobacteria (γ)
Rhodoferax antarcticus ANT.BR	GU233447	1	99	Proteobacteria (β)
Sulfuricurvum kujiense DSM 16994	CP002355	1	98	Proteobacteria (ε)
Cloacibacillus sp. CL-84	JQ809697	1	92	Synergistetes
Unknown Actinomycete (MC 13)	X68464	1	90	Actinobacteria

Fig. 1. Phylogenetic relationships among bacterial 16S rRNA gene sequences amplified from pooled community DNA obtained from the dairy wastewaters in June. Bootstrap values >70% are noted at the branch junctions.

quite surprising given the fact that these Gram-positive bacteria are regularly detected in cattle manures and wastewaters (Klein et al., 2010a; Dungan et al., 2012). The non-detects could be related to limited 16S rRNA sequence coverage or a PCR amplification bias. Some early research on enterococci in cattle found that *E. fecalis* was abundant in the feces of preruminating calves but was rare in older dairy cows (Devriese et al., 1992). Bacteroidetes were the second most abundant sequences identified, with members belonging to *Bacteroides* and *Meniscus*. Similar to our June results, Ravva et al. (2011) found that Firmicutes was the predominant phylum in fresh dairy manure, followed by Bacteroidetes.

The phylogenetic relationships among the bacterial clones from August and October once again demonstrated the predominance of Firmicutes in the wastewater samples (Fig. 2 and 3). Compared with June, additional genera other than *Clostridium* were identified within Firmicutes, including *Tissierella*, *Oscillibacter*, *Desulfotomaculum*, *Faecalibacterium*, *Proteocatella*, and *Ruminococus*. Members of Proteobacteria (δ and γ subdivisions only) also dominated the sequence matches. Some of the more interesting matches within Proteobacteria were those with purple sulfur bacteria (PSB), represented by high sequence similarities to *Thiocapsa roseopersicina* (96–99%), *Thiolamprovum pedioforme* (92–100%), and *Thiococcus pfennigii* (94%) of the family Chromatiaceae. The highest number of clone sequences were associated with *T. pedioforme*, suggesting that it was the dominant PSB in the ponds that we characterized. Goh et al. (2009) examined facultative swine waste lagoons and found that they were dominated by PSB affiliated with *T.*

pedioforme. Purple sulfur bacteria were also noted in many of the dairy pond samples by the very dark pink to red color of the wastewater (data not shown), but it is not fully understood what conditions induce their growth (Chen et al., 2003; McGarvey et al., 2009). The presence of PSB in manure wastewaters is of practical significance because they can oxidize H_2S and some volatile organic compounds (Visscher and Van Gemerden, 1991; Caumette, 1993; Zaar et al., 2003), which has been linked to a reduction in offensive odors (Holm and Vennes, 1970).

Of the 167 clone sequences representing Archaea, seven genera were found to be closely related (91–100% sequence similarity) to isolates obtained from sediments and feces (Fig. 4). Most of the putative sequence matches (98%) represented members from the class Methanomicrobia. The matches that dominated the samples were *Methanosaeta concilii*, *Methanocorpusculum sinense*, and *Methanosarcina mazei*, representing 58, 23, and 7% of the clone sequences, respectively. Species belonging to *Methanoculleus*, *Methanospirillum*, *Methanogenium*, and *Methanobrevibacter* represented <10% of the clone sequences. Although members of *Methanobrevibacter*, *Methanoculleus*, and *Methanosarcina* are commonly isolated from the rumen (Janssen and Kirs, 2008), *Methanobrevibacter thaueri* was the only putative match in our study that has been previously detected in bovine rumen contents (Skillman et al., 2006). Based on our results, it would appear that the origin of methanogens in the wastewater ponds was not from the dairy cattle rumen. In addition, the fact that only 10 species were represented by the clone sequences does suggest low archaeal richness within the wastewaters. In a study of microbial communities in a swine manure storage pit,

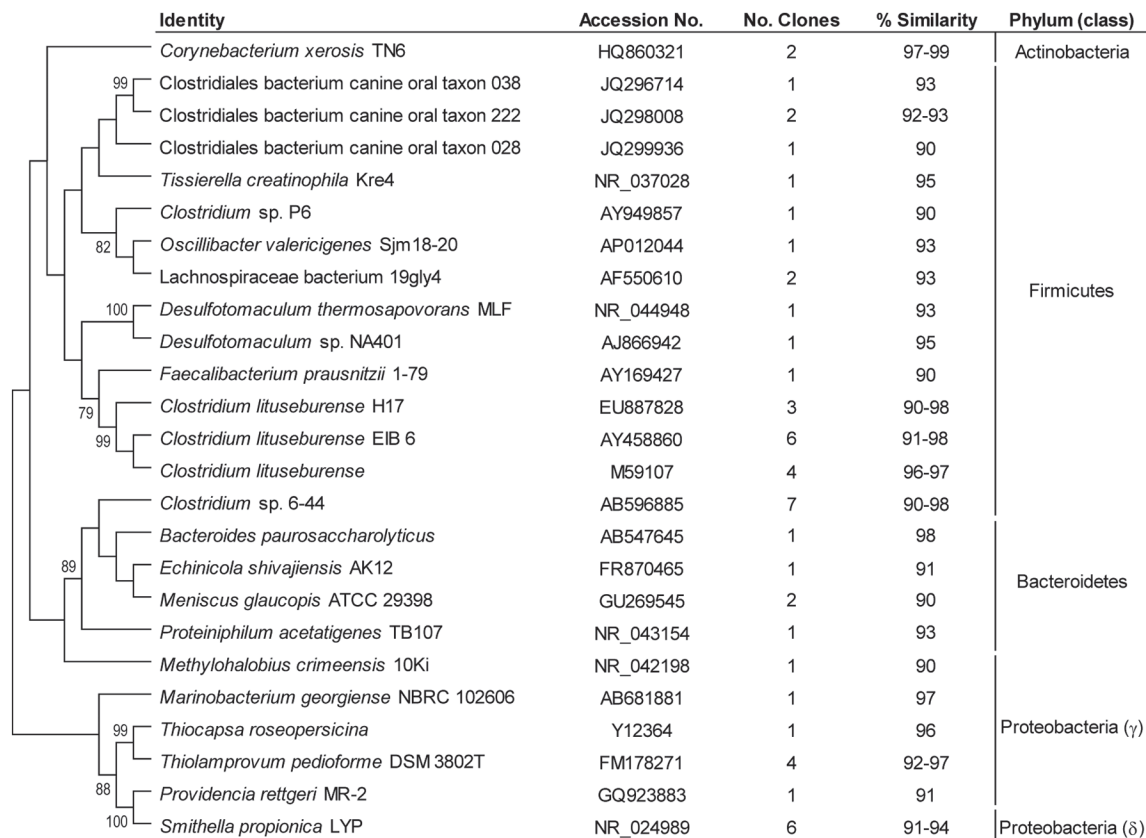| Identity	Accession No.	No. Clones	% Similarity	Phylum (class)
Corynebacterium xerosis TN6	HQ860321	2	97-99	Actinobacteria
Clostridiales bacterium canine oral taxon 038	JQ296714	1	93	Firmicutes
Clostridiales bacterium canine oral taxon 222	JQ298008	2	92-93	
Clostridiales bacterium canine oral taxon 028	JQ299936	1	90	
Tissierella creatinophila Kre4	NR_037028	1	95	
Clostridium sp. P6	AY949857	1	90	
Oscillibacter valericigenes Sjm18-20	AP012044	1	93	
Lachnospiraceae bacterium 19gly4	AF550610	2	93	
Desulfotomaculum thermosapovorans MLF	NR_044948	1	93	
Desulfotomaculum sp. NA401	AJ866942	1	95	
Faecalibacterium prausnitzii 1-79	AY169427	1	90	
Clostridium lituseburense H17	EU887828	3	90-98	
Clostridium lituseburense EIB 6	AY458860	6	91-98	
Clostridium lituseburense	M59107	4	96-97	
Clostridium sp. 6-44	AB596885	7	90-98	
Bacteroides paurosaccharolyticus	AB547645	1	98	Bacteroidetes
Echinicola shivajiensis AK12	FR870465	1	91	
Meniscus glaucopis ATCC 29398	GU269545	2	90	
Proteiniphilum acetatigenes TB107	NR_043154	1	93	
Methylohalobius crimeensis 10Ki	NR_042198	1	90	
Marinobacterium georgiense NBRC 102606	AB681881	1	97	Proteobacteria (γ)
Thiocapsa roseopersicina	Y12364	1	96	
Thiolamprovum pedioforme DSM 3802T	FM178271	4	92-97	
Providencia rettgeri MR-2	GQ923883	1	91	
Smithella propionica LYP	NR_024989	6	91-94	Proteobacteria (δ)

Fig. 2. Phylogenetic relationships among bacterial 16S rRNA gene sequences amplified from pooled community DNA obtained from the dairy wastewaters in August. Bootstrap values >70% are noted at the branch junctions.
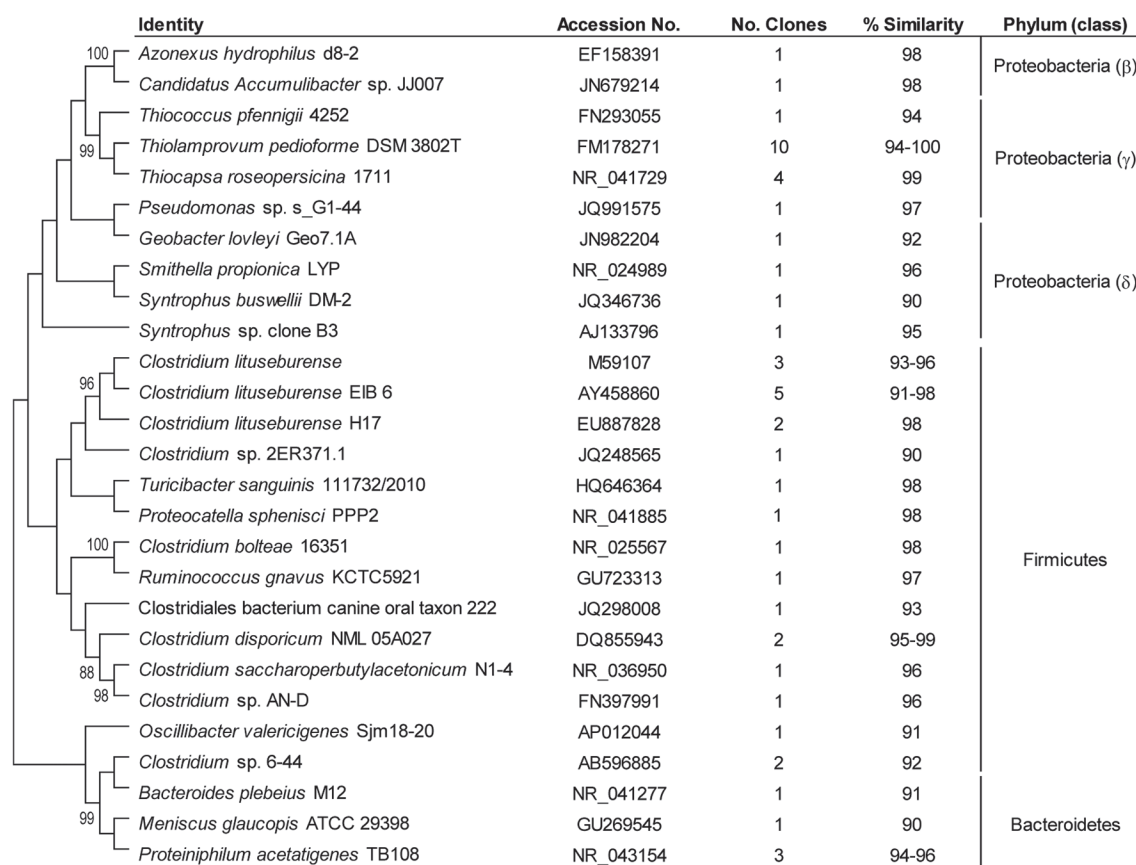
Identity	Accession No.	No. Clones	% Similarity	Phylum (class)
Azonexus hydrophilus d8-2	EF158391	1	98	Proteobacteria (β)
Candidatus Accumulibacter sp. JJ007	JN679214	1	98	
Thiococcus pfennigii 4252	FN293055	1	94	Proteobacteria (γ)
Thiolamprovum pedioforme DSM 3802T	FM178271	10	94-100	
Thiocapsa roseopersicina 1711	NR_041729	4	99	
Pseudomonas sp. s_G1-44	JQ991575	1	97	
Geobacter lovleyi Geo7.1A	JN982204	1	92	
Smithella propionica LYP	NR_024989	1	96	Proteobacteria (δ)
Syntrophus buswellii DM-2	JQ346736	1	90	
Syntrophus sp. clone B3	AJ133796	1	95	
Clostridium lituseburense	M59107	3	93-96	Firmicutes
Clostridium lituseburense EIB 6	AY458860	5	91-98	
Clostridium lituseburense H17	EU887828	2	98	
Clostridium sp. 2ER371.1	JQ248565	1	90	
Turicibacter sanguinis 111732/2010	HQ646364	1	98	
Proteocatella sphenisci PPP2	NR_041885	1	98	
Clostridium bolteae 16351	NR_025567	1	98	
Ruminococcus gnavus KCTC5921	GU723313	1	97	
Clostridiales bacterium canine oral taxon 222	JQ298008	1	93	
Clostridium disporicum NML 05A027	DQ855943	2	95-99	
Clostridium saccharoperbutylacetonicum N1-4	NR_036950	1	96	
Clostridium sp. AN-D	FN397991	1	96	
Oscillibacter valericigenes Sjm18-20	AP012044	1	91	
Clostridium sp. 6-44	AB596885	2	92	
Bacteroides plebeius M12	NR_041277	1	91	Bacteroidetes
Meniscus glaucopis ATCC 29398	GU269545	1	90	
Proteiniphilum acetatigenes TB108	NR_043154	3	94-96	

Fig. 3. Phylogenetic relationships among bacterial 16S rRNA gene sequences amplified from pooled community DNA obtained from the dairy wastewaters in October. Bootstrap values >70% are noted at the branch junctions.

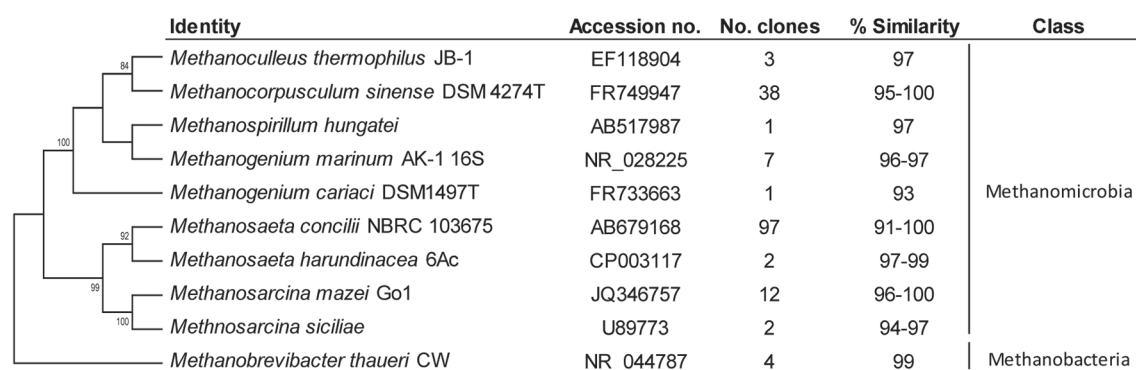| Identity	Accession no.	No. clones	% Similarity	Class
Methanoculleus thermophilus JB-1	EF118904	3	97	Methanomicrobia
Methanocorpusculum sinense DSM 4274T	FR749947	38	95-100	
Methanospirillum hungatei	AB517987	1	97	
Methanogenium marinum AK-1 16S	NR_028225	7	96-97	
Methanogenium cariaci DSM1497T	FR733663	1	93	
Methanosaeta concilii NBRC 103675	AB679168	97	91-100	
Methanosaeta harundinacea 6Ac	CP003117	2	97-99	
Methanosarcina mazei Go1	JQ346757	12	96-100	
Methnosarcina siciliae	U89773	2	94-97	
Methanobrevibacter thaueri CW	NR_044787	4	99	Methanobacteria

Fig. 4. Phylogenetic relationships among archaeal 16S rRNA gene sequences amplified from pooled community DNA obtained from the dairy wastewaters (data from all months combined). Bootstrap values >70% are noted at the branch junctions.

only eight archaeal phylotypes were identified, with most DNA sequences (i.e., 61%) being closely related to uncultured clones from swine manure (Snell-Castro et al., 2005).

In conclusion, a culture-independent approach was used to increase our understanding of microbial communities in dairy wastewaters, which are often difficult to analyze along with other fecal and environmental materials. The results from our phylogenetic analyses revealed that the dairy wastewaters contained a variety of genera and species belonging to the domains Bacteria and Archaea. However, because a limited number of 16S rRNA gene sequences were obtained, it is unlikely that a thorough analysis of the microbial populations in the wastewaters was achieved. Furthermore, the non-detection of specific organisms within the wastewaters (e.g., pathogens) may also have occurred because they were either not present or there was a PCR amplification bias. While the latter two issues are generally beyond control, it is essential that an appropriate number of sequences are analyzed to ensure proper coverage of the microbial community diversity. This could have been accomplished in the current study by generating a higher number of clones and/or using a high-throughput sequencing technique.

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