Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk

S. N. Masiello, N. H. Martin, A. Trmčić, M. Wiedmann, and K. J. Boor¹

Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, NY 14850

ABSTRACT

The presence of coliform bacteria in pasteurized fluid milk typically indicates that product contamination occurred downstream of the pasteurizer, but it may also indicate pasteurization failure. Although coliform detection is frequently used as a hygiene indicator for dairy products, our understanding of the taxonomic and phenotypic coliform diversity associated with dairy products is surprisingly limited. Therefore, using Petrifilm Coliform Count plates (3M, St. Paul, MN), we isolated coliforms from high-temperature, short-time (HTST)-pasteurized fluid milk samples from 21 fluid milk processing plants in the northeast United States. Based on source information and initial characterization using partial 16S rDNA sequencing, 240 nonredundant isolates were obtained. The majority of these isolates were identified as belonging to the genera *Enterobacter* (42% of isolates), *Hafnia* (13%), *Citrobacter* (12%), *Serratia* (10%), and *Raoultella* (9%); additional isolates were classified into the genera *Buttiauxella*, *Cedecea*, *Kluysvera*, *Leclercia*, *Pantoea*, and *Rahnella*. A subset of 104 representative isolates was subsequently characterized phenotypically. Cold growth analysis in skim milk broth showed that all isolates displayed at least a 2-log increase over 10 d at 6°C; the majority of isolates ($n = 74$) displayed more than a 5-log increase. In total, 43% of the representative isolates displayed lipolysis when incubated on spirit blue agar at 6°C for 14 d, whereas 71% of isolates displayed proteolysis when incubated on skim milk agar at 6°C for 14 d. Our data indicate that a considerable diversity of coliforms is found in HTST-pasteurized fluid milk and that a considerable proportion of these coliforms have phenotypic characteristics that will allow them to cause fluid milk spoilage.

Key words: coliform, HTST-pasteurized milk, milk spoilage, cold growth

INTRODUCTION

Preventing postpasteurization contamination (PPC) with spoilage microorganisms remains a major challenge for dairy processors (Ralyea et al., 1998; Ranieri and Boor, 2009; Martin et al., 2011). Along with *Pseudomonas* spp., coliform bacteria are frequently isolated PPC contaminants in pasteurized fluid milk (Martin et al., 2011). Coliforms are defined as aerobic or facultatively anaerobic, gram-negative, non-spore-forming rods capable of fermenting lactose, resulting in gas and acid production within 48 h at 35°C (Feng et al., 2002; Nörnberg et al., 2010). Traditionally, coliforms were considered to be represented by 4 genera: *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* (Bergey et al., 1939). Today, over 20 bacterial genera include strains that have phenotypic characteristics that classify them as coliforms (Imhoff, 2005). Detection of coliforms plays an important role in the dairy industry because coliforms are frequently used as hygiene indicators, and there are clear regulatory limits for the presence of coliforms in finished dairy products. For example, the US Pasteurized Milk Ordinance limits the number of coliforms in pasteurized grade “A” milk to ≤ 10 cfu/mL (FDA, 2011).

Coliform bacteria that are psychrotolerant and capable of growing at refrigerated storage temperatures are of particular concern for the dairy industry, as psychrotolerant growth can result in physical degradation and unacceptable sensory characteristics of the product due to the production of lipolytic and proteolytic enzymes (Nörnberg et al., 2010). This is also supported by a recent 10-year study of HTST fluid milk quality in New York State (NYS); milk samples that tested positive for coliforms on the initial day of refrigerated shelf-life had significantly lower sensory scores on d 14 of refrigerated shelf-life compared with samples that were not positive for coliforms on the initial day (Martin et al., 2012). Postpasteurization contamination is not isolated to the United States; for example, previous research found 40% of fluid milk samples taken from Norway and Sweden had PPC with coliforms (Eneroth et al., 1998).

Received April 20, 2015.

Accepted September 14, 2015.

¹Corresponding author: kjb4@cornell.edu

Although coliform testing continues to play an important role in the dairy industry, available data on the phenotypic and genotypic coliform diversity found in dairy products are surprisingly limited. Most reported studies characterized only small sets of coliform isolates and typically these isolates were obtained from only a few processing facilities. For example, researchers in Norway and Sweden assessed coliform levels from 3 different dairy processing plants (Eneroth et al., 1998), whereas other researchers utilized as little as 6 to as many as 75 coliform isolates to characterize dairy product-associated coliform bacteria (Juven et al., 1981; Wessels et al., 1989). An improved understanding of the diversity and spoilage potentials of fluid milk associated coliforms is vital to improve fluid milk quality. For example, better baseline data on coliform diversity associated with fluid milk may facilitate development and validation of more robust and faster detection methods and may facilitate source tracking of coliform contamination. Therefore, the objectives of this study were (1) to characterize the taxonomic diversity of psychrotolerant coliforms in pasteurized fluid milk obtained from a substantial number of processing plants, and (2) to characterize representative isolates for relevant phenotypic characteristics (i.e., cold growth capability and proteolysis and lipolysis during growth at refrigeration temperatures).

MATERIALS AND METHODS

Sample Collection and Handling

Pasteurized milk samples were collected from 2010 to 2011 from fluid milk processing facilities located in the northeast United States (NYS, Maine, Pennsylvania, Vermont, New Hampshire, and Massachusetts). All participating processing facilities were enrolled in the Voluntary Shelf-Life (VSL) program, which is administered by the Cornell University Milk Quality Improvement Program (MQIP; Martin et al., 2012). Processing capacity of facilities ranged from <1 million to >600 million lbs of fluid milk annually. Samples collected represented packaged pasteurized products that were processed at each facility via HTST pasteurization [at a minimum of 72°C (161°F), 15 s], including whole fat (minimum 3.25% milk fat), reduced fat (1.5 or 2% milk fat), low fat (1% milk fat), and nonfat (<0.2% milk fat) milk in quart (946 mL), half gallon (1.9 L), or gallon (3.8 L) containers. Containers used for packaging included high-density polyethylene jugs, paperboard cartons, or glass bottles. All milk samples were transported to the MQIP laboratory in coolers packed with ice packs or ice. Samples were held at 4°C until initial testing in the laboratory 24 to 48 h after

sample collection. Of the 29 total fluid milk processing plants enrolled in the 2010–2011 VSL program, 21 plants had positive coliform samples and were included in the study reported here.

Coliform Isolation from Milk Samples

Upon arrival at the laboratory, samples were inverted 25 times within 7 s and for each milk sample ~400 mL of milk was aseptically distributed into each of 4 sterile 500-mL glass bottles. Bottles were stored at 6°C until the appropriate test day for each bottle. Samples were tested for microbiological and sensory quality on the initial day as well as at d 7, 10, and 14 postprocessing (Martin et al., 2012). The initial day was defined as the first day of testing, which varied from 0 to 6 d postprocessing due to the plant processing schedule. Samples from a subset of processors with histories of consistently manufacturing high-quality products were also tested at d 17 and 21 postprocessing (Martin et al., 2012). Coliform testing was performed on each test day by inoculating milk samples in duplicate on separate Petrifilm Coliform Count plates (3M, St. Paul, MN); plates were subsequently incubated at 32°C for 24 h according to the manufacturer's instructions. For each sample date (e.g., d 7) with a sample positive for coliforms, 2 coliform isolates were selected from the Petrifilm Coliform Count plates and streaked for purity on brain heart infusion (BHI) agar (Difco, Franklin Lakes, NJ), which was subsequently incubated at 32°C for 24 h. Isolates were confirmed as coliforms by inoculation in brilliant green bile broth (Difco); only isolates confirmed as coliforms were retained and further characterized. Pure cultures were grown overnight in BHI broth at 32°C and then frozen in 15% glycerol at –80°C and stored until further characterization. Before further characterization as described below, isolates were streaked for isolation from frozen stock onto BHI agar and grown at 32°C for 24 h. Detailed information on all isolates is available through Food Microbe Tracker (<http://www.foodmicrobetracker.com>).

Genus Identification Using Sequencing of Partial 16S rDNA

All isolates were characterized by sequencing a 616-bp fragment of the 16S rRNA gene as described previously (Huck et al., 2008). The resulting 16S rDNA sequence data were used to identify isolates to the genus level. Genus identification was performed using the Ribosomal Database Project classifier (Cole et al., 2005); genus identification was assigned based on a seqmatch score (S_{ab}) of 0.90 or higher (Cole et al., 2005). For 5 isolates that did not produce 16S rDNA

gene products with the protocol reported by Huck et al. (2008), genomic DNA was obtained using the QIAmp DNA Mini Kit (Qiagen, Venlo, the Netherlands) and used for subsequent PCR and sequencing of the 16S rRNA gene as described by Huck et al. (2008).

Partial 16S rDNA data were also used to classify isolates into 16S rDNA sequence types (**16SrDNA-ST**). Two isolates were classified into the same 16SrDNA-ST if they shared an identical partial 16S rDNA sequence. The 16SrDNA-ST data were used to select isolates for subsequent phenotypic characterization (i.e., cold growth and proteolytic and lipolytic capabilities, as detailed below). If ≤ 10 isolates were grouped into a given 16SrDNA-ST, a single isolate was selected for phenotypic characterization; if there were >10 isolates within a given 16SrDNA-ST, multiple isolates were chosen from that sequence type. With this approach, 104 representative isolates were selected for phenotypic characterization.

Phylogenetic Analysis of Coliform Isolates

To confirm genus identifications for all isolates, a 16S rDNA-based parsimony phylogenetic tree was constructed using PAUP (version 4, Sinauer Associates Inc., Sunderland, MA); this tree included the sequences obtained here as well as all type strains of coliforms that were included in the Ribosomal Database Project database (<https://rdp.cme.msu.edu/>). Isolates were confirmed as belonging to a specific genus if they fell within the same 16SrDNA-ST as the type strain sequence.

Cold Growth Analysis During Refrigerated Storage at 6°C

For cold growth analysis, an isolated colony for a given isolate was inoculated into a tube containing 5 mL of BHI broth (Difco), followed by incubation at 32°C for 24 h. To enumerate the bacteria present after the 24 h incubation, 1 mL of the culture was aliquoted into a 2-mL Eppendorf tube and centrifuged for 10 min at $95,589 \times g$. After removal of the supernatant, bacterial pellets were resuspended with 1 mL of PBS (Difco). A 1-mL aliquot of each suspension was serially diluted with phosphate buffer and a 100- μ L aliquot of each sample was plated in duplicate onto SPC (Difco) agar plates (50 μ L per plate) with an AP5000 spiral plater (Advanced Instruments Inc., Norwood, MA). The remaining volume of the isolate suspension was stored at -20°C for less than 24 h before use. Results from enumeration were used to dilute each remaining suspension with phosphate buffer to a final concentration of 10^2 to 10^3 cfu/mL. A 1-mL aliquot of the final

dilution (at 10^2 to 10^3 cfu/mL) was then inoculated into 4 mL of sterile skim milk broth (**SMB**; Difco); 100 μ L of the inoculated SMB was immediately spiral plated onto 2 SPC agar plates (50 μ L per plate) for d 0 bacterial enumeration. Inoculated SMB was subsequently incubated at 6°C for 10 d and bacterial enumeration was performed at d 2, 4, 6, 8, and 10.

Characterization of Proteolytic and Lipolytic Activity in Bacterial Isolates Grown at 6°C

The 104 isolates selected for cold growth analyses were further analyzed for ability to produce extracellular proteases and lipases during growth at 6°C. Production of extracellular proteolytic enzymes was determined on skim milk agar (**SMA**; Difco) and production of extracellular lipolytic enzymes was determined on spirit blue agar (**SB**; Difco) containing an additional prepared lipase reagent representing a mixture of tributyrin and polysorbate 80 (Difco). Single bacterial colonies were streaked on the respective plates. After incubation at 6°C for 14 d, isolates were scored for enzymatic activity on a 3-point scale. For SMA plates, a score of (–) was given to isolates with no visible clearing around the streak area, (+) was given to isolates with noticeable clearing around the streak area, and (++) was given to isolates with an obvious clear zone and opaque zone surrounding the streak area (Frank and Yousef, 2004). For SB plates with the added lipase reagent, a score of (–) was given to isolates with no visible clearing around the streak area, (+) was given to isolates with a small zone of clearing (≤ 2 mm) around the streak area with the remainder of the plate remaining blue, and (++) was given to isolates where the clearing around the streak area was >2 mm (Immanuel et al., 2008).

Data Analyses

All data were managed in Excel (version 2007, Microsoft Corp., Redmond, WA). Coliform bacteria count data were log-transformed before analysis and enzymatic activity levels were coded 1 to 3, with 1 = (–), 2 = (+), and 3 = (++). Excel was used to compute the Simpson's Index of Diversity (**SID**; Simpson, 1949) to quantify overall species and 16SrDNA-ST diversity among the 240 nonredundant isolates (isolates were considered redundant if they shared the same plant, date, sample, and genus). The SAS statistical software (version 9.3, SAS Institute Inc., Cary, NC) was used to perform Fisher's exact tests to determine whether the proportion of isolates that showed different levels of enzymatic activity differed among genera. The JMP Pro statistical software (version 10.0, SAS Institute Inc.) was used to plot bacterial growth over 10 d at 6°C

Table 1. Coliform genera isolated from HTST-pasteurized fluid milk samples

Genus	No. of isolates	No. of nonredundant isolates ¹	No. of 16SrDNA-ST ²	No. of plants where a given genus was isolated ³
<i>Enterobacter</i>	175	100	19	12
<i>Hafnia</i>	52	31	8	6
<i>Citrobacter</i>	45	28	27	12
<i>Serratia</i>	39	24	13	6
<i>Raoultella</i>	38	22	4	5
<i>Buttiauxella</i>	15	11	10	5
<i>Kluyvera</i>	13	9	5	5
<i>Pantoea</i>	11	6	5	3
<i>Cedecea</i>	7	4	2	2
<i>Rahnella</i>	5	4	3	4
<i>Leclercia</i>	2	1	1	1
Total	402	240	97	—

¹Nonredundant isolates are defined as isolates that did not share the same plant, date, sample, and genus as any other isolate.

²16SrDNA-ST = 16SrDNA sequence type; 2 isolates were defined as having the same 16SrDNA-ST if they shared an identical partial 16S rDNA sequence.

³Isolates were obtained from coliform positive samples collected from 21 fluid milk processing plants.

for different isolates representing the most commonly isolated genera.

RESULTS

Coliform Bacteria Isolated from HTST-Pasteurized Fluid Milk Represent Diverse Enterobacteriaceae Genera

A total of 402 coliform isolates obtained from fluid milk samples collected from 21 different fluid milk processing plants in the northeast United States (Table 1; Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-9728>) were initially characterized by sequencing a 616-bp 16S rDNA fragment. The 16S rDNA sequence data were used to (1) classify isolates to the genus level, and (2) assign a 16S rDNA sequence type (16SrDNA-ST) to each isolate; 2 isolates were classified into the same 16SrDNA-ST if they shared an identical 16S rDNA sequence over the 616 bp sequenced. Genus-level characterization classified the isolates into 11 different *Enterobacteriaceae* genera, including *Enterobacter*, *Hafnia*, *Citrobacter*, *Serratia*, *Raoultella*, *Buttiauxella*, *Kluyvera*, *Pantoea*, *Cedecea*, *Rahnella*, and *Leclercia* (Table 1). The 402 isolates could be classified further into 97 different 16SrDNA-ST. The genera that included the largest number of 16SrDNA-ST were *Citrobacter* (27 sequence types), *Enterobacter* (19 sequence types), *Serratia* (13 sequence types), *Buttiauxella* (10 sequence types), and *Hafnia* (8 sequence types) (Table 1). The most commonly isolated 16SrDNA-ST within the 402 isolates was *Enterobacter* sp. 16SrDNA-ST 40 (115 isolates; Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-9728>). As multiple isolates from

the same fluid milk sample and time in shelf life were analyzed in several cases, classification data were also used to define a set of nonredundant isolates; isolates were considered redundant if they shared the same plant, date, sample, and genus. This approach yielded 240 nonredundant coliform isolates (Table 1), which were used to assess the frequency of different coliform genera in HTST-pasteurized fluid milk samples.

To assess the overall genus diversity, SID was calculated based on the number of nonredundant isolates classified into each of the 11 genera; SID at the genus level was 0.78. As the SID ranges from 0 to 1 with a larger value indicating greater diversity (Simpson, 1949), our data suggest that the isolates obtained here represent considerable diversity. Hence, phenotypic characterization of these isolates should provide a good representation of the diversity of coliform genera typical for HTST-pasteurized fluid milk in the northeast United States.

Five Enterobacteriaceae Genera Represent the Majority of Coliform Bacteria Isolated from HTST-Pasteurized Fluid Milk Samples

The most commonly isolated genera from the pasteurized fluid milk samples examined in this study were *Enterobacter* [42% of total isolates (100/240)], *Hafnia* [13% of total isolates (31/240)], *Citrobacter* [12% of total isolates (28/240)], *Serratia* [10% of total isolates (24/240)], and *Raoultella* [9% of total isolates (22/240); Table 1]. Combined, these genera accounted for 85% of the total coliforms isolated from pasteurized fluid milk samples. These 5 genera were also found across processing plants, with both *Citrobacter* and *Enterobacter* iso-

Table 2. Frequency of common coliform genera throughout fluid milk shelf life¹

Genus	No. (%) of isolates obtained by day ^{2,3}						Total no. of isolates
	Initial day	d 7	d 10	d 14	d 17	d 21	
<i>Enterobacter</i>	2 (100)	15 (45)	26 (45)	33 (45)	12 (60)	12 (63)	100
<i>Hafnia</i>	0 (0)	6 (18)	10 (17)	14 (19)	1 (5)	0 (0)	31
<i>Citrobacter</i>	0 (0)	5 (15)	9 (16)	11 (15)	2 (10)	1 (5)	28
<i>Serratia</i>	0 (0)	6 (18)	4 (7)	8 (11)	3 (15)	3 (16)	24
<i>Raoultella</i>	0 (0)	1 (3)	9 (16)	7 (10)	2 (10)	3 (16)	22
Total number	2	33	58	73	20	19	205

¹Common genera were defined as those represented by 5 or more nonredundant isolates (nonredundant isolates were defined as isolates that did not share the same plant, date, sample, and genus with any other isolate).

²Days are ± 1 d.

³Percentage values represent the percent of isolates within a given day that represent the different genera; values may not add up to 100% due to rounding.

lated from samples collected from 12 of the 21 processing plants that had coliform-positive samples (Table 1). Isolation of different genera over shelf life did not show any apparent patterns; for example, *Enterobacter* was consistently the most commonly isolated genus across d 7, 10, 14, 17, and 21, representing between 45 and 63% of isolates obtained on the different days of shelf life (Table 2).

The diversity of coliform genera isolated from samples representing a given plant also showed considerable range: 6 different genera were found in 3/21 plants, 5 different genera were found in 2/21 plants, 4 different genera were found in 1/21 plants, 3 different genera were found in 6/21 plants, 2 different genera were found in 6/21 plants, and only 1 genus was found in 3/21 processing plants. Among the plants included here, samples were typically collected over 2 or sometimes 3 visits to a given plant with 4 to 6 mo between visits. Interestingly, for 10 of the study plants, isolates representing the same genus were obtained from samples collected over sepa-

rate visits (Table 3). For 3 plants (plants A, F, and S; see Table 3), isolates with the same 16SrDNA-ST were obtained from samples collected over 2 separate visits, providing preliminary evidence for a persistent coliform source in these facilities. Overall, characterization to 16SrDNA-ST allowed for considerable discrimination among the 240 nonredundant isolates, as supported by an SID of 0.85.

All Coliform Isolates Tested Displayed a 2-Log Increase in Bacterial Growth over 10 Days at 6°C

Across the 104 representative coliform isolates chosen for cold growth analysis at 6°C for 10 d (Table 4), the average bacterial counts at d 0, 2, 4, 6, 8, and 10 were 2.08 ± 0.57 , 2.76 ± 0.86 , 4.11 ± 1.66 , 5.70 ± 2.29 , 7.66 ± 2.15 , and 8.23 ± 1.92 mean $\log_{10}$ cfu/mL, respectively. Based on the development of bacterial numbers over 10 d at 6°C, we calculated, for each bacterial isolate, the parameter “bacterial growth at 6°C over 10 d,” which

Table 3. Plants where identical coliform genera and 16SrDNA-ST were isolated from HTST-pasteurized fluid milk samples collected on separate sampling visits

Plant	Re-isolated genus	16SrDNA-ST ¹ (no. of isolates) for isolates representing the re-isolated genus		
		Visit A	Visit B	Visit C
A	<i>Enterobacter</i>	40 (3), 43 (1), 49 (2)	40 (2), 58 (1)	NA ²
B	<i>Enterobacter</i>	40 (5)	44 (1), 52 (8), 53 (1), 56 (1), 57 (1)	NA
E	<i>Cedecea</i>	13 (2)	12 (4)	NA
F	<i>Raoultella</i>	81 (4)	80 (3), 81 (4)	NA
H	<i>Citrobacter</i>	38 (2)	14 (1), 15 (2), 16 (1)	NA
O	<i>Raoultella</i>	80 (6)	83 (12), 84 (1)	NA
P	<i>Buttiauxella</i>	10 (1)	4 (1), 9 (1), 5 (1)	NA
Q	<i>Citrobacter</i>	17 (1)	0	21 (1), 26 (1)
	<i>Enterobacter</i>	0	40 (51), 43 (4), 46 (1)	54 (2)
	<i>Serratia</i>	95 (1), 97 (1)	89 (17), 90 (1), 91 (1), 92 (1)	0
S	<i>Enterobacter</i>	40 (6)	40 (2), 43 (1)	NA
T	<i>Enterobacter</i>	49 (11), 48 (3)	40 (4)	NA

¹16SrDNA-ST = 16SrDNA sequence type; 2 isolates are defined as having the same 16SrDNA-ST if they share an identical partial 16S rDNA sequence.

²Not applicable.

was defined as log bacterial numbers at d 10 minus the log bacterial numbers at d 0. Average bacterial growth of all coliform isolates tested at 6°C was 5.88 ± 1.62 logs. Of the 104 representative isolates tested, 74 isolates had growth greater than 5 logs over 10 d at 6°C; the remaining isolates all showed between 2 and 5 logs of growth. The most substantial average growth at 6°C was observed for isolates representing the genera *Rahnella*, *Serratia*, and *Buttiauxella* (7.98, 7.31, and 6.86 logs, respectively; Table 4). The average bacterial growth over 10 d at 6°C of each of the 5 most commonly isolated genera (*Citrobacter*, *Enterobacter*, *Hafnia*, *Raoultella*, and *Serratia*) exceeded 5.10 logs. Comparisons of the growth over 10 d at 6°C for different isolates within the 5 most common genera showed considerable diversity of growth within certain genera (Figure 1). Isolates within the genus *Citrobacter* had the largest range of bacterial growth, spanning over 5 logs from 2.00 to 7.13 logs (Figure 1). *Serratia* and *Enterobacter* isolates showed log growth at 6°C ranging from 4.64 to 8.24 logs and 2.77 to 6.99 logs, respectively. *Raoultella* and *Hafnia* isolates showed the smallest range of bacterial growth, ranging from 4.00 to 6.51 logs and 5.07 to 7.60 logs, respectively (Figure 1).

Coliform Bacteria Isolated from HTST-Pasteurized Fluid Milk Samples Differed in Lipolysis and Proteolysis Phenotypes

The 104 representative isolates tested for cold growth were also analyzed for lipolytic and proteolytic activity

after incubation at 6°C for 14 d. Among the 104 isolates tested for lipolysis, 59 isolates scored (–) (no visible clearing around streak area on SB), 29 isolates scored (+) [small zone of clearing (≤ 2 mm) around streak area on SB], and 16 isolates scored (++) (clearing > 2 mm on SB) (Table 4). We detected a significant association between lipolytic activity [with isolates defined as showing lipolytic activity if they were scored as a (+) or (++)] and genus of the isolates tested ($P < 0.0001$; Fisher's exact test), indicating that the proportion of isolates that show lipolytic capabilities differs between coliform genera. Only *Serratia* isolates showed strong lipolysis (++) , with the majority of the representative *Serratia* (93%) isolates characterized showing this lipolysis phenotype. For both the genus *Enterobacter* and the genus *Pantoea*, the majority of representative isolates (70 and 80%, respectively) scored as lipolytic (+) on SB; for all other genera, the majority of isolates showed no evidence of lipolysis (–).

Among the 104 representative isolates tested for proteolysis, 30 isolates scored (–) (no visible clearing around the streak area on SMA), 64 isolates scored as (+) (noticeable clearing around the streak area on SMA), and 10 isolates scored as (++) (an obvious clear zone and opaque zone surrounding the streak area on SMA) (Table 4). Similar to the analysis of lipolytic activity, we found a significant association between the proteolytic activity [defined as a (+) or (++)] and genera of the isolates tested ($P = 0.01$; Fisher's exact test), indicating that the proportion of isolates that show proteolytic capabilities differs between coliform genera. The genera *Serratia* and *Buttiauxella* had the

Table 4. Phenotypic characteristics of 104 representative coliform isolates tested for cold growth as well as lipolytic and proteolytic capabilities

Genus	No. of isolates tested ¹	Δ growth [log d 10 – log d 0]	Day 14 lipolysis score ²			Day 14 proteolysis score ³		
			(–)	(+)	(++)	(–)	(+)	(++)
<i>Citrobacter</i>	27	5.22	22	5	0	4	22	1
<i>Enterobacter</i>	20	5.12	6	14	0	7	13	0
<i>Serratia</i>	17	7.31	0	1	16	4	6	7
<i>Buttiauxella</i>	10	6.86	10	0	0	0	8	2
<i>Hafnia</i>	9	6.57	9	0	0	7	2	0
<i>Kluyvera</i>	5	5.18	4	1	0	2	3	0
<i>Pantoea</i>	5	6.57	1	4	0	2	3	0
<i>Raoultella</i>	5	5.17	4	1	0	3	2	0
<i>Rahnella</i>	3	7.98	3	0	0	0	3	0
<i>Cedecea</i>	2	3.47	0	2	0	0	2	0
<i>Leclercia</i>	1	2.87	0	1	0	1	0	0
Total	104	—	59	29	16	30	64	10

¹16S rDNA sequence data were used to select isolates for phenotypic characterization (cold growth, proteolytic and lipolytic capabilities).

²Lipolysis was tested on spirit blue agar: (–) = no visible clearing around streak area; (+) = small zone of clearing around streak area (≤ 2 mm) but remainder of the plate remains blue; (++) = clearing around the streak area greater than 2 mm.

³Proteolysis was tested on skim milk agar: (–) = no visible clearing around streak area; (+) = noticeable clearing around streak area; (++) = obvious clear zone and opaque zone surrounding the streak area.

largest percentage of representative isolates score as (++) , with 41 and 20% of isolates, respectively.

DISCUSSION

Although detection of coliforms is frequently used as an indicator of the hygienic quality of HTST-pasteurized fluid milk, our understanding of the taxonomic and phenotypic diversity of coliforms associated with this product produced in contemporary processing plants is limited. This study showed that a considerable

diversity of coliforms can be found in fluid milk and we defined some of the most common coliform genera found in HTST-pasteurized fluid milk. Coliforms isolated from HTST-pasteurized milk in general showed considerable growth capabilities at 6°C and virtually all coliforms isolated here should be considered “psychrotolerant” (defined here as having the ability to grow at refrigeration temperatures). Overall, our data suggest that the PPC population of coliform bacteria is composed of diverse members capable of cold growth as well as lipolysis and proteolysis. Thus, these bacteria

Figure 1. Log growth from d 0 to 10 of refrigerated storage at 6°C for each isolate within commonly isolated genera from pasteurized fluid milk. Log growth = log bacterial numbers at d 10 – log bacterial numbers at d 0.

can likely lead to loss of product quality due to spoilage and sensory defects.

Coliform Bacteria Isolated from HTST-Pasteurized Milk Samples Are Diverse

Eleven different coliform bacteria genera were represented by the isolates obtained from HTST-pasteurized fluid milk samples in the northeast United States. These data suggest that postpasteurization contaminants from fluid milk represent a diverse collection of coliform bacteria rather than a homogeneous population. In previous work that characterized gram-negative bacteria obtained from bulk tank raw milk samples stored at 4°C, researchers isolated coliform bacteria representing the genera *Citrobacter*, *Enterobacter*, *Hafnia*, and *Serratia* from samples collected in the United States in 1997 (Jayarao and Wang, 1999) and representing the same genera (*Citrobacter*, *Enterobacter*, *Hafnia*, and *Serratia*) from samples collected in Ireland in 2005 (Kagkli et al., 2007). These genera match 4 of the 5 most commonly isolated coliform genera reported here. When specifically assessing pasteurized milk and dairy products collected in 1988 in South Africa, researchers isolated coliform bacteria representing the genera *Citrobacter*, *Enterobacter*, *Serratia*, *Hafnia*, and *Cedecea* (Wessels et al., 1989), consistent with our current results. Hence, similar coliform genera appear to be consistently isolated from dairy-associated samples collected in different studies and in different countries. Interestingly, Såde and colleagues (2013) isolated *Hafnia* spp., *Pantoea* spp., *Rahnella* spp., *Buttiauxella* spp., and *Serratia* spp. from modified-atmosphere packed meats and poultry collected in Finland at the end of chilled storage at 6°C, suggesting that similar psychrotolerant coliforms are present in different types of food products.

Three coliform genera (*Raoultella*, *Leclercia*, and *Kluyvera*) were isolated here but have not been reported in the key studies on coliforms in dairy products discussed above (Wessels et al., 1989; Jayarao and Wang, 1999; Kagkli et al., 2007). The genus *Raoultella* was previously classified as part of the genus *Klebsiella* (a coliform group commonly found associated with dairy plant equipment; Drancourt et al., 2001; Tang et al., 2009; Zadoks et al., 2011). *Raoultella* spp. may thus have been reported as *Klebsiella* in previous studies; interestingly none of the isolates obtained here were identified as *Klebsiella*. Conversely, isolation of *Leclercia* spp. and *Kluyvera* spp. was unexpected because of their limited association with food products thus far in previous research. The genus *Leclercia* was formerly part of the genus *Escherichia* and has been documented

as having associations with clinical human infections (Temesgen et al., 1997; Hess et al., 2008). Little is known about the ecology and diversity of the genus *Kluyvera*, which was formerly identified as enteric group 8, except that it is a coliform bacteria associated with the environment as well as clinical human infections (Farmer et al., 1981; Sarria et al., 2001; Carter and Evans, 2005). Importantly though, only 2 and 13 of the 240 nonredundant coliform isolates characterized here represented the genera *Leclercia* and *Kluyvera*. Additionally, the *Leclercia* isolates were all obtained from a single visit to one dairy plant and therefore may be the result of a factor unique to that plant.

Coliform Isolates Obtained from Pasteurized Fluid Milk Can Survive and Grow at 6°C

Previous research has suggested that coliform bacteria are poor competitors at low temperatures (Anand and Griffiths, 2011). However, in the absence of competition with other bacteria for nutrients, coliform bacteria have been found to grow at a wide range of temperatures, including refrigeration temperatures (Stead, 1986; Anand and Griffiths, 2011). Therefore, pasteurized fluid milk kept at refrigeration temperature may be an ideal environment for these spoilage organisms, particularly if the milk is not contaminated with any other psychrotolerant bacteria. The ability of coliforms to grow in commercial pasteurized milk is also supported by a few previous studies, although limited data are available on the specific gram-negative organisms that caused fluid milk spoilage, except for a few studies on psychrotolerant *Pseudomonas* spp., which are often considered key organisms linked to fluid milk spoilage (Schröder et al., 1982; Eneroth et al., 2000; Dogan and Boor, 2003; Martin et al., 2012). For example, a recent 10-yr study of HTST-pasteurized fluid milk quality in NYS found that 11% (111/1,008) of fluid milk samples showed coliform counts of more than 10 cfu/mL before d 14 of refrigerated storage (Martin et al., 2012). Additionally, a 1982 study of PPC of fluid milk in 5 United Kingdom dairies (Schröder et al., 1982) also found that 100% of isolates from spoiled milk (showing off-flavor or bitterness as early as at 10 d of storage at 5°C) were gram-negative rods (Schröder et al., 1982). A 1999 study of PPC of fluid milk in both a Swedish and a Norwegian dairy found that close to 20% of all pasteurized milk samples were positive for gram-negative psychrotolerant bacteria by 11 d of storage at 7°C (Eneroth et al., 2000). Although isolates from the 2 previous studies (Schröder et al., 1982; Eneroth et al., 2000) were not further characterized and thus may include noncoliform gram-negatives (e.g., *Pseudomonas*

spp.), these data further support the importance of gram-negative spoilage organisms in HTST-pasteurized fluid milk.

Our data also showed that a large proportion of the coliforms characterized showed considerable growth capabilities at 6°C, with 71% of isolates showing >5 logs of growth over 10 d. This is consistent with previous data, which showed that the majority of coliform genera isolated from milk and dairy products in South Africa were capable of growth on SPC agar incubated at 7°C for 10 d (Wessels et al., 1989); specifically, 100% of isolates representing *Citrobacter freundii* (13), *Enterobacter agglomerans* (10), *Serratia marcescens* (3), and *Hafnia alvei* (3) grew on SPC agar after 10 d at 7°C (Wessels et al., 1989). Our data also showed that some coliform genera included isolates that differed considerably in their ability to grow at 6°C in SMB. This may represent overall phenotypic diversity within a genus or may reflect distinct characteristics for different species within a given genus. For example, in a previous study, Wessels and colleagues (1989) reported that 100% of *Enterobacter agglomerans* and 89% of *Enterobacter cloacae* isolates were capable of psychrotrophic growth.

PPC Coliform Isolates Obtained from HTST-Pasteurized Fluid Milk Varied in Lipolytic and Proteolytic Capabilities

As sensory defects in fluid milk resulting from lipolysis and proteolysis of the product have direct effects on both overall milk quality as well as consumer acceptance (Ma et al., 2000; Santos et al., 2003), we further characterized representative coliform isolates obtained here for proteolytic and lipolytic capabilities when grown at 6°C. The release of free fatty acids from triglycerides in the milk, known as lipolysis, results in a “rancid” flavor defect of the product (Shipe et al., 1978). The accumulation of small peptides as a result of proteolysis of milk results in a flavor defect that is described as “bitter” or “astringent” (Harwalkar et al., 1989; Ma et al., 2000). Our results showed that isolates representing 11 different coliform genera identified had varying capabilities of lipolysis; 57% of the isolates showed no lipolytic reaction, whereas 43% showed lipolysis, scoring (+) or (++). This observation differs from previous research (Juven et al., 1981; Wessels et al., 1989), which indicated limited lipolytic capabilities of coliform bacteria isolated from dairy products and raw milk in Israel and South Africa. For example, one study showed that among 75 *Enterobacteriaceae* isolates from dairy products tested for lipolysis, only 7 isolates (9%; 3/17 *Enterobacter cloacae*; 2/3 *Serratia marcescens*; 1/14 *Klebsiella oxytoca*; 1/2 *Klebsiella pneumoniae*) showed lipolytic activity on Victoria blue

butterfat agar (Wessels et al., 1989). These differences in the frequency of lipolysis phenotype among dairy isolates from different studies may be due, at least in part, to different methods of measuring lipolysis or to the small isolate sets characterized in some of these previous studies. For example, in one study examining the changes in refrigerated milk caused by psychrotolerant strains of *Enterobacteriaceae* isolated from raw milk, researchers found that none of the 6 strains produced any lipolytic activity on Tween 80 agar (Juven et al., 1981). Our study reported here not only tested >15 times more isolates, allowing for better representation of strain diversity, but also used spirit blue agar to detect lipolytic activity. This is relevant, as previous work has suggested that the lipolytic activity detected with either Tween 80 or tributyrin as the substrate is not correlated (Plou et al., 1998), and both tributyrin and polysorbate (Tween) 80 were substrates in the spirit blue agar lipase reagent that was used here. We surmise that lipolysis activity determined using the spirit blue agar lipase reagent is more relevant to dairy products than lipolysis activity detected only with Tween 80, as the reagent contains both tributyrin (butyric acid is a component of milk fat) and Tween 80 (containing oleic acid, another component of milk fat) (Garton, 1963; MacGibbon and Taylor, 2006).

Similar to the lipolysis results, we found that proteolytic activity differed between and within the coliform genera in the current study; nearly 30% of the isolates tested showed no observed proteolysis, whereas only 10% of the isolates, predominantly representing the genus *Citrobacter*, showed a strong proteolytic reaction. By comparison, previous studies (Juven et al., 1981; Wessels et al., 1989) reported limited proteolytic capabilities for *Enterobacteriaceae* isolated from dairy products. For example, one study (Juven et al., 1981) reported that none of 6 psychrotolerant *Enterobacteriaceae* isolates obtained from raw milk (collected in Israel) exhibited proteolytic activity when evaluated on skim milk agar. Similarly, Wessels and colleagues (1989) reported that *Enterobacteriaceae* strains isolated from milk and dairy products (collected in South Africa) were generally nonproteolytic; the only species identified as proteolytic in this study were *E. cloacae*, *K. oxytoca*, and *Serratia rubidua*. Overall, our results suggest that proteolytic activity may be more common among dairy-associated coliforms than reported in previous studies.

CONCLUSIONS

Our data indicate that psychrotolerant coliforms introduced as PPC in fluid milk represent considerable taxonomic diversity. In addition, a considerable propor-

tion of these coliform isolates were characterized by the ability to exhibit lipolytic and proteolytic activity. As contamination with psychrotolerant coliforms has been shown to influence the sensory characteristics and consumer acceptability of fluid milk, continued efforts are clearly needed to reduce PPC with coliforms. Future efforts are needed to specifically define the sources of coliform PPC, particularly because our data suggested that some coliform types (either specific genera or 16SrDNA-ST) were repeatedly isolated in a given facility, suggesting point source introduction of coliforms in some plants. Additionally, future work is needed to explore possible differences in cold growth and enzymatic activity capabilities within multiple coliform genera and subtypes. Overall, general hygienic issues within a fluid milk processing plant may lead to not only the introduction of coliform bacteria, but to potential PPC with a diverse group of coliform contaminants capable of having a direct effect on pasteurized milk quality and the consumer's sensory experience.

ACKNOWLEDGMENTS

This project was supported by the New York State Milk Promotion Advisory Board (contract #C200796) through the New York State Department of Agriculture and Markets (Albany, NY). The authors thank the management and employees of all participating processing plants; Cornell University Cooperative Extension personnel for obtaining samples; Laura Strawn (Virginia Polytechnic Institute and State University, Blacksburg) for critical review of the manuscript; as well as members of the Cornell University Milk Quality Improvement Program for laboratory support.

REFERENCES

- Anand, S. K., and M. W. Griffiths. 2011. *Enterobacteriaceae*. Pages 67–71 in *Encyclopedia of Dairy Sciences*. 2nd ed. Fuquay, J. W., P. F. Fox, and P. L. H. McSweeney, ed. Academic Press, San Diego, CA.
- Bergey, D. H., R. S. Breed, E. G. D. Murray, and A. P. Hitchens. 1939. *Enterobacteriaceae*. Pages 587–959 in *Bergey's Manual of Determinative Bacteriology*. 5th ed. The Williams and Wilkins Company, Baltimore, MD.
- Carter, J. E., and T. N. Evans. 2005. Clinically significant *Kluyvera* infections: A report of seven cases. *Am. J. Clin. Pathol.* 123:334–338.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The ribosomal database project (RDP-II): Sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294–D296.
- Dogan, B., and K. J. Boor. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. *Appl. Environ. Microbiol.* 69:130–138.
- Drancourt, M., C. Bollet, A. Carta, and P. Rousselier. 2001. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int. J. Syst. Evol. Microbiol.* 51:925–932.
- Eneroth, Å., S. Ahrne, and G. Molin. 2000. Contamination of milk with gram-negative spoilage bacteria during filling of retail containers. *Int. J. Food Microbiol.* 57:99–106.
- Eneroth, Å., A. Christiansson, J. Brendehaug, and G. Molin. 1998. Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. *Int. Dairy J.* 8:829–834.
- Fairbairn, D. J., and B. A. Law. 1986. Proteinases of psychrotrophic bacteria: Their production, properties, effects and control. *J. Dairy Res.* 53:139–177.
- Farmer, J. J., G. R. Fanning, G. P. Huntley-Carter, B. Holmes, F. W. Hickman, C. Richard, and D. J. Brenner. 1981. *Kluyvera*, a new (redefined) genus in the family *Enterobacteriaceae*: Identification of *Kluyvera ascorbata* sp. nov. and *Kluyvera cryocrescens* sp. nov. in clinical specimens. *J. Clin. Microbiol.* 13:919–933.
- FDA. 2011. Standards for grade “A” milk and milk products. Pages 27–30 in *Grade “A” Pasteurized Milk Ordinance*. Standards for grade “A” milk and milk products. US Department of Health and Human Services, Public Health Service, Washington, DC.
- Feng, P., S. D. Weagant, M. A. Grant, and W. Burkhardt. 2002. Enumeration of *Escherichia coli* and the coliform bacteria. Chapter 4 in *Bacteriological Analytical Manual*. 8th ed. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD. Accessed Jan. 20, 2015. <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm>.
- Frank, J. F., and A. E. Yousef. 2004. Test for groups of microorganisms. Pages 227–248 in *Standard Methods for the Examination of Dairy Products*. 17th ed. H. M. Wehr and J. F. Frank, ed. Am. Public Health Assoc., Washington, DC.
- Garton, G. A. 1963. The composition and biosynthesis of milk lipids. *J. Lipid Res.* 4:237–254.
- Harwalkar, V. R., B. Boutin-Muma, H. Cholette, R. McKellar, D. Emmons, and G. Klassen. 1989. Isolation and partial purification of astringent compounds from ultra-high temperature sterilized milk. *J. Dairy Res.* 56:367–373.
- Hess, B., A. Burchett, and M. K. Huntington. 2008. *Leclercia ad-carboxylata* in an immunocompetent patient. *J. Med. Microbiol.* 57:896–898.
- Huck, J. R., M. Sonnen, and K. J. Boor. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *J. Dairy Sci.* 91:1218–1228.
- Imhoff, J. 2005. *Enterobacteriales*. Pages 587–850 in *Bergey's Manual of Systematic Bacteriology*. D. Brenner, N. Krieg, J. Staley, G. Garrity, D. Boone, P. De Vos, M. Goodfellow, F. Rainey, and K. H. Schleifer, ed. Springer, New York, NY.
- Immanuel, G., P. Esakkiraj, A. Jebadhas, P. Iyapparaj, and A. Palavesam. 2008. Investigation of lipase production by milk isolate *Serratia rubidaea*. *Food Technol. Biotechnol.* 46:60–65.
- Jayarao, B. M., and L. Wang. 1999. A study on the prevalence of gram-negative bacteria in bulk tank milk. *J. Dairy Sci.* 82:2620–2624.
- Juven, B., S. Gordin, I. Rosenthal, and A. Laufer. 1981. Changes in refrigerated milk caused by enterobacteriaceae. *J. Dairy Sci.* 64:1781–1784.
- Kagkli, D. M., M. Vancanneyt, P. Vandamme, C. Hill, and T. M. Cogan. 2007. Contamination of milk by enterococci and coliforms from bovine faeces. *J. Appl. Microbiol.* 103:1393–1405.
- Ma, Y., C. Ryan, D. Barbano, D. Galton, M. Rudan, and K. Boor. 2000. Effects of somatic cell count on quality and shelf-life of pasteurized fluid milk. *J. Dairy Sci.* 83:264–274.
- MacGibbon, A. K. H., and M. W. Taylor. 2006. Composition and structure of bovine milk lipids. Pages 1–42 in *Advanced Dairy Chemistry*, Volume 2. 3rd ed. P. F. Fox and P. L. McSweeney, ed. Springer, New York, NY.
- Martin, N. H., N. R. Carey, S. C. Murphy, M. Wiedmann, and K. J. Boor. 2012. A decade of improvement: New York state fluid milk quality. *J. Dairy Sci.* 95:7384–7390.
- Martin, N. H., M. L. Ranieri, S. C. Murphy, R. D. Ralysa, M. Wiedmann, and K. J. Boor. 2011. Results from raw milk microbiologi-

- cal tests do not predict the shelf-life performance of commercially pasteurized fluid milk. *J. Dairy Sci.* 94:1211–1222.
- Nörnberg, M. F., R. S. Friedrich, R. D. Weiss, E. C. Tondo, and A. Brandelli. 2010. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. *Int. J. Dairy Technol.* 63:41–46.
- Plou, F., M. Ferrer, O. Nuero, M. Calvo, M. Alcalde, F. Reyes, and A. Ballesteros. 1998. Analysis of tween 80 as an esterase/ lipase substrate for lipolytic activity assay. *Biotechnol. Tech.* 12:183–186.
- Ralyea, R. D., M. Wiedmann, and K. J. Boor. 1998. Bacterial tracking in a dairy production system using phenotypic and ribotyping methods. *J. Food Prot.* 61:1336–1340.
- Ranieri, M. L., and K. Boor. 2009. Short communication: Bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. *J. Dairy Sci.* 92:4833–4840.
- Säde, E., A. Murros, and J. Björkroth. 2013. Predominant enterobacteria on modified-atmosphere packaged meat and poultry. *Food Microbiol.* 34:252–258.
- Santos, M. V., Y. Ma, Z. Caplan, and D. M. Barbano. 2003. Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. *J. Dairy Sci.* 86:1601–1607.
- Sarria, J. C., A. M. Vidal, and R. C. Kimbrough 3rd.. 2001. Infections caused by *Kluyvera* species in humans. *Clin. Infect. Dis.* 33:E69–E74.
- Schröder, M. J. A., C. M. Cousins, and C. H. McKinnon. 1982. Effect of psychrotrophic post-pasteurization contamination on the keeping quality at 11 and 5°C of HTST-pasteurized milk in the UK. *J. Dairy Res.* 49:619–630.
- Shipe, W., R. Bassette, D. Deane, W. Dunkley, E. Hammond, W. Harper, D. Kleyn, M. Morgan, J. Nelson, and R. Scanlan. 1978. Off flavors of milk: Nomenclature, standards, and bibliography. *J. Dairy Sci.* 61:855–869.
- Simpson, E. H. 1949. Measurement of diversity. *Nature* 163:688.
- Stead, D. 1986. Microbial lipases: Their characteristics, role in food spoilage and industrial uses. *J. Dairy Res.* 53:481–505.
- Tang, X., S. H. Flint, R. J. Bennett, J. D. Brooks, and R. H. Morton. 2009. Biofilm growth of individual and dual strains of *Klebsiella oxytoca* from the dairy industry on ultrafiltration membranes. *J. Ind. Microbiol. Biotechnol.* 36:1491–1497.
- Temesgen, Z., D. R. Toal, and F. R. Cockerill. 1997. *Leclercia ad-eccarboxylata* infections: Case report and review. *Clin. Infect. Dis.* 25:79–81.
- Wessels, D., P. J. Jooste, and J. F. Mostert. 1989. Psychrotrophic, proteolytic and lipolytic properties of enterobacteriaceae isolated from milk and dairy products. *Int. J. Food Microbiol.* 9:79–83.
- Zadoks, R. N., H. M. Griffiths, M. A. Munoz, C. Ahlstrom, G. J. Bennett, E. Thomas, and Y. H. Schukken. 2011. Sources of *Klebsiella* and *Raoultella* species on dairy farms: Be careful where you walk. *J. Dairy Sci.* 94:1045–1051.