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Genetic Diversity and Spoilage Potentials among *Pseudomonas* spp. Isolated from Fluid Milk Products and Dairy Processing Plants

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Degradation of milk components through various enzymatic activities associated with the contamination of dairy products by *Pseudomonas* spp. can reduce the shelf life of processed milk. Reliable methods for differentiating among *Pseudomonas* spp. strains are necessary to identify and eliminate specific sources of bacterial contamination from dairy processing systems. To that end, we assessed the genetic diversity and dairy product spoilage potentials among a total of 338 *Pseudomonas* spp. isolates from raw and pasteurized milk and from environmental samples collected from four dairy processing plants. The majority of isolates were identified as *P. fluorescens* and *P. putida* by API 20 NE. A total of 42 different ribotype patterns were identified among a subset of 81 isolates. The presence of many different ribotypes within this collection indicates high genetic diversity among the isolates and suggests multiple origins of contamination within the processing plant and in dairy products. The extracellular enzyme activity patterns among *Pseudomonas* isolates appeared to be associated with ribotypes. Isolates with the same ribotype frequently had the same extracellular protease, lecithinase, and lipase activities. For example, isolates grouped in ribotype 55-S-6 had the highest extracellular protease activity, while those in ribotypes 50-S-8 and 72-S-3 had the highest extracellular lipase activities. We conclude that ribotyping provides a reliable method for differentiating *Pseudomonas* strains with dairy food spoilage potential.

Bacterial spoilage causes significant economic losses for the food industry. Product contamination with psychrotrophic microorganisms is a particular concern for the dairy industry as dairy products are distributed at temperatures permissive for the growth of these organisms. The diverse microbes that may be categorized as psychrotrophic are ubiquitous in nature and can be isolated from soil, water, and vegetation (8). As psychrotrophic bacteria typically enter processed dairy products through postpasteurization contamination in the milk processing plant (21, 23), these microbes may account for only a small fraction of the initial flora of processed milk. Bacterial spoilage ensues when growth conditions during refrigerated storage allow psychrotrophic microbes to increase in number and to become the dominant microflora (9).

Currently, the predominant microorganisms limiting the shelf life of processed fluid milk at 4°C are *Pseudomonas* spp. (9, 11, 29). In addition to the ability of *Pseudomonas* spp. to grow to high numbers during refrigerated storage, many of these strains also produce heat-stable extracellular lipases, proteases, and lecithinases which can further contribute to milk spoilage (6, 8, 26, 27). Many of these enzymes remain active, even following thermal processing steps that can destroy the organisms that produce these enzymes (13, 20, 27). Degradation of milk components through various enzymatic activities can reduce the shelf life of processed milk. For example, digestion of casein by proteases can lead to a bitter flavor and the clotting and gelation of milk. Lipases hydrolyze tributyrin and milk fat to yield free fatty acids, which cause milk to taste rancid, bitter, unclean, and soapy. Lecithinase degrades milk fat globule membranes and increases the susceptibility of milk fat to the action of lipases (8, 10, 26). The hydrolytic products of milk fats and proteins decrease the organoleptic quality of fluid milk products.

Not all *Pseudomonas* spp. strains are equally capable of producing degradative defects in processed fluid milk samples (11, 32). Reliable methods for differentiating *Pseudomonas* spp. strains with food spoilage potential from strains that are less destructive are necessary to identify and eliminate the environmental contamination sources of the destructive strains. Traditional microbiological techniques for bacterial identification, such as observation of growth patterns on selective and differentiative media, biochemical reactions, and microscopy, often lack discriminatory power and usually are ineffective in establishing a causal relationship between contamination of the finished product and the environmental source. Furthermore, results can be difficult to reproduce. Genotypic methods such as ribotyping offer enhanced discriminatory power (30). Ribotyping has been demonstrated to rapidly and reproducibly type bacterial isolates to the genus, species, and strain levels (4, 5).

Our objectives in this study were as follows: (i) to assess the genetic diversity of *Pseudomonas* spp. in milk and dairy processing environments and (ii) to determine the association between genetic types (ribotypes) of *Pseudomonas* spp. and their potentials to cause spoilage (i.e., production of proteolytic and lipolytic enzymes). Our results show high diversity among *Pseudomonas* spp. in dairy plants. We also found a strong association between *Pseudomonas* spp. ribotype and spoilage capacity.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *Pseudomonas* spp. isolates from raw and pasteurized milk samples were obtained as part of the shelf life testing

TABLE 1. Number of *Pseudomonas* isolates and ribotypes obtained from each dairy processing plant

Sample source	Dairy A		Dairy B		Dairy C		Dairy D		Total	
	No. of isolates	No. of ribotypes[a]	No. of isolates	No. of ribotypes	No. of isolates	No. of ribotypes	No. of isolates	No. of ribotypes	No. of isolates	No. of ribotypes
Processed milk	28	1 (3)	60	6 (9)	57	5 (12)	56	8 (12)	201	21 (36)
Raw milk	12	3 (6)	10	4 (5)	10	3 (3)	15	5 (6)	47	15 (20)
Environment	4	3 (3)	30	5 (5)	23	5 (5)	33	6 (12)	90	17 (25)
Total	54	7 (12)	100	14 (19)	90	13 (20)	104	17 (30)	338	42 (81)

[a] Numbers in parentheses indicate the number of isolates selected for ribotyping.

program of the Cornell University Milk Quality Improvement Program (2). Raw and processed milk samples and environmental samples from the four processing plants involved in this program were collected over a 15-month period. Each processing plant was visited at least three times. Processed milk samples were collected during the first and second visits, raw milk was collected during the second visit, and environmental samples were collected during the third visit.

The numbers of viable bacteria in pasteurized and raw milk samples were determined by standard plate counts (SPC) and psychrotrophic plate counts (PPC) (19). Pasteurized milk samples were plated at days 0, 7, and 14 after receipt. For each sample, 10 to 15 *Pseudomonas*-like colonies were picked randomly from representative SPC plates and streaked to purification on plate count agar (PCA; Difco, Detroit, Mich.). Environmental samples were collected from the processing environment (floor, drains, pipes, filling bowl interior, pasteurized-milk storage tanks, and valves) by using sterile sponges moistened with neutralizing buffer (Hardy Diagnostics, Santa Maria, Calif.). Environmental samples were transported to the laboratory in sterile stomacher bags, stored at 4°C, and analyzed within 24 h. A volume of 25 to 50 ml of phosphate buffer was added to the bags, and 10-fold serial dilutions were plated onto cetrimide-fucidin-cephaloridine (Oxoid, Basingstoke, United Kingdom)-supplemented *Pseudomonas* agar base (Oxoid) plates. Ten to fifteen colonies were chosen randomly from each sample. All single colonies were initially characterized according to Gram staining results and oxidase and catalase activities. The colonies that were confirmed as putative *Pseudomonas* spp. (gram negative, oxidase positive, and catalase positive) were further characterized as described below.

Phenotypic characterization. All isolates were characterized by using API 20 NE strips (BioMerioux Vitek, Inc., Hazelwood, Mo.) according to the manufacturer's instructions. Species identifications were obtained by using the API database. Oxidase reactions of the isolates were determined using BBLDrySlide oxidase slides (Becton Dickinson Company, Sparks, Md.) according to the manufacturer's instructions. For catalase activity, 1.5% H_2O_2 was dropped onto a bacterial smear that had been placed on a glass slide. Gas production indicated a positive reaction.

Extracellular-enzyme production. To determine the production of extracellular proteases, lipases, and lecithinases, *Pseudomonas* colonies were streaked to isolation on PCA and then single colonies were transferred to agar plates containing the appropriate substrates. Production of extracellular proteolytic enzymes was determined on PCA (Difco) containing 1% skim milk powder (skim milk agar) (31). After incubation at 30°C for 72 h, the plates were flooded with 1 N HCl for observation of clearance zones formed by protease-positive strains. For each isolate, clearance zones around two to five colonies were measured and the ratio of the clearance zone diameter to the colony diameter was calculated. The assay was repeated at least three times, and the mean ratios and standard deviations are reported. Production of extracellular lipase was assessed using single-layer agar (31) consisting of 5% (wt/vol) clarified butterfat (16) and Victoria blue B blended into tryptic soy agar (Difco) (1:7,500 dilution). After being incubated at 30°C for up to 5 days, the plates were observed for the presence of colonies surrounded by dark blue zones. Lipase activity was assessed by noting the color intensity surrounding the colonies. Following visual inspection, the color intensity was categorized on a scale of 1 to 4, where 1 represents light blue and 4 represents very dark blue. Production of extracellular lecithinase was determined on PCA containing 10% egg yolk emulsion (Sigma, St. Louis, Mo.). After incubation at 30°C for up to 5 days, the diameters of the opaque rings surrounding lecithinase-positive colonies were measured for two to three representative colonies for each isolate. The ratio of the size of the opaque zone to that of the colony was calculated. The mean ratios and standard deviations from three assays are reported.

In addition to the 338 isolates from this study, a total of 27 *Pseudomonas* isolates were selected from a previous study for the enzyme assays (32).

Ribotyping. Isolates with different API 20 NE codes were selected for ribotyping. Colonies obtained from different samples but with identical API codes were treated as independent isolates.

Bacteria were grown on brain heart infusion agar (Becton Dickinson) overnight at 30°C and ribotyped by using the restriction enzyme *Eco*RI in a RiboPrinter (Qualicon Inc., Wilmington, Del.) as described previously (5).

Sensory analyses. Sensory analyses of the pasteurized milk samples were performed by 10 trained members of a sensory panel as follows. Milk samples were mixed by inversion, and then, in dim light, 60 ml of each sample was poured into 148-ml plastic cups, which were capped with plastic lids and presented at 15°C to panel members. Panelists evaluated the milk according to the procedures of Chapman et al. (7). Milk flavor was scored on a scale from 1 to 10, where any rating below 6 was considered poor, 6 to 7 was fair, and 8 to 10 was good. The overall scores were assigned with a predominant sensory criticism. Unsalted crackers and spring water were provided to cleanse the palate. Panelists were provided monetary incentives to encourage participation through the completion of the experiments. The Compusense *five* (Compusense Inc., Guelph, Canada) computerized data collection system was used to determine the order of presentation of the samples and to collect the data.

RESULTS

Ecology of *Pseudomonas* in dairy processing plants. The genetic diversity of *Pseudomonas* spp. isolated from four dairy processing plants was studied. A total of 338 putative *Pseudomonas* isolates were obtained from raw milk, processed milk, and environmental samples (Table 1). All 338 isolates were characterized using API 20 NE strips. Based on the API 20 NE results, 51% of the isolates were identified as *P. fluorescens*, 14% were identified as *P. putida*, and 25% were identified as either *P. fluorescens* or *P. putida*. The API 20 NE system was unable to identify 10% of the isolates to the species level.

Ribotyping was completed for 81 isolates with different API codes. The resulting ribotypes are shown in Table 2. Initially, ribotype patterns with similarity coefficients of >0.93, as calculated by the Riboprinter's software by use of band positions and intensities, were considered identical and were grouped together as one ribogroup with the same designation. Further refinement of these groupings was performed by visual evaluation of closely related ribotype patterns. A total of 42 different ribotypes were found among the 81 isolates tested (Table 1).

Eight of 42 (19%) ribotypes were found in more than one dairy, while the remaining 81% were found only in one dairy. None of the raw milk isolates was of the same ribotype as that of pasteurized milk isolates in any of the four dairies. Except for one ribotype, 72-S-3, the ribotype patterns of the isolates from milk collected in the first plant visit did not resemble the patterns from isolates collected in the second plant visit. In dairy plant D, ribotype 112-S-2 was found in both processed milk samples and in environmental samples, including those collected from the valve, floor, and drain. However, in the

TABLE 2. Sources, ribotypes, and enzyme activities of *Pseudomonas* isolates

Dairy and visit	Strain	API 20 NE identification[a]	Ribotype	Sample source (day of plating)	Enzyme activity[b]		
					Protease	Lecithinase	Lipase
Dairy A							
Second[c]	D3-105	*P. fluorescens*	407-S-3	Processed milk (7)	2.8 ± 0.3	1.6 ± 0.1	3
	D3-111	*P. fluorescens*	407-S-3	Processed milk (7)	2.6 ± 0.1	1.6 ± 0.0	3
	D3-121	*P. fluorescens*	407-S-3	Processed milk (14)	2.8 ± 0.1	1.5 ± 0.1	3
	D3-221	*P. fluorescens*	48-S-6	Raw milk	2.9 ± 0.1	1.4 ± 0.1	1
	D3-222	*P. fluorescens*	48-S-6	Raw milk	2.7 ± 0.0	1.3 ± 0.1	1
	D3-223	*P. putida*[d]	82-S-6	Raw milk	3.6 ± 0.5	—	3
	D3-224	*P. fluorescens*	82-S-6	Raw milk	3.6 ± 0.2	—	3
	D3-227	*P. fluorescens*	55-S-6	Raw milk	3.7 ± 0.1	—	3
	D3-229	*P. putida*[d]	82-S-6	Raw milk	3.7 ± 0.4	—	3
Third	D3-381	*P. fluorescens*	542-S-7	Floor	2.1 ± 0.2	2.1 ± 0.2	1
	D3-382	*P. fluorescens*	424-S-8	Floor	2.8 ± 0.3	2.2 ± 0.1	1
	D3-384	*P. fluorescens*[e]	542-S-8	Floor	—	—	—
Dairy B							
First	D3-046	*P. fluorescens*[e]	51-S-5	Processed milk (7)	—	—	—
	D3-047	*P. fluorescens*[e]	51-S-5	Processed milk (7)	—	—	—
	D3-050	*P. fluorescens*[e]	51-S-5	Processed milk (7)	—	—	—
	D3-061	*P. putida*	384-S-2	Processed milk (14)	—	—	—
Second	D3-313	*P. fluorescens*	524-S-5	Processed milk (1)	3.0 ± 0.1	—	3
	D3-314	*P. putida*[d]	57-S-5	Processed milk (7)	—	—	1
	D3-321	*P. putida*[d]	57-S-5	Processed milk (7)	—	—	2
	D3-326	*P. aeroginosa*[d]	524-S-7	Processed milk (7)	1.7 ± 0.3	2.0 ± 0.2	1
	D3-309	*P. putida*[d]	82-S-6	Processed milk (14)	1.6 ± 0.1	—	2
	D3-153	*P. fluorescens*[f]	408-S-1	Raw milk	2.4 ± 0.3	1.6 ± 0.2	—
	D3-156	*P. fluorescens*	424-S-8	Raw milk	1.4 ± 0.4	2.2 ± 0.1	1
	D3-157	*P. fluorescens*	408-S-3	Raw milk	—	2.2 ± 0.2	—
	D3-158	*P. putida*	82-S-4	Raw milk	—	—	—
	D3-161	*P. fluorescens*[k]	408-S-3	Raw milk	—	2.3 ± 0.3	1
Third	D3-352	*P. fluorescens*	50-S-8	Floor	2.5 ± 0.4	2.4 ± 0.2	4
	D3-367	*P. putida*	537-S-7	Floor	—	—	—
	D3-363	*P. putida*	537-S-5	Inside filling bowl	2.5 ± 0.1	1.6 ± 0.1	2
	D3-364	*P. fluorescens*[e]	537-S-6	Inside filling bowl	—	—	—
	D3-375	*P. putida*	82-S-4	Drain	—	—	—
Dairy C							
First	D3-016	*P. fluorescens*	48-S-7	Processed milk (7)	2.7 ± 0.1	1.8 ± 0.1	1
	D3-017	*P. fluorescens*[e]	375-S-5	Processed milk (7)	—	—	4
	D3-018	*P. fluorescens*[e]	375-S-5	Processed milk (7)	—	—	4
	D3-021	*P. fluorescens*	48-S-7	Processed milk (7)	2.6 ± 0.2	1.8 ± 0.2	1
	D3-029	*P. putida*	72-S-3	Processed milk (7)	—	—	4
	D3-032	*P. putida*	377-S-5	Processed milk (14)	—	—	—
	D3-034	*P. putida*	377-S-5	Processed milk (14)	—	—	—
Second	D3-147	*P. fluorescens*[e]	72-S-3	Processed milk (1)	—	—	4
	D3-133	*P. fluorescens*[e]	72-S-3	Processed milk (7)	—	—	4
	D3-168	*P. fluorescens*	72-S-3	Processed milk (14)	—	—	4
	D3-170	*P. fluorescens*[e]	72-S-3	Processed milk (14)	—	—	4
	D3-175	*P. fluorescens*	408-S-8	Processed milk (14)	3.2 ± 0.3	2.3 ± 0.3	4
	D3-148	*P. fluorescens*	112-S-6	Raw milk	3.0 ± 0.2	1.3 ± 0.0	3
	D3-149	*P. fluorescens*	409-S-3	Raw milk	3.2 ± 0.3	1.5 ± 0.3	3
	D3-167	*P. putida*	384-S-2	Raw milk	—	—	—
Third	D3-328	*P. fluorescens*	536-S-6	Floor	—	—	—
	D3-331	*P. fluorescens*	57-S-8	Floor	2.8 ± 0.1	1.8 ± 0.2	2
	D3-338	*P. putida*	536-S-7	Drain	—	—	—
	D3-348	*P. fluorescens*	536-S-8	Overhead pipe filler	—	1.53 ± 0.0	—
	D3-350	*P. fluorescens*	112-S-2	Overhead pipe filler	3.3 ± 0.1	1.5 ± 0.1	1
Dairy D							
First	D3-077	*P. fluorescens*	384-S-3	Processed milk (7)	2.5 ± 0.2	2.6 ± 0.2	2
	D3-086	*P. fluorescens*	97-S-4	Processed milk (7)	2.7 ± 0.4	1.9 ± 0.1	4
	D3-092	*P. fluorescens*	543-S-6	Processed milk (14)	1.9 ± 0.0	2.5 ± 0.2	1
	D3-094	*P. fluorescens*	97-S-4	Processed milk (14)	1.3 ± 0.0	2.0 ± 0.3	2

Continued on following page

TABLE 2—*Continued*

Dairy and visit	Strain	API 20 NE identification[a]	Ribotype	Sample source (day of plating)	Enzyme activity[b]		
					Protease	Lecithinase	Lipase
Second	D3-182	*P. fluorescens*[g]	112-S-2	Processed milk (7)	2.8 ± 0.1	1.4 ± 0.1	1
	D3-183	*P. fluorescens*[e]	409-S-6	Processed milk (7)	—	—	—
	D3-184	—	409-S-6	Processed milk (7)	—	—	—
	D3-187	*P. putida*[h]	409-S-8	Processed milk (7)	—	—	—
	D3-193	*P. fluorescens*	416-S-8	Processed milk (7)	3.4 ± 0.4	1.8 ± 0.2	1
	D3-195	*P. fluorescens*	422-S-2	Processed milk (14)	2.5 ± 0.4	—	—
	D3-196	*P. fluorescens*	422-S-2	Processed milk (14)	2.6 ± 0.2	—	—
	D3-197	*P. fluorescens*	422-S-2	Processed milk (14)	2.7 ± 0.3	—	—
	D3-180	*P. fluorescens*	57-S-8	Raw milk	3.0 ± 0.2	1.7 ± 0.0	2
	D3-181	*P. fluorescens*	409-S-5	Raw milk	3.0 ± 0.3	1.4 ± 0.0	1
	D3-210	*P. putida*[d]	57-S-5	Raw milk	—	—	1
	D3-211	*P. fluorescens*[e]	48-S-6	Raw milk	—	1.8 ± 0.5	—
	D3-212	*P. fluorescens*	48-S-6	Raw milk	2.4 ± 0.1	1.6 ± 0.2	4
	D3-213	*P. fluorescens*[e]	72-S-3	Raw milk	—	—	4
Third	D3-266	*P. fluorescens*	524-S-1	Floor	2.6 ± 0.1	3.0 ± 0.3	1
	D3-267	*P. fluorescens*	112-S-2	Floor	2.9 ± 0.3	1.4 ± 0.0	1
	D3-269	*P. fluorescens*	112-S-2	Floor	3.0 ± 0.2	1.4 ± 0.2	1
	D3-270	*P. fluorescens*[i]	112-S-2	Floor	2.6 ± 0.1	1.4 ± 0.0	1
	D3-274	*P. fluorescens*[j]	537-S-2	Floor	—	—	—
	D3-276	*P. fluorescens*[g]	112-S-2	Valve	3.0 ± 0.1	1.3 ± 0.1	1
	D3-283	*P. fluorescens*	112-S-2	Valve	3.3 ± 0.3	1.4 ± 0.1	1
	D3-286	*P. fluorescens*	112-S-2	Drain	2.7 ± 0.2	1.3 ± 0.2	1
	D3-287	*P. fluorescens*	57-S-8	Drain	3.0 ± 0.1	1.8 ± 0.0	2
	D3-293	*P. fluorescens*[j]	82-S-6	Drain	2.7 ± 0.1	—	3
	D3-294	*P. fluorescens*	536-S-4	Drain	3.3 ± 0.5	2.30 ± 0.2	1
	D3-296	*P. putida*	82-S-6	Pipe lines	2.0 ± 0.3	—	2

[a] Species were assigned by best likelihood.
[b] Enzyme activities ± standard deviations are reported as follows: mean ratio reflecting protease activity = clearance zone diameter/colony size; mean ratio reflecting lecithinase activity = opaque-zone diameter/colony size; lipase activity = mean color intensity; —, not detectable.
[c] *Pseudomonas* spp. could not be isolated on the first visit.
[d] Another possibility is *P. fluorescens.*
[e] Another possibility is *P. putida.*
[f] Another possibility is *Burkholderia cepacia.*
[g] Other possibilities are *Burkholderia pseudomallei*, *P. aeruginosa*, and *Chromobacterium violaceum.*
[h] Other possibilities are *P. fluorescens* and *Ralstonia pichettii.*
[i] Other possibilities are *P. stutzeri* and *P. putida.*
[j] Another possibility is *P. aeruginosa.*
[k] Other possibilities are *B. cepacia* and *Ochrobactrum anthropi*.

other three dairies, no connection was established between isolates from processed milk samples and those from environmental samples. In two of the dairies, the same ribotypes (82-S-4 and 57-S-8) were isolated from raw milk samples and environmental samples.

From dairy plant A, seven different ribotypes were isolated from raw milk, processed milk, and environmental samples (Table 1). *Pseudomonas* spp. could not be isolated from processed milk samples obtained during the first plant visit (the major contaminating organisms in these milk samples were *Acinetobacter* spp.). *Pseudomonas* spp. were isolated from raw milk and processed milk samples obtained during the second plant visit. None of the raw milk or environmental ribotypes matched the ribotypes from processed milk. Only one ribotype was isolated from the processed milk samples. As we were unable to isolate *Pseudomonas* from samples collected during the first visit, samples were also obtained during three additional plant visits following the second visit. No *Pseudomonas* was isolated from the additional samples. The SPC from milk obtained during those three visits were also very low, averaging 500 CFU/ml at 14 days postprocessing. Only four *Pseudomonas* colonies were isolated from environmental samples.

In dairy plant B, 14 different ribotypes were isolated from raw and processed milk and environmental samples (Table 1). Ribotype 82-S-4 was isolated from both raw milk and environmental (drain) samples. A total of 15 colonies were isolated from the milk samples collected during the first sampling period that had been plated at 7 days postprocessing. All of these were identified as *P. fluorescens* by API 20 NE (Table 2). The three strains selected bore ribotype 51-S-5. Another 15 colonies were isolated from the samples plated on day 14, and all of these were identified as *P. putida* by API 20 NE (Table 2). Of these, one isolate bore ribotype 384-S-2.

In dairy plant C, 13 different ribotypes were isolated from raw and processed milk and environmental samples (Table 1). Ribotype 72-S-3 was isolated from processed milk samples obtained during both the first and second plant visits.

Seventeen different ribotypes were isolated from raw and processed milk and environmental samples collected from dairy plant D (Fig. 1). Ribotype 112-S-2 was observed among isolates from processed milk, floor, valve, and drain samples. Ribotype 57-S-8 was isolated from both raw milk and environmental (drain) samples.

Spoilage potentials of *Pseudomonas* and relationships between ribotypes and enzyme activities. All *Pseudomonas* isolates were evaluated for production of extracellular protease,

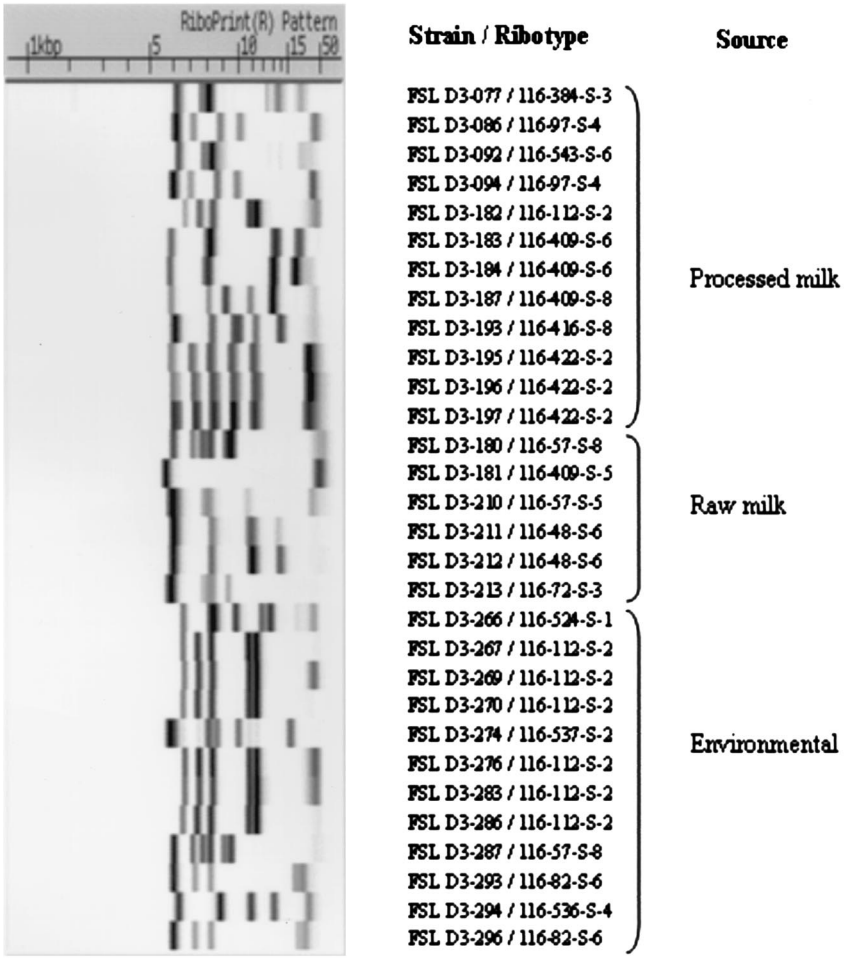

FIG. 1. Riboprints of *Pseudomonas* isolates obtained from dairy processing plant D.

lipase, and lecithinase. Of the 338 *Pseudomonas* isolates, 51% were protease positive, 47% were lecithinase positive, and 67% were lipase positive. Lipolytic and proteolytic activities varied among the *Pseudomonas* strains. Most of the *P. fluorescens* strains were positive for all enzyme activities (69%), while most *P. putida* strains (87.5%) were negative for all enzyme activities. Isolates that were characterized as either *P. fluorescens* or *P. putida* by API 20 NE were mostly protease negative (93%); however, the majority of these isolates (75%) had lipase activity. No clear differences emerged among the enzyme activities between the milk isolates and the environmental isolates (Table 2).

Isolates bearing the same ribotype generally had similar extracellular enzymatic profiles (Table 3). The extracellular enzymatic activities and ribotypes were also closely related. Specifically, ribotypes 50-S-8 and 72-S-3 were composed of isolates with the highest extracellular lipase activities while the highest extracellular protease activity was seen in isolates grouped in ribotype 55-S-6 (Fig. 2).

In general, the presence or absence of both extracellular lecithinase and lipase activities appeared to be correlated in this collection of isolates. Forty-five percent of the isolates had both lecithinase and lipase activities, while 31% had neither activity. On the other hand, 22% of the isolates had only lipase activity and just 2% of the isolates had lecithinase activity but no lipase activity.

Effect of *Pseudomonas* on the flavor of milk samples. For sensory analysis, milk was scored on a scale from 1 to 10, where any rating below 6 was considered poor, 6 to 7 was fair, and 8 to 10 was good. The overall scores were assigned with a predominant criticism. Table 4 presents the SPC, mean flavor scores of processed milk samples, and percentages of enzymatically active *Pseudomonas* isolates from the samples.

In general, sensory scores were considered "good" at 7 days postprocessing, with an average score of 7.5. Two products also retained good flavor scores (7.6 and 7.5) at 14 days postprocessing. In general, good flavor scores were associated with low numbers of bacteria. All rancid flavors were associated with the presence of lipase-producing *Pseudomonas*. We conclude that, with the exception of two samples examined in this study, the flavor defects of processed milk were associated with both high numbers of bacteria and the presence of degradative enzymatic activities among the contaminating *Pseudomonas* strains. One of the two exceptions was the plant B milk sample from the first visit which coagulated at 14 days postprocessing even though the *Pseudomonas* isolates obtained from the processed milk samples had none of the degradative enzymatic activities measured in this study. Despite the apparent absence

TABLE 3. Ribotypes and enzyme activity profiles of *Pseudomonas* isolates

Ribotype	No. of isolates	Enzyme activity profile			Source(s)[a] and/or reference
		Protease	Lecithinase	Lipase	
48-S-6	10	+	+	+	A2, D2; 32
	2	−	+	−	D2; 32
48-S-7	12	+	+	+	C1; 32
	1	+	+	−	32
50-S-8	3	+	+	+	B3; 32
51-S-5	5	−	−	−	B1; 32
55-S-6	2	+	−	+	A2; 32
57-S-5	4	−	−	+	B1, D2; 32
57-S-8	3	+	+	+	C3, D2, D3
	1	−	−	−	32
72-S-3	8	−	−	+	C1, D2, 32
	1	−	−	−	32
82-S-4	3	−	−	−	B2, B3; 32
82-S-6	7	+	−	+	A2, B1, D3; 32
	1	+	−	−	32
97-S-4	3	+	+	+	D1; 32
111-S-5	2	+	+	+	32
112-S-2	8	+	+	+	C3, D1, D3
375-S-5	2	−	−	+	C1
377-S-5	2	−	−	−	C1
384-S-2	2	−	−	−	B1, C2
407-S-3	3	+	+	+	A1
408-S-3	2	−	+	+	B2
409-S-6	2	−	−	−	D1
422-S-2	3	+	−	−	D1
424-S-8	2	+	+	+	A3, B2

[a] Letters indicate dairy plants A, B, C, and D, and numbers indicate samples from processed milk (1), raw milk (2), or the plant environment (3).

of degradative *Pseudomonas* spp., the overall numbers of bacteria were high in this product (1.3×10^8 CFU/ml). Milk samples from dairy plant D (first visit) were positive for all enzyme activities tested and the SPC was high (1.9×10^8 CFU/ml), but the sensory panelists reported no serious flavor defects.

DISCUSSION

Our goals were to determine the association between *Pseudomonas* ribotypes and the organisms' milk spoilage potentials and to examine the genetic diversity of *Pseudomonas* spp. in milk and in the environment of dairy processing plants.

Among the 338 *Pseudomonas* isolates examined, 51% were identified as *P. fluorescens*. These results are consistent with previous observations indicating that *P. fluorescens* is the predominant organism in refrigerated milk. The presence of this microbe in processed milk samples likely reflects the presence of *P. fluorescens* in the dairy processing environment as well as its short generation time at refrigeration temperatures (8, 11, 17).

Ribotyping of *Pseudomonas* isolates revealed 42 different ribotype patterns among 81 isolates. This large number of different ribotypes indicates considerable ecologic diversity among *Pseudomonas* spp. within the dairy processing environment. This diversity contributes to the different spoilage patterns of milk products among the four dairy plants. Specifically, as shown in Table 2, 81% of ribotypes were found only in one plant. These results suggest multiple origins of *Pseudomonas* spp. that may be isolated from dairy processing plants.

Previous research has shown that gram-negative psychrotrophic bacteria such as *Pseudomonas* do not survive commercial pasteurization (8). Therefore, the presence of gram-negative bacteria in freshly pasteurized milk generally indicates postpasteurization contamination (8). In some cases, however, bacteria such as the normally heat-sensitive *Pseudomonas* can survive the heat treatments used in conventional pasteurization of milk if initial bacterial populations are extremely large (14). In this study, isolates from raw milk samples had different ribotypes than those of isolates from pasteurized milk samples, which suggests that processed milk contaminants were not survivors of pasteurization.

Processing environments commonly contribute to postpasteurization contamination of pasteurized milk products; specifically, filling machines are an important source of contami-

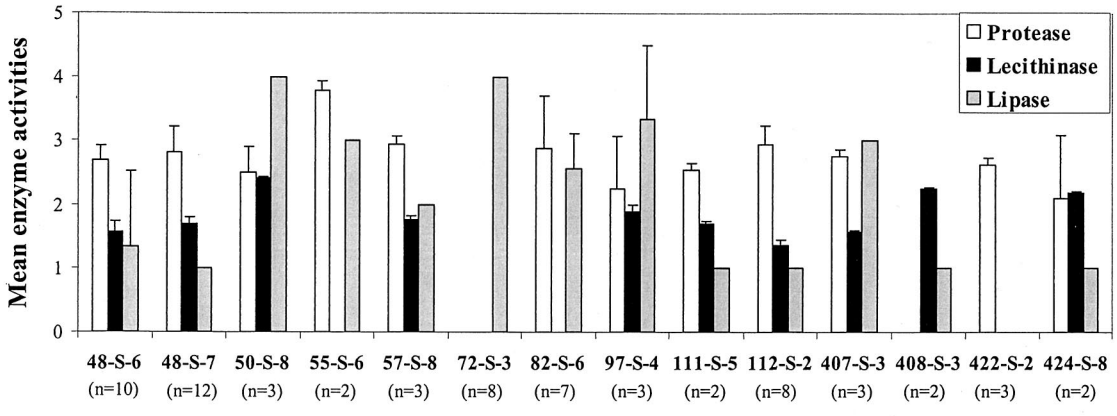

FIG. 2. Relationship between ribotypes and enzyme activities of *Pseudomonas* spp. Mean activities and standard deviations are reported as follows: mean ratio reflecting protease activity = clearance zone diameter/colony size; mean ratio reflecting lecithinase activity = opaque zone diameter/colony size; lipase activity = mean color intensity; n = the number of isolates within a ribotype.

TABLE 4. SPC, mean flavor scores, and percentage of enzymatically active *Pseudomonas* isolates from processed milk samples

Visit	Sample source and day of plating	SPC/ml	No. of *Pseudomonas* isolates tested	% of *Pseudomonas* isolates with enzyme activity			Flavor score	Sensory result(s)
				Protease	Lecithinase	Lipase		
First	Dairy B							
	Day 7	7.1×10^6	15	0	0	0	7.8	Lacks freshness, light oxidized
	Day 14	1.3×10^8	16	0	0	0	1	Coagulated
	Dairy C							
	Day 7	1.2×10^7	15	13	80	100	6.4	Lacks freshness, rancid
	Day 14	2.6×10^8	15	0	80	80	1	Rancid
	Dairy D							
	Day 7	1.5×10^4	14	100	100	100	8.4	Lacks freshness
	Day 14	1.9×10^8	14	100	100	100	7.7	Lacks freshness
Second	Dairy A							
	Day 7	2×10^3	15	100	100	100	8.7	No criticism
	Day 14	5.3×10^4	13	100	100	100	7.6	Lacks freshness
	Dairy B							
	Day 7	6×10^7	14	36	78	100	1.3	Rancid
	Day 14	4.7×10^8	14	64	21	71	NT[a]	NT
	Dairy C							
	Day 7	2.7×10^7	14	0	0	100	5.1	Fruity or fermented, lacks freshness
	Day 14	1.4×10^8	12	67	58	100	2.5	Rancid
	Dairy D							
	Day 7	2.4×10^5	13	38	38	38	8.2	Cooked, lacks freshness
	Day 14	3.2×10^8	15	100	0	0	NT	NT

[a] NT, not tasted due to presence of coliforms.

nation of pasteurized milk (12, 15, 23, 25). The same ribotype was found in the processed milk samples and environmental samples of dairy plant D, which suggests that the processing environment was likely to have been the source of the contamination of the finished product. In the other three plants, none of the ribotypes isolated from environmental samples were identical to those found in finished products. Given the genetic diversity of the *Pseudomonas* spp. characterized in this study, the most likely explanation for this finding is that we did not sample the environmental source(s) responsible for postprocessing contamination of these milk products or that the isolates picked from the environmental sampling plates did not represent the full diversity of the strains present in the samples. In plant A, we could not isolate *Pseudomonas* spp. from the processed milk samples obtained during three plant visits. The numbers of *Pseudomonas* spp. obtained from environmental sources in the same plant were also very low. These findings provide further evidence that the processing environment serves as a probable source of contamination of processed milk, as a low level of environmental contamination would likely result in low levels of contamination of the finished product.

With the exception of ribotype 72-S-3 isolated in dairy plant C, different ribotypes were identified among isolates collected during different plant visits. One likely explanation for this observation is the high level of diversity among *Pseudomonas* isolates in these plants. Another possibility might be a lack of long-term colonization for the gram-negative *Pseudomonas* spp. in these dairy processing environments. In contrast, long-term existence in food processing environments has been reported for some gram-positive organisms (18, 22, 28). For example, Svensson et al. (28) reported that the same random amplified polymorphic DNA type of mesophilic *Bacillus subtilis* was isolated from a dairy plant over a 2-year period. Unnerstad et al. (reviewed in reference 1) reported that the same *Listeria monocytogenes* strain persisted in a dairy plant for at least 7 years. The long-term persistence of *L. monocytogenes* in poultry and seafood processing environments also has been reported (18, 22). A short-term existence for gram-negative microbes in contrast to the likelihood of a long-term colonization by gram-positive bacteria might indicate a fundamental difference in the ecology of these groups of organisms in the food processing environment.

Spoilage potentials of the *Pseudomonas* strains. SPC, PPC, and coliform counts are methods that are frequently used to monitor milk quality (3). However, poor correlations exist among the SPC, the PPC, the coliform count, and product shelf life (14). With the exception of the determination of the presence of very high initial bacterial counts, simple measurement of bacterial numbers present in freshly pasteurized products does not provide useful predictive insight into product shelf life. Craven and Macauley (11) showed that the presence of large populations of *Pseudomonas* spp. generally resulted in shorter shelf lives for pasteurized milk than if products were contaminated with other types of organisms. However, they also reported that some samples predominantly containing *Pseudomonas* spp. had shelf lives that were equal to or greater than those of samples predominantly containing other types of microorganisms. The growth rates and metabolic activities of contaminating organisms at refrigeration temperatures are the two most important factors that influence product shelf life (14).

Our results showed that 69% of the *P. fluorescens* strains were positive for all enzyme activities, while most *P. putida*

strains (87.5%) were negative for all enzyme activities. These results correspond with those of Wiedmann et al. (32) in that the majority of the *P. fluorescens* strains have extracellular protease, lipase, and lecithinase activity while *P. putida* strains are usually negative for all enzyme activities. We conclude that *P. fluorescens* is an important spoilage organism in processed milk products. Our results show that the lecithinase and lipase activities of pseudomonads are usually associated. Seventy-six percent of the isolates either had both lecithinase and lipase activity or neither lecithinase nor lipase activity.

Ribotyping is very useful for the characterization of spoilage microflora. In this study, isolates that had the same ribotype, even if obtained from different sources, usually had the same extracellular-enzyme profiles (Table 3). We found considerable variation in the lipolytic and proteolytic activities of the *Pseudomonas* strains; however, isolates with the same ribotype usually had similar extracellular-enzyme activities (Fig. 2).

Effect of *Pseudomonas* on the flavor of milk samples. Growth of psychrotrophic bacteria after pasteurization is an important factor in milk lipolysis and proteolysis. Flavor defects generally develop in processed milk when the bacterial population of the milk rises to $\geq 10^7$ CFU/ml (24). Off-flavors generally develop in three stages. First, milk loses its freshness. Then, it is perceived as stale. Finally, its products develop rancid, fruity, and bitter flavors. Bitter flavors usually accompany protein degradation. Soapy and rancid flavors usually are a result of lipid breakdown (8). Our results indicate that sensory characteristics depend on both the number of bacteria and their enzymatic activities. For example, although all *Pseudomonas* isolates obtained from plant A were positive for all three enzyme activities, no major flavor defects were reported, probably because of the low SPC of the samples. All rancid flavors were associated with lipase production capability among the *Pseudomonas* isolates. Although *Pseudomonas* isolates obtained from the first visit to plant B had no enzyme activity, milk coagulated by day 14 postprocessing. This defect was probably the consequence of protease production by other, non-*Pseudomonas* bacteria. Although *Pseudomonas* spp. are the main concern with regard to proteolytic degradation of milk, other proteolytic organisms such as *Achromobacter*, *Aeromonas*, *Flavobacterium*, and *Xanthomonas* spp. can be found in milk and can cause proteolytic degradation and milk coagulation (11, 26). *Pseudomonas* spp. isolates from milk samples obtained during the first visit to plant D were positive for all enzyme activities, but pronounced flavor defects were not detected despite the high SPC. The most likely explanation for this phenomenon is that the presence of the *Pseudomonas* strains able to cause spoilage was low relative to the presence of other organisms with lower spoilage potentials.

Conclusions. We found that ribotyping is a discriminatory and useful typing method for differentiating *Pseudomonas* spp. capable of decreasing the shelf lives of fluid milk products. Ribotyping revealed a large genomic diversity among *Pseudomonas* spp. isolated from these dairy plants. Our results indicate that there is a relationship between bacterial ribotypes and their spoilage potentials. Ribotyping is a convenient and reliable method for identifying strains with food spoilage potential.

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