Phenotypic characterization and PCR-ribotyping of *Pseudomonas fluorescens* isolates, in tracking contamination routes in the production line of pasteurized milk

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Summary

Contamination sites of *Pseudomonas fluorescens* were traced in the production line for milk pasteurization in a large dairy plant in Shiraz, Iran. Samples of raw and pasteurized milk were collected at six sites along the line. All milk samples were incubated at 7°C until the aerobic plate count had reached 10^6-10^7 cfu mL^{-1}. Colonies were picked randomly and identified. No growth of gram negative psychrotrophic bacteria (GNP) was detected in the immediately pasteurized milk samples (just after the pasteurization), during long incubation at 7°C. Recontamination most often occurred in the filling step. In this study 34.1 and 4.9% of the milk packages showed contamination with GNP and *P. fluorescens*, respectively. Twenty three *P. fluorescens* isolates were examined for phenotypic characteristics and 16S-23S PCR ribotyping. Phylogenetic analysis was conducted on the phenotypic and genotypic characteristics. The *P. fluorescens* isolates were shown to belong to 6 biotypes (B1-B6). The predominance of a particular ribotype was often observed for a given biotype, although there were two ribotypes in each of the B2 and B6 biotypes. The 16S-23S PCR-ribotyping technique allowed differentiation between the isolates. Based on this method, the isolates belonged to 5 subtypes. Phylogenetic analysis based on 16S-23S PCR-ribotyping and phenotypic characterization could be helpful in tracking contamination routes in the production line for milk pasteurization.

Key words: Milk, Recontamination, *Pseudomonas fluorescens*, PCR-ribotyping, Phenotyping

Introduction

Raw milk delivered to the dairy plant contains gram negative psychrotrophic bacteria (GNP) which can grow and multiply rapidly in chill-stored raw milk (Chambers, 2002). These bacteria are killed during pasteurization, but as a result of the post-pasteurization contamination around 50% of the retail packages can be spoiled by GNP after prolonged chill storage (Ternström *et al*., 1993; Dogan and Boor, 2003; Lafarge *et al*., 2004; Blackburn, 2006). Furthermore, they produce the majority of heat stable extracellular proteases, lipases and phospholipases secreted into raw milk during preprocessing storage. Many of these enzymes can survive pasteurization (72°C for 15 s) and even ultra-high-temperature treatments (138°C for 20 s or 149°C for 10 s) and can consequently reduce the eating quality and shelf life of processed dairy products (Griffiths *et al*., 1981; Cousin, 1982; Sørhaug and Stepaniak, 1997; Wiedmann *et al*., 2000; Boor and Murphy, 2002; Fromm and Boor, 2004). Some important lipases in milk are the lecithinases or other phospholipases, which are able to disrupt the integrity of the milk fat globule membrane, and expose the fat to degradation by native milk lipases, resulting in physical degradation of the emulsion in milk (Munsch-Alatossava and Alatossava, 2006).

Pseudomonas spp. are by far the most common spoilage-causing psychrotrophic

bacteria. Different *Pseudomonas* spp. have been isolated from raw milk, of which *P. fluorescens* is the most frequent (Kraft and Rey, 1979). Ternström *et al*. (1993) observed that *P. fluorescens* made up more than 55.6% of all the bacteria isolated from raw milk. Gennarl and Dragotto (1992) showed that *P. fluorescens* was present in 84% of the raw milk samples.

Phenotypic methods have proven useful for quantifying and describing bacteria causing dairy product spoilage; however, precise location of the sources of these spoilage organisms in the processing environment or on the farm requires reliable, differential strain identification strategies. Currently accessible phenotypic speciation strategies for the most common dairy product spoilers, i.e., *Pseudomonas* spp., frequently yield uncertain results (Munsch-Alatossava and Alatossava, 2006). Further, the simple identification of the same genus and species by standard methods in both environmental samples and in the finished product does not clearly establish a causal relationship (Wiedmann *et al*., 2000; Giraffa and Neviani, 2001; Huck *et al*., 2007). To solve this problem, in this study, we attempted to characterize *P. fluorescens* isolated from the production line of pasteurized milk by using phenotypic and genotypic markers and to conduct phylogenetic analysis on *P. fluorescens* to understand the degree of relatedness within the species. Consequently it could be helpful in finding contamination routes in the pasteurized milk production line.

Materials and Methods

This study was conducted in a large dairy plant in Shiraz, Iran. Three times (occasions I, II and III) raw and pasteurized milk samples were taken out at six sites along the production line, i.e. (A) raw milk from the silo tank, (B) just before pasteurization, (C) just after pasteurization, (D) from the pasteurized milk reservoir, (E) just before the filling machine and (F) sealed milk packages (Fig. 1). In each occasion, three sample series were collected at 30 min intervals and each sampling was arranged in such a way that the milk from one silo tank was followed through the whole line. Milk samples were collected in small sterile tubes. The sampling valve was swabbed just before sampling with 70% ethanol. The first 150 mL of milk through the sampling valve was removed. At least two consumer packages were picked up from the line every 30 min during each occasion (more than 8 packages per each sampling occasion). All milk samples were kept cold during transportation to the laboratory. All milk samples were stored at 7°C until the counting on tryptic soy agar (TSA, Merck, Darmstadt, Germany) modified by adding 1% dextrose, incubated at 30°C for 3 days, had reached 10^6-10^7 colony forming units (cfu) per mL. Samples were also cultured on violet red bile glucose agar (VRBG, Merck, Darmstadt, Germany) and/or pseudomonas selective agar (PSA; C.F.C Supplement, Cetrimide 10 µg/mL, Fucidin 10 µg/mL, Cephaloridine 50 µg/mL; HiMedia Laboratories, Mumbai, India) was incubated at 26°C for 3 days (intended for *Pseudomonas fluorescens* isolation).

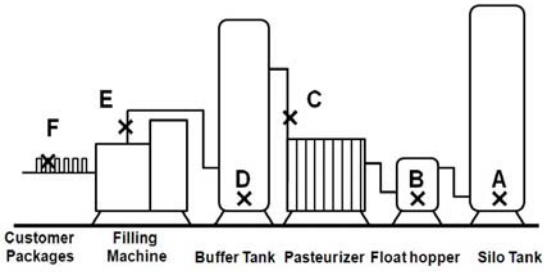


Fig. 1: Sampling sites along the production line for pasteurized milk

The day after sampling, 100 µL of each milk sample was serially diluted and cultured on modified TSA and on VRBG agar and then the count was followed until it reached the critical bacterial load (10^7 cfu mL^{-1}). The milk from the same package was examined 1, 4, 7 and 10 days after sampling. The critical aerobic plate count (10^7 cfu mL^{-1}) was usually reached after about three days for unpasteurized milk and after 7 days for pasteurized milk. The samples were rejected if the critical load was not reached within 10 days.

Isolation and identification

For identification of *P. fluorescens*,

colonies were randomly chosen from the agar plates containing 20-200 colonies. For each sample, a maximum of three suspected colonies from the VRBG agar and three from the PSA were subcultured on the pseudomonas selective agar plates until pure cultures were achieved. All gram negative, glucose oxidative and oxidase positive isolates with fluorescent pigment were considered for final identification by molecular method (Forbes *et al*., 2007). Further phenotypic characterizations were conducted using API 20NE identification kite (BioMérieux, Hazelwood, MO).

Air, water and environmental samples

For air sampling, four open Petri dishes of the VRBG agar and the PSA were placed at the processing area near the filling machine for 20 min. The Petri dishes were closed and incubated at 26°C for 72 h (Sveum *et al*., 1992; Salustiano *et al*., 2003). Two sterile cotton swabs were used to take samples of condensed water on the surfaces of the filling machine. The swabs were directly put into tubes containing 250 mL of sterile skim milk which were incubated at 7°C for a maximum of 10 days. Also, samples of water (5 mL) used in the cleaning system of the pipes were inoculated in 250 mL sterile skim milk and incubated at 7°C for a maximum of 10 days (Eneroth *et al*., 2000a). The procedure described previously was followed for finding *P. fluorescens.*

Packaging material

Before the filling procedure started in each sampling occasion, five empty packages (1 L size) were formed and sealed in the filling machine. Then these empty packages were opened aseptically, filled with sterile milk and resealed aseptically. These packages were incubated at 7°C and followed the procedure for finding *P. fluorescens* (Eneroth *et al*., 2000b).

Molecular method for identification of *P. fluorescens*

All gram negative, glucose oxidative and oxidase positive isolates were chosen for final identification by molecular method. Amplification of DNA 16S specific region of *P. fluorescens* as described by Scarpellini *et al*. (2004) was performed using the primer set 16SPSEfluF and 16SPSER (Table 1). Template DNA was prepared by boiling 200 µL of bacterial suspension in distilled water (OD 600 = 0.6) for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000 g, 10 min, 5°C); the supernatants were subsequently kept on ice or at -20°C. Three micro liters of template DNA suspension was used for each reaction. The PCR were performed in a volume of 50 µL containing 3 µL template DNA, 5 µL of 10 × PCR buffer, 200 µM of each dNTPs, 2 mM of $MgCl_2$, 0.5 µM of each primer and 1.5 U of *Taq* Polymerase. The DNA was amplified by the following thermal profile: 2 min at 94°C; 35 cycles consisting of 94°C for 45 s, 54°C for 45 s, 68°C for 2 min; final extension of 68°C for 2 min; and final cooling at 4°C. Following amplification 7 µL of the products were analyzed by electrophoresis at 100 V in (1% agarose gel, 0.2 µg mL^{-1} of ethidium bromide) in TAE buffer. As described by Scarpellini *et al*. (2004), amplification of a single DNA fragment of 850 bp was sought (Fig. 2).

Phenotypic characterization

Phenotypic characterization of *P. fluorescens* isolates was determined using the API 20 NE identification kit. *P. fluorescens* isolate was inoculated into 0.85% NaCl; the turbidity was adjusted to 0.5 MacFarland standard (BioMérieux). The inoculums were distributed into the API 20NE test strips, which were incubated at

Table 1: Primer sets

Primer	Sequence (5'-3')	Target	Annealing temp (°C)	Product size (bp)
16SPSEfluF	TGCATTCAAAACTGACTG	*P. fluorescens*	54	850
16SPSER	AATCACACCGTGGTAACCG			
23SR7	GGTACTTAGATGTTTCAGTTC	for PCR-ribotyping	45	_[a]
16SR2	TTGTACACACCGCCCGTCA			

[a]Products with different numbers and sizes

30°C, and read after 24 and 48 h. For each isolate, the test was repeated twice to determine the validity of the API 20 NE profiles generated.

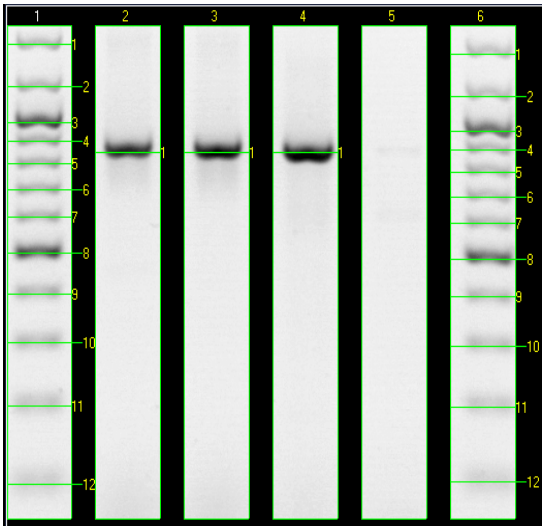


Fig. 2: Agarose gel showing the species-specific amplification of the 850 bp fragment for *Pseudomonas. fluorescens* isolates (Lane 3 and 4), Lane 2: a positive control, *P. fluorescens* ATCC 13525, Lane 5: negative control, Lanes 1 and 5 Marker Gene Ruler 100 bp (MBI Fermentas), In lanes 1 and 6, Bands 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 are 1.5, 1.2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 Kbp sizes, respectively

The production of extracellular proteolytic enzymes was determined on plate count agar (PCA, Merck, Darmstadt, Germany), supplemented with 1% skim milk powder. The *P. fluorescens* isolate was streaked on two agar plates, and each plate was incubated at 7 and 26°C for 168 and 48 h, respectively. The appearance of a clear zone surrounding the colonies was interpreted as a positive proteolytic reaction (Wiedmann *et al*., 2000; Dogan and Boor, 2003). Production of extracellular lecithinase was determined by streaking of *P. fluorescens* isolates on PCA containing 10% egg yolk emulsion. The opaque rings surrounding the colonies were considered as a lecithinase-positive reaction after incubation at 7 and 26°C for 7 and 2 days, respectively (Dogan and Boor, 2003). The tests were repeated twice. The proteolytic and lipolytic (PL) profile was established by using the following rules: A score of 1 and 2 was given to proteolytic reaction at 7 and 26°C, respectively. Nonproteolytic isolates were scored 0. Isolate scores for proteolytic activity at 7 and 26°C were summed to form a proteolytic profile. For example, a *P. fluorescens* isolate with score 3 indicated that the isolate was proteolytic at both 7 and 26°C. The same was done for lipolytic profiles.

PCR-ribotyping of *P. fluorescens*

The method described by Wang and Jayarao (2001) was used to conduct 16S-23S PCR-ribotyping. The assay for each isolate was replicated on at least two separate occasions to verify the reproducibility of the PCR amplified DNA fingerprinting patterns. The primers of 23SR7 and 16SR2 (Table 1) were used for PCR. PCR-ribotyping was performed in a 25-µL volume for each reaction containing 3 µL of genomic DNA, 1 µL of dNTPs (100 µM), 0.6 µL of each primer (3 µM), 2.5 µL of 10 × PCR buffer, 1 µL of $MgCl_2$ (50 µM), 0.5 µL of *Taq* DNA polymerase (2.5 U), and 16.4 µL of distilled water. An initial denaturing step of 95°C for 3 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min except for an extension step of 4 min during the last cycle. A negative control contained all reagents except template DNA. After amplification, 7 µL of the PCR product was electrophoresed in a 2% agarose gel containing 1.5 µg mL^{-1} ethidium bromide using TAE buffer at 100 V for 2 h. Isolates with identical banding patterns were grouped into the same PCR-ribotype.

Phylogenetic analysis

Phenotypic relatedness among different isolates of *P. fluorescens* was determined using API 20 NE profiles. Genotypic relatedness was determined using 16S-23S PCR ribotypes. Phenotypic and genotypic characters were scored as discrete variables (0 or 1; 0, when the character was negative or missing; 1, when character was positive or present). The genetic similarity was calculated using the Jaccard's coefficient according to this formula (Sneath and Sokal, 1973; Johnsen *et al*., 1996; Martins *et al*., 2006):

Sii' is the Jaccard's similarity index

$Sii = a / (a + b + c)$

Where:
a: is the number of bands present in the two genotypes
b: is the number of polymorphic bands present in genotype i only
c: is the number of polymorphic bands present in genotype i' only

The same method was used for calculating phenotypic similarity between different biotypes according to API 20NE profiles. Cluster analysis was carried out using the unweighted pair group method using arithmetic averages (UPGMA) provided by the NTSYSpc software program ver. 2.02e (Applied Biostatistics Inc., 1986-1998).

Results

All milk samples were cultured on the day after arrival to the laboratory. This was set to be the initial plate count. The unpasteurized milk samples had a median value of the initial aerobic plate count at approximately 1.1×10^6 cfu mL^{-1} (Table 2, sampling sites A, B and raw milk delivery truck). The count range was from 1.3×10^5 to 3.1×10^6 cfu mL^{-1}. Milk collected at the sampling sites A-B, and raw milk samples from the delivery truck, within the same sampling run, had approximately the same initial count. All unpasteurized milk samples showed growth on the VRBG agar plates incubated at 26°C and at 37°C (intended for *Pseudomonas* and *Enterobacteriaceae* growth, respectively), indicating the presence of gram negative bacteria. The initial plate count of gram negative bacteria were approximately 1.1×10^5 cfu mL^{-1} for incubation at 26°C and 6.6×10^4 cfu mL^{-1} for incubation at 37°C (Table 2).

The pasteurized milk samples had an initial aerobic plate count varying between 3.3×10^3 and 2.1×10^5 cfu mL^{-1}. There were no big differences in the initial aerobic total plate count between the milk samples at the sampling sites C-E. In initial plating, no pasteurized milk sample, except 7 customer packages (7 out of 41, 17%) had any growth on the VRBG agar plates incubated at 26°C or 37°C. Thirty four percent of the customer packages (14 out of 41) showed contamination with gram negative psychrotrophic bacteria (GNP) after incubation at 7°C for more than 7 days. No contamination with GNP was detected in the immediately pasteurized milk samples (site C, just after the pasteurizer) during 10 days incubation at 7°C.

Unpasteurized milk had reached the critical bacterial loads of 10^6-10^7 cfu mL^{-1} after approximately three days of storage at 7°C. GNP bacteria were isolated from all samples (sampling site A, B and delivery truck). *P. fluorescens* was often present in the incubated unpasteurized milk samples but it was not the dominating spoilage microflora. Members of *Enterobacteriaceae* were isolated from all of the raw milk samples. Totally, 23 *P. fluorescens* were isolated from different samples. Most of them (20 out of 23, 87%) were isolated from unpasteurized milk and condensed water on the filling machine. *P. fluorescens* was isolated from none of the air and water samples. Bacterial growth was observed in 20% (3 out of 15) of packaging material samples and only one *P. fluorescens* isolate was detected in them.

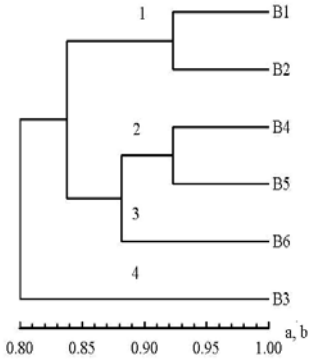
In the present study, biotypes based on API 20NE profiles were used to subtype *P. fluorescens*. The 23 isolates of *P. fluorescens* were shown to belong to 6 biotypes (Fig. 3). The isolates showed differences in utilization of nitrate, arginine, gelatin and adipate. Phylogenetic analyses based on biotypes of *P. fluorescens* resulted in placing the 6 biotypes into 4 clusters. Clusters 1, 2, 3 and 4 had 13, 6, 3, and 1 isolates, respectively. Clusters 1 and 2 accounted for 82.6% of the isolates. Each of these 2 clusters consists of 2 biotypes. Biotypes B1 and B2 in cluster 1 only showed the difference in utilization of arginine and in cluster 2, the two biotypes had a difference in adipate utilization. The predominance of a particular ribotype was often observed for a given biotype in the clusters. Biotype B1, B4, B5 and B3 only showed R4, R2, R5 and R1 ribotyping profile, respectively.

The *P. fluorescens* isolates were examined for their PL activity at 7 and 26°C. Based on PL activity at two different temperatures, the 23 isolates belonged to 4 PL profiles (Table 3). The results showed that 87% of isolates were observed to be

Table 2: Bacterial contamination along the production line for pasteurized milk. Values for the initial plate count and presence of gram negative bacteria, gram positive bacteria and *Pseudomonas fluorescens* after milk samples incubation at 7°C, are shown

	Raw milk delivery truck	Silo tank (site A)	Before the pasteurizer (site B)	After the pasteurizer (site C)	Buffer tank (site D)	From the filling machine (site E)	Consumer package (site F)	(site M)[c]	Packaging material
Number of samples	10	18	18	18	18	18	41	18	15
Initial aerobic plate count[a] (TSA, 30°C, 3d)	1.1×10^6 (1.3×10^5-3.1×10^6)	9.7×10^5 (5.4×10^5-2.7×10^6)	1.1×10^6 (1.6×10^5-1.8×10^6)	1.6×10^4 (3.5×10^3-5.5×10^4)	4.1×10^4 (3.3×10^3-8.9×10^4)	4.2×10^4 (8×10^3-5.5×10^4)	8.6×10^4 (7.6×10^3-2.1×10^5)	n.d.[d]	n.d.
Initial gram negative plate count[a] at 26°C, 3d (VRBG)	9.1×10^4 (4.8×10^4-1.3×10^5)	1.1×10^5 (5.2×10^4-2.5×10^5)	1.2×10^5 (3.2×10^4-4.1×10^5)	<10 (<10)	<10 (<10)	<10 (<10)	<10 (<10)	n.d.	n.d.
Initial gram negative plate count[a] at 37°C, 1d (VRBG)	7.2×10^4 (6.8×10^4-9×10^4)	6.6×10^4 (6.1×10^4-7×10^4)	5.9×10^4 (2.8×10^4-9.3×10^4)	<10 (<10)	<10 (<10)	<10 (<10)	<10 (<10)	n.d.	n.d.
Presence of gram negatives[b]	100%	100%	100%	0%	11%	16%	34.1%	100%	20%
Presence of *P. flurescens*[b]	40%	42%	60%	0%	5.5%	0%	4.9%	33.3%	6.6%
Presence of gram positives[b]	100%	100%	100%	100%	100%	100%	100%	100%	20%

[a]Colony forming units per mL (cfu mL^{-1}), median value and range within parentheses, [b]Samples were incubated at 7°C until the aerobic plate count reached 10^6 to 10^7 cfu mL^{-1} of milk. Maximum three colonies per sample were randomly picked and identified, [c]Samples of condensed water on the surfaces of the filling machine, [d]Not determined



	Isolates			Biochemical reactions[c]				PL types and biotypes[d]			
	API 20NE Profiles	No.	%	NIT	ARG	GEL	ADI	PL 00	PL 03	PL 32	PL 33
B1	1157555	10	43.5	+	+	+	–		R4 (1)	R4 (1)	R4 (8)
B2	1156555	3	13	+	–	+	–				R4 (1), R3 (2)
B4	157555	2	8.7	–	+	+	–				R2 (2)
B5	157575	4	17.5	–	+	+	+				R5 (4)
B6	147555	3	13	–	+	–	–	R5 (1), R4 (2)			
B3	1156575	1	4.3	+	–	+	+				R1 (1)

Fig. 3: Unrooted phylogenetic tree showing relationship among different *Pseudomonas fluorescens* isolates based on API 20 NE profiles. [a, b]A measure of relatedness between isolates of *P. fluorescens* on a scale of 0.8 to 1 (0.8, 80% related; 1, 100% related). A total of 6 biotypes (B1 to B6) are grouped into 4 clusters (1 to 4). [c]Biochemical reactions: NIT: Nitrate, ARG: Arginine, GEL: Gelatin, ADI: Adipate, +: positive for substrate utilization, –: negative for substrate utilization. [d]PL types and ribotypes: PL types are indicated by numbers 00 to 33, while ribotypes include subtypes R1 to R5. Figures in parentheses indicate the number of isolates

Table 3: Proteolytic and lecithinase activity profiles of *Pseudomonas fluorescens* isolates

PL[a] profile	Proteolytic reaction		Lecithinase reaction		Isolates No. (%)
	7°C	26°C	7°C	26°C	
PL-00	-	-	-	-	3 (13)
PL-33	+	+	+	+	17 (74)
PL-03	-	-	+	+	1 (4.3)
PL-32	+	+	-	+	2 (8.7)

[a]PL: Proteolytic and lecithinase activity profiles. The first digit of number is representative of proteolytic profile and the second digital is representative of lecithinase activity profile

proteolytic and/ or lipolytic at 7 and 26°C. Nineteen out of the 23 isolates (82.6%) were proteolytic at both 7 and 26°C, and other isolates were nonproteolytic at both incubation temperatures. Eighty seven and 78.3% of the isolates were lipolytic at 26 and 7°C, respectively (Table 3). This study showed that the effect of temperature on production of proteinases and lipases by *P. fluorescens* was not significant ($P<0.05$) (Table 4). In all biotypes except for B1, the same PL profiles were observed (Fig. 3).

Table 4: Effect of temperature on production of proteinases and lecithinases by *Pseudomonas fluorescens*

Temperature	Percentage of isolates	
	Proteinases	Lecithinases
7°C	82.6[a1]	78.3[a]
26°C	82.6[a]	87[a]

[1]The same letter in each column indicates statistical non significance at 0.05 level

Each of the 23 *P. fluorescens* isolates was subtyped using the 16S-23S PCR ribotyping technique twice. With 16-23S PCR ribotyping, DNA fragments of 860, 830, 710, 650, 540, 310 and 200 bp were amplified (Fig. 4 and Table 5). The 23 isolates belonged to 5 subtypes (R1 to R5), of which R4 (56.5%) was predominant (Table 5 and Fig. 5). Phylogenetic analysis of *P. fluorescens* was performed to estimate the genetic relatedness between *P. fluorescens* isolates based on 16-23S PCR ribotyping results. The 23 isolates of *P. fluorescens* were placed into 4 clusters (Fig. 5). Cluster 4 consists of two ribotypes, R3 and R4. This cluster accounted for 65.2% of the isolates. Isolates grouped under each of the subtype R1, R2 and R3 belonged to the same biotype and PL profile, but different biotypes and/or PL profiles were observed in subtypes R4 and R5.

Discussion

A basic thought in this study was to look for *P. fluorescens* as the most frequent psychrotrophic bacteria (Kraft and Rey, 1979) that grow to high numbers in chill-stored milk. One single psychrotroph bacterium with a generation time of 4-5 h (Montville and Matthews, 2005) could then, in theory, reach about 10^7 cfu mL^{-1} within 7 to 10 days of storage at 7°C; therefore, the

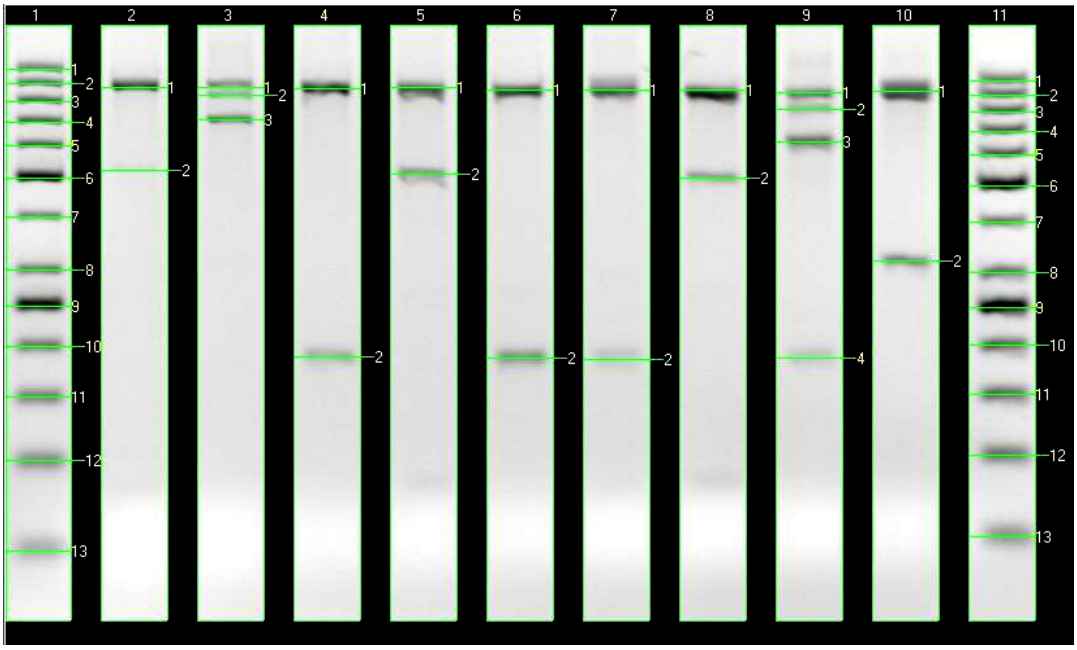
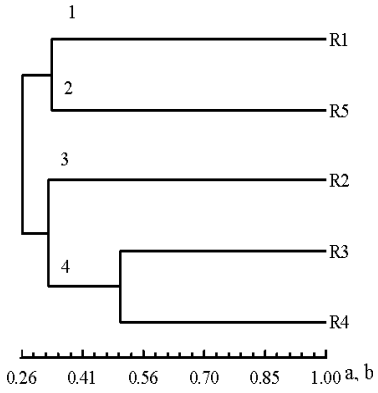


Fig. 4: PCR-ribotyping of *Pseudomonas fluorescens* isolated from the production line of pasteurized milk. Lanes 2, 3 and 4 give an example of different PCR-ribotypes. Lanes 2, 5 and 8 are three isolates of the same PCR-ribotype. Lanes 4, 6 and 7 are three isolates of the same PCR-ribotype. Lanes 1 and 11 marker gene ruler 50 bp MBI Fermentas, In lanes 1 and 11, Bands 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 are 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15 and 0.05 Kbp sizes, respectively

Table 5: 16S-23S PCR ribotyping patterns of *Pseudomonas fluorescens* isolates

16S-23S PCR ribotyping	Fragment size (bp)						
	860	830	710	650	540	310	200
R1	+	-	-	-	+	-	-
R2	+	+	+	-	-	-	-
R3	+	-	-	-	-	-	+
R4	+	+	-	+	-	-	+
R5	+	-	-	-	-	+	-



	Isolates		PL types and biotypes[c]			
	No.	%	PL00	PL03	PL32	PL33
R1	1	4.4				B2 (1)
R5	5	21.7	B6 (1)		B5 (1)	B5 (3)
R2	2	8.7				B4 (2)
R3	2	8.7				B2 (2)
R4	13	56.5	B6 (2)	B1 (1)	B1 (1)	B1 (8), B2 (1)

Fig. 5: Unrooted phylogenetic tree showing relationship among different *Pseudomonas fluorescens* isolates based on 16-23S PCR Ribotype profiles. [a]A measure of relatedness between isolates of *P. fluorescens* on a scale of 0.26 to 1 (0.26, 26% related; 1, 100% related). [b]A total of 5 ribotypes (R1 to R5) are grouped into 4 clusters (1 to 4). [c]PL types and biotypes: PL types are indicated by numbers 00 to 33, while biotypes are indicated as B1–B6. Figures in parentheses indicate the number of isolates

initial plate count of the fresh milk is of less interest. Since low levels of bacteria can be difficult to detect, and the traditional plate counts of the fresh product can be misleading, the procedure to incubate milk samples at 7°C before isolation and identification is necessary for the detection of organisms able to grow to high numbers in refrigerated milk (Eneroth *et al*., 1998).

In this study, the mean initial aerobic and gram negative plate counts of raw milk samples observed were higher than those reported by Hogan *et al*. (1988), Eneroth *et al*. (1998) and Jayarao and Wang (1999). This is related to hygienic practice during the production, storage and handling of milk from farms to the dairy plant (Hogan *et al*., 1988; Murphy and Boor, 2000; Boor and Murphy, 2002; Chambers, 2002; Blackburn, 2006). All the GNP present in the raw milk were killed during pasteurization, but still, a varying number of the milk packages were spoiled by GNP. Thus, recontamination does occur (Eneroth *et al*., 1998). In the present study the milk samples from site C (just after pasteurization) were free from GNP able to grow in milk. This means that, the pasteurization and the cleaning system of the pipes and tanks were working properly. However, post-pasteurization contamination by GNP occurred in the pasteurized milk reservoir (site D) and the filling machine (site E). In our study 34.1 and 4.9% of the milk packages showed contamination with GNP and *P. fluorescens*, respectively. Eneroth *et al*. (1998) isolated GNP and pseudomonads from 40% of milk packages, but they did not differentiate pseudomonads at species level. The filling procedure is an open process and allows the milk to come in contact with the surrounding air and its aerosols; condensed water on the machinery may also find its way into the milk, and the packaging material might be contaminated (Phillips and Griffiths, 1990). In the survey, 20% of packaging material was contaminated with GNP bacteria and *P. fluorescens* was isolated from them.

Recontamination by GNP bacteria during the filling process has previously been reported (Eneroth *et al*., 1998; Ralyea *et al*., 1998). Because of a relatively large opening on the top that was frequently used for sampling and milk level monitoring during the process, the pasteurized milk reservoir (site D) in this dairy plant was semi open and it could be a critical contamination point. Although GNP bacteria was detected in the air samples, *P. fluorescens* was not isolated from them. Salustiano *et al*. (2003) reported that suction of the air by an air sampler machine showed a microbial number up to 10 times higher than those determined by open Petri dish technique and using an air sampler is better for recovering microorganisms from the air.

The phenotypic characterization and API 20NE profiles of our *P. fluorescens* isolates have been reported by other researchers (Wang and Jayarao, 2001; Munsch-Alatossava and Alatossava, 2006). Phenotypic methods used in detecting the most common dairy product spoilers, i.e., *Pseudomonas* spp., frequently yield uncertain results. Furthermore, the simple identification of the same genus and species by standard methods in both environmental samples and in the finished product does not clearly establish a causal relationship (Wiedmann *et al*., 2000; Giraffa and Neviani, 2001; Martins *et al*., 2005). Munsch-Alatossava and Alatossava (2006) identified GNP bacteria in raw milk with API 20NE and BIOLOG GN2 identification systems. They reported that the results were system-dependent. The results of the numerical profile analyses by API 20NE proposed that some strains might be members of *Stenotrophomonas*, *Burkholderia* and *Acinetobacter* genera; however, the identity of many isolates remained doubtful or controversial (Munsch-Alatossava and Alatossava, 2006). Kostman *et al*. (1992) and Dasen *et al*. (1994) reported PCR-ribotyping as a rapid and accurate method for typing *Burkholderia* (*Pseudomonas*) *cepacia*. Wang and Jayarao (2001) used this method in genotypic characterization of *Pseudomonas* strains isolated from bulk tank milk. Our survey showed that the 16S-23S PCR ribotyping technique allowed differentiation of *P. fluorescens*. The predominance of a particular ribotype was often observed for a given biotype in the clusters. From 6 detected biotypes, B1, B4, B5 and B3 only showed R4, R2, R5 and R1 ribotyping profile, respectively. Although within each of the biotypes B2 and B6 two ribotypes were found, suggesting the discriminatory ability of the PCR-ribotyping technique in tracking bacteria and finding exact contamination routes in the production line of pasteurized milk may be limited. Our results showed that 87% of *P. fluorescens* isolates were observed to be proteolytic and lipolytic at 7 and 26°C, so they can cause defects in raw and pasteurized milk due to proteolysis of milk proteins and lipolysis of milk fat. Munsch-Alatossava and Alatossava (2006) and Wang and Jayarao (2001) reported differences in proteolytic and lipolytic activity of *P. fluorescens* isolates.

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