

## Characterisation of the thermostable protease AprX in strains of *Pseudomonas fluorescens* and impact on the shelf-life of dairy products: preliminary results

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### Abstract

Bacterial proteases are involved in food spoilage and shelf-life reduction. Among the bacterial proteases, a predominant role in spoilage of dairy products seems to be played by the thermostable metallo-protease AprX, which is produced by various strains of *Pseudomonas fluorescens*. Differences in AprX enzyme activity among different strains were highlighted, but the most proteolytic strains were not identified. In this study, the presence of the *aprX* gene was evaluated in 69 strains isolated from food matrices and 18 reference strains belonging to the *P. fluorescens* group, which had been previously typed by the multi locus sequence typing method. Subsequently, a subset of reference strains was inoculated in ultra-high temperature milk, and the expression of the *aprX* gene was evaluated at 22 and 6°C. On the same milk samples, the proteolytic activity was then evaluated through Azocasein and trinitrobenzenesulfonic acid solution assays. Finally, to assess the applicability of the former assay directly on dairy products the proteolytic activity was tested on industrial *ricotta* samples using the Azocasein assay. These results demonstrate the spread of *aprX* gene in most strains tested and the applicability of Azocasein assay to monitor the proteolytic activity in dairy products.

### Introduction

Thermostable protease activity was indicated as one of the main factors responsible of dairy products spoilage, such as gelation of ultra-high temperature (UHT) milk, with consequences on the shelf-life and significant economic losses for the food industry.

Proteases are predominantly active against the casein fraction, which causes gelation of UHT milk and/or the formation of bitter off-flavors. The gelation of UHT milk derives from the formation of complexes between the  $\kappa$ -casein and  $\beta$ -lactoglobulin that are denatured as a result of the heat treatment. Gelation is triggered by enzymatic processes of protein degradation (Rauh *et al.*, 2014; Datta and Deeth, 2001). The proteolysis during storage of UHT milk seems to be a consequence of the interaction between the plasminogen-plasmin system (endogenous protease of the milk) with proteases of bacterial origin. Among the bacterial proteases involved in spoilage processes a predominant role seems to be played by the thermostable metallo-protease AprX, which is produced by various strains of the species *Pseudomonas fluorescens*. The AprX protease, produced and released by bacteria in milk, is resistant to heat and is able to maintain unaltered its activity even after heat treatments that milk may undergo during its processing such as pasteurization, UHT treatment, and cheese-making (Ismail and Nielsen, 2010; Frohbieter *et al.*, 2005). Such enzyme activities could also have a role during cheese ripening for the formation of flavors in seasoned cheeses, and aromatic characteristics of milk cultures of the *P. fluorescens* group are strain-dependent (Morales *et al.*, 2005; Carraro *et al.*, 2011).

To evaluate proteolytic activity, several assays were developed and applied in *P. aeruginosa* (Kessler and Safrin, 2014) and some of them tested for AprX activity on *P. fluorescens* strains (Dufour *et al.*, 2008; Marchand *et al.*, 2009a). The AprX protein and its encoding gene have been studied extensively (Liu *et al.*, 2007; Maunsell *et al.*, 2006; Nicodeme *et al.*, 2005; Woods *et al.*, 2001; Liao and McCallus, 1998). Differences in AprX enzyme activities were highlighted, but the identification of the most proteolytic strains was not completely elucidated (Dufour *et al.*, 2008; Marchand *et al.*, 2009a, 2009b). This is due partially to the unreliability of methods currently used for the identification of *P. fluorescens* strains. Recently, *P. fluorescens* was recognized as a species group (Mulet *et al.*, 2010) and a molecular typing approach was developed and applied on several *P. fluorescens* reference and field strains (Andreani *et al.*, 2014). The determination of the genetic diversity among species and strains belonging to the *P. fluorescens* group provides an accurate method for strain identification.

In the present study the presence of the *aprX* gene was evaluated in 69 strains isolated from food matrices and 18 reference strains belonging to the *P. fluorescens* group. All strains were previously typed by multi locus sequence typing (MLST) method and allocated in a *subgroup* as genetically related to a specific reference type strain.

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Key words: *Pseudomonas fluorescens*; Spoilage; Dairy products; AprX.

Received for publication: 19 July 2016.

Revision received: 16 November 2016.

Accepted for publication: 19 November 2016.

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Italian Journal of Food Safety 2016; 5:6175

doi:10.4081/ijfs.2016.6175

In a selected group of reference strains, the activity and gene expression of AprX were tested in milk. Finally, the applicability of Azocasein assay directly on dairy products was evaluated on industrial *ricotta* samples. The results demonstrated the spread of *aprX* gene in most of the strains tested and the applicability of trinitrobenzenesulfonic acid solution (TNBS) and Azocasein tests to monitor the proteolytic activity in dairy products.

### Materials and Methods

#### Bacterial strains

*P. fluorescens* group strains are listed in Table 1. For each strain, the sequence type (ST) and the *subgroup* obtained by MLST analysis (Andreani *et al.*, 2014) are reported. Strains were conserved at -80°C in Tryptic Soy Broth [TSB; Oxoid, Basingstoke, UK; with 50% v/v glycerol (Sigma-Aldrich, Saint Louis, MO, USA)].

#### DNA and RNA extraction

For DNA extraction, a single colony from a fresh culture on CFC *Pseudomonas* Agar Base (CFC PAB; Oxoid) was re-suspended in 100  $\mu$ L of nuclease-free water, vortexed at high speed for 5 seconds and incubated at 95°C for 10 minutes. The tube was vortexed again and centrifuged for 2 minutes at 14,000 rpm. The supernatant was transferred to a fresh tube and stored at -20°C (Martino *et al.*, 2011).

For RNA extraction, a single pure colony of each strain was inoculated in triplicate (giving 3 biological replicates for each strain) in 3 mL MBM Broth [0.7% K<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% trisodium citrate, 0.01% MgSO<sub>4</sub>, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2% glucose] and kept at 22°C for

**Table 1. Species, subgroups, food origin, sequence type, presence of *AprX* gene and proteolytic activity in plate of *Pseudomonas fluorescens* strains.**

Strain	Species	Subgroup	Source	ST	<i>AprX</i> gene	Proteolytic activity		
						6°C	22°C	31°C
DSM 17152T	<i>Pseudomonas gessardii</i>	<i>P. fluorescens</i> subgroup	Mineral water	7	+	+	++	-
DSM 15294T	<i>Pseudomonas brenneri</i>	<i>P. fluorescens</i> subgroup	Natural mineral water	8	+	+	++	-
DSM 17967T	<i>Pseudomonas mandelii</i>	<i>P. mandelii</i> subgroup	Mineral water	9	+	+	+	-
DSM 17150T	<i>Pseudomonas jessenii</i>	<i>P. jessenii</i> subgroup	Mineral water	10	+	-	+	-
DSM 16610T	<i>Pseudomonas koreensis</i>	<i>P. koreensis</i> subgroup	Agricultural soil	11	+	+++	+	++
DSM 17489T	<i>Pseudomonas orientalis</i>	<i>P. fluorescens</i> subgroup	Spring water	12	+	+++	++	-
DSM 18928T	<i>Pseudomonas synxantha</i>	<i>P. fluorescens</i> subgroup	Cream	13	+	++	+	-
DSM 18862T	<i>Pseudomonas azotoformans</i>	<i>P. fluorescens</i> subgroup	Paddies	14	+	+++	+++	-
DSM 6252T	<i>Pseudomonas lundensis</i>	<i>P. fragi</i> subgroup	Prepacked beef	15	+	-	-	-
DSM 14020T	<i>Pseudomonas rhodesiae</i>	<i>P. fluorescens</i> subgroup	Natural mineral water	16	+	++	+++	-
DSM 11331T	<i>Pseudomonas veronii</i>	<i>P. fluorescens</i> subgroup	Mineral water	17	+	++	++	-
DSM 17149T	<i>Pseudomonas libanensis</i>	<i>P. fluorescens</i> subgroup	Spring water	18	+	+++	++	-
DSM50415	<i>Pseudomonas fluorescens</i>	<i>P. fluorescens</i> subgroup	Soil	19	+	++	++	+
CECT124T	<i>Pseudomonas corrugata</i>	<i>P. corrugata</i> subgroup	Tomato: pith necrosis	20	+	-	++	++
CECT229T	<i>Pseudomonas marginalis</i>	<i>P. fluorescens</i> subgroup	<i>Cichorium intybus</i> (endive)	21	+	-	+++	-
CECT378T	<i>Pseudomonas fluorescens</i>	<i>P. fluorescens</i> subgroup	Pre-filter tanks, town water works	22	+	++	++	-
CECT446T	<i>Pseudomonas fragi</i>	<i>P. fragi</i> subgroup	Unknown	23	+	+++	+++	-
CECT4470T	<i>Pseudomonas chlororaphis</i>	<i>P. chlororaphis</i> group	Plate contaminant	24	+	++	++	++
ps_1		<i>P. fluorescens</i> subgroup	Mozzarella cheese	25	+	+	++	-
ps_2		<i>P. fluorescens</i> subgroup	Blue mozzarella cheese	26	+	++	++	-
ps_3		<i>P. fragi</i> subgroup	Mozzarella cheese	27	+	-	-	-
ps_4		<i>P. fluorescens</i> subgroup	Mozzarella cheese	28	+	-	+++	-
ps_5		<i>P. fluorescens</i> subgroup	Mozzarella cheese	29	+	-	++	-
ps_6		<i>P. fluorescens</i> subgroup	Blue mozzarella cheese	30	+	++	++	-
ps_7		<i>P. koreensis</i> subgroup	Mozzarella cheese	31	+	+++	+++	-
ps_8		<i>P. fluorescens</i> subgroup	Mozzarella cheese	32	+	+++	+++	-
ps_9		<i>P. fluorescens</i> subgroup	Mozzarella cheese	33	-	++	++	-
ps_10		<i>P. fluorescens</i> subgroup	Mozzarella cheese	34	+	++	++	-
ps_11		<i>P. fluorescens</i> subgroup	Mixed salad	35	+	+	++	-
ps_12		<i>P. fragi</i> subgroup	Butter	36	+	-	-	-
ps_13		<i>P. fluorescens</i> subgroup	Blue mozzarella cheese	37	+	++	++	-
ps_14		<i>P. koreensis</i> subgroup	Pork	38	+	-	+	+
ps_15		<i>P. fluorescens</i> subgroup	Salmo trutta marmoratus (trout)	39	+	++	++	-
ps_16		<i>P. fluorescens</i> subgroup	Salmo trutta fario (trout)	40	+	+++	+++	++
ps_17		<i>P. fragi</i> subgroup	Ricotta	41	+	++	+	-
ps_18		<i>P. fluorescens</i> subgroup	UHT milk	42	+	++	++	++
ps_19		<i>P. fragi</i> subgroup	UHT milk	43	-	-	+	++
ps_20		<i>P. fluorescens</i> subgroup	UHT milk	44	+	+++	++	+
ps_21		<i>P. koreensis</i> subgroup	Mozzarella cheese	45	+	+	+	-
ps_22		<i>P. fluorescens</i> subgroup	Blue mozzarella cheese	46	+	+	++	-
ps_23		<i>P. koreensis</i> subgroup	Mozzarella cheese	47	+	-	++	-
ps_24		<i>P. fluorescens</i> subgroup	Cheese	48	+	-	++	-
ps_25		<i>P. fluorescens</i> subgroup	UHT milk	49	+	-	+	-
ps_26		<i>P. koreensis</i> subgroup	Human	50	+	++	++	++
ps_27		<i>P. fluorescens</i> subgroup	Mixed salad	51	+	++	++	-
ps_28		<i>P. koreensis</i> subgroup	Mixed salad	52	+	++	+++	+++
ps_29		<i>P. fluorescens</i> subgroup	Mixed salad	53	+	+++	+++	-
ps_30		<i>P. fluorescens</i> subgroup	Mixed salad	54	+	+	+++	-
ps_31		<i>P. fluorescens</i> subgroup	Mixed salad	55	+	++	+++	-
ps_32		<i>P. chlororaphis</i> group	Mixed salad	56	+	-	-	-
ps_33		<i>P. fluorescens</i> subgroup	Mixed salad	57	+	++	++	-
ps_34		<i>P. fluorescens</i> subgroup	Mixed salad	58	+	+++	+++	-

Continued on next page.

24 h to reach  $10^8$  CFU/mL. Then, cultures were diluted five times in 3 mL of milk. After 24h of incubation at 22°C or five days at 5°C, 1 mL of culture was then extracted using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNase treatment was performed using the Qiagen RNase-Free DNase Set (Qiagen). RNA was eluted in 30 µL of RNase-free H<sub>2</sub>O. Purified DNA and RNA were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One microgram of total RNA for each sample was reverse transcribed to cDNA using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To evaluate DNA contamination in RNA samples treated with DNase, no-RT (reverse-transcriptase) samples were also checked in PCR amplification.

### Polymerase chain reaction amplification

Primers for *aprX* gene amplification were designed using PriFi software (<http://cgi-www.daimi.au.dk/cgi-chili/PriFi>; Fredslund *et al.*, 2005) using as template the alignment of *aprX* sequences from complete genomes of *P. fluorescens* group already available in Genbank (<http://www.ncbi.nlm.nih.gov/genome>). The sequence of primers was Pse\_AprXF1 CAGACCCTGACCCACGARATCGG and Pse\_AprXR1 TGAGGTTGATCTCTGGTTCTGGG. The *tpoD* housekeeping gene was used as positive control for DNA and RNA extraction using primers reported in Andreani *et al.* (2014).

PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler

(Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 20 µL of amplification mix containing 1U of GoTaq polymerase (Promega, Madison, WI, USA), 1X GoTaq Buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleotide triphosphate (dNTP), 250 mM each primer and 5 ng of genomic DNA as template. The reaction mixture was subjected to the following thermal cycle: an initial step at 94°C for 2 min to activate the polymerase and 35 cycles each of denaturation at 94°C for 20 seconds, annealing of the primers at 60°C for 30 seconds and extension at 72°C for 1 minute and a final step of extension at 72°C for 7 min. Amplified products were analysed by electrophoresis on 1.8% agarose-Tris-acetate-EDTA (TAE) gels, stained with SYBR® Safe DNA Gel Stain (Invitrogen) and visualized on a UV transilluminator (Gel Doc XR™; Biorad, Hercules, CA, USA).

Table 1. Continued from previous page.

Strain	Species	Subgroup	Source	ST	<i>AprX</i> gene	Proteolytic activity		
						6°C	22°C	31°C
ps_35		<i>P. fluorescens</i> subgroup	Mixed salad	59	+	+++	++	-
ps_36		<i>P. fluorescens</i> subgroup	Mixed salad	60	+	+++	++	-
ps_37		<i>P. clororaphis</i> subgroup	Mixed salad	61	+	+	++	+++
ps_38		<i>P. clororaphis</i> subgroup	Mixed salad	62	+	+	++	+++
ps_39		<i>P. fluorescens</i> subgroup	Mixed salad	63	+	++	++	+
ps_40		<i>P. fluorescens</i> subgroup	Ricotta	64	+	+++	++	-
ps_48		<i>P. fragi</i> subgroup	Pork	72	+	++	+++	+
ps_50		<i>P. fragi</i> subgroup	Pork	74	+	++	+++	+++
ps_51		<i>P. clororaphis</i> group	Pork	75	+	++	+++	+++
ps_54		<i>P. fragi</i> subgroup	Pork	78	+	++	++	+
ps_55		<i>P. koreensis</i> subgroup	Pork	79	+	-	++	+++
ps_56		<i>P. koreensis</i> subgroup	Sashimi	80	+	+	++	+++
ps_57		<i>P. fluorescens</i> subgroup	Sashimi	81	+	++	+++	++
ps_58		<i>P. corrugata</i> subgroup	Sashimi	82	+	-	++	++
ps_59		<i>P. fluorescens</i> subgroup	Sashimi	83	+	+++	+++	++
ps_60		nd	Sashimi	nd	+	+++	+++	+++
ps_61		<i>P. fluorescens</i> subgroup	Sashimi	84	+	+	+++	++
ps_62		nd	Sashimi	nd	+	+	+++	+++
ps_63		nd	Sashimi	nd	+	++	+++	++
ps_64		<i>P. fluorescens</i> subgroup	Sashimi	85	+	-	+++	+
ps_65		<i>P. koreensis</i> subgroup	Rocket	86	+	++	+++	+++
ps_66		<i>P. koreensis</i> subgroup	Rocket	87	+	++	+++	+++
ps_67		<i>P. koreensis</i> subgroup	Valerian	88	+	++	+++	+++
ps_68		<i>P. corrugata</i> subgroup	Valerian	89	+	-	+++	+++
ps_69		<i>P. mandelii</i> subgroup	Dairy-product	90	+	-	+	+
ps_70		<i>P. fragi</i> subgroup	Dairy-product	91	+	+	++	-
ps_71		<i>P. fragi</i> subgroup	UHT milk	92	-	+	+	+
ps_72		<i>P. fragi</i> subgroup	Dairy-product	93	+	-	++	+
ps_73		<i>P. fluorescens</i> subgroup	Dairy-product	94	-	-	+	-
ps_74		<i>P. fragi</i> subgroup	Dairy-product	95	+	++	+	+
ps_75		<i>P. fluorescens</i> subgroup	Blue mozzarella cheese	29	+	-	++	-
ps_76		<i>P. fluorescens</i> subgroup	Mozzarella cheese	26	+	++	++	-
ps_77		<i>P. fluorescens</i> subgroup	Meat	96	+	-	++	-
ps_78		<i>P. fluorescens</i> subgroup	Mozzarella cheese	97	+	-	+	-
ps_79		<i>P. fluorescens</i> subgroup	Meat	98	+	-	-	-

ST, sequence type; UHT, ultra-high temperature; nd, not determined.

## Proteolytic activity in plate

The reference and field strains were tested for their proteolytic activity by agar diffusion assays at 6°C for 10 days, at 22 and 31°C for 7 days. All the strains were revitalized through a 72-hours-preinoculum in TSB at 22°C. A dilution of 10<sup>5</sup> CFU/mL was applied for the subsequent tests. The extracellular protease activity evaluation was conducted on Nutrient Agar (NA; Biokar diagnostics, Paris, France) with 2% UHT milk observing a clear zone around the colonies. The presence of a clear zone around the colonies after incubation was indicative for proteolysis.

## Quantification of extracellular proteolytic activity (Azocasein assay)

Proteolytic activity of bacterial strains was quantified using Azocasein (Sigma-Aldrich) as substrate. One hundred µL of a 3% (w/v) Azocasein stock solution were added to 100 µL of cell free supernatant fluid and 300 µL of 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.5. The negative control was set up with 100 µL of not inoculated milk. The mixture was incubated at 37°C for 1 h and the reaction was stopped by adding 500 µL of 20% (w/v) trichloroacetic acid (TCA). The sample was centrifuged at 12,000 g for 10 min and absorbance of the supernatant was measured at 366 nm using Multiskan GO UV/Vis spectrophotometer (Thermo Fisher Scientific). Absorbance of the blank (500 µL not incubated sample plus 500 µL of TCA 20%), and of the negative control were subtracted from sample absorbance. The results are reported as OD<sub>366</sub>.

## Quantification of proteolysis (trinitrobenzenesulfonic acid solution test)

The experimental set-up made it possible to calculate the net proteolytic activity produced during 2 weeks of storage at 37°C after a heat treatment simulating UHT process and storage (as described in Marchand *et al.*, 2008, 2009a). Hydrolysis of proteins was measured by the determination of the release of α-amino

groups by the trinitrobenzenesulfonic acid (TNBS) method. The free amino groups react with the TNBS reagent (Sigma-Aldrich) at pH 9.2 in the dark. A yellow-orange colour develops and its intensity is determined by absorption measurements at 420 nm. The degree of proteolysis is calculated from the increase in absorption after 2 weeks of storage at 37°C and expressed as mmol of glycine equivalents mL<sup>-1</sup> milk, using glycine (2.5, 2.25, 2, 1.75, 1.5, 1.25, 1, 0.75, 0.5, 0.25 e 0 mM; Sigma-Aldrich) to create a standard curve. The experiment was repeated twice, first in macro method (experiment A using a 7800 UV/Vis spectrophotometer; JASCO, Easton, MD, USA) and second time in micro method (experiment B using a Multiskan GO UV/Vis spectrophotometer (Thermo Fisher Scientific).

## Proteolytic activity in industrial ricotta samples

Of *ricotta* samples, 10 g were inoculated with 1 mL of a 10<sup>8</sup> cell/mL of a fresh culture of *P. fluorescens* group strains growth in TSB in sterile 50 mL tubes and were maintained at refrigeration temperature for seven days. The Azocasein assay was carried out in order to evaluate the proteolytic activity of the inoculated *ricotta* in comparison to three industrial *ricotta* samples, belonging to the same lot, collected and analyzed 24 h from production. One gr of *ricotta* was sampled in duplicate from each of the four inoculated samples and the three fresh *ricotta* samples and diluted in 5 mL of Phosphate-buffered saline (PBS). After mixing, 2 mL were centrifuged at 12,000 rpm for 5 minutes, then 100 µL of the supernatant was used for the Azocasein assay as previously described.

## Results

### Distribution of the *aprX* gene in

### *Pseudomonas fluorescens* group

The distribution of the *aprX* gene was evaluated on 18 reference and 69 field strains and the result is reported in Table 1. Only for four strains (4.6%) the PCR amplification of *aprX* gene, repeated twice, gave a negative result. All these four strains resulted positive to the amplification of the *rpoD* gene. The four strains belonged to the *P. fluorescens* subgroup (ps\_9 and ps\_73) or to the *P. fragi* subgroup (ps\_19 and ps\_71).

### Proteolytic activity of

### *Pseudomonas fluorescens* strains in plate

Proteolytic activity was observed at least at one temperature condition in 17 reference strains and 65 fields strains (for a total of 94.2%), indicating that proteolysis is a common spoilage mechanism for *P. fluorescens* group strains. The non-proteolytic strains are *P. lundensis* DSM6252T, ps\_3, ps\_12, ps\_32, ps\_79. The complete data are reported in Table 1.

### Proteolytic activity and *aprX* gene expression in milk samples inoculated with *Pseudomonas fluorescens*

The experiment was set up to simulate the conditions during UHT milk production (as described in Marchand *et al.*, 2008, 2009a) and a schematic representation of the analyses is reported in Figure 1. Briefly, 100 µL (10<sup>7</sup> cells) of a fresh culture of each strain was inoculated in 10 mL of UHT milk and grown for 24 hours. An aliquot of 100 µL of the culture was then inoculated in 10 mL of UHT milk and grown for 24 hours. Double growth in milk until exponential phase was done to adapt bacterial strains to milk. The culture was then diluted to 10<sup>3</sup> cfu/mL in UHT milk and incubated at 6°C for five days. At the end of the incubation, the Azocasein test was performed as described in Materials and Methods to measure the global proteolytic activity (thermoremanent and non-

Table 2. Proteolytic activity and *AprX* gene expression in *Pseudomonas fluorescens* group references strains.

Type strains	Subgroups	Proteolytic activity in plate*			<i>AprX</i> gene expression°		Azocasein OD <sub>366</sub>		TNBS# mM glycine		Azocasein <sup>§</sup> Ricotta OD <sub>366</sub>
		6°C	22°C	31°C	22°C	6°C	Pre-HT	Post-HT	Experiment A	Experiment B	
DSM 17489T	<i>P. orientalis</i>	+++	++	-	+++	++	0.28	0.22	3.69	11.85	1.02
DSM 6252T	<i>P. lundensis</i>	-	-	-	---	---	0.02	0.01	1.92	6.72	nd
DSM 14020T	<i>P. rhodesiae</i>	++	+++	-	+++	---	0.07	0.04	1.71	24.09	0.34
DSM 17149T	<i>P. libanensis</i>	+++	++	-	+++	---	0.37	0.17	-	-	1.38
CECT378T	<i>P. fluorescens</i>	++	++	-	++	---	0.1	0.03	3.15	7.24	1.01
CECT446T	<i>P. fragi</i>	+++	+++	-	+++	---	0.28	0.18	-	-	nd
CECT229T	<i>P. marginalis</i>	-	+++	-	---	---	0.01	0.02	-	-	nd

TNBS, trinitrobenzenesulfonic acid solution; HT, high-temperature. \*, ++ or +++ depending on the size of the clear zone around the colonies; °+ or - is positive or negative result in the amplification of each triplicate; #experiment A was performed in macromethods, Experiment B in micromethods; §reported the average between the OD of the duplicates. nd, not determined.



thermoresistant proteases). The culture was then heat-treated (10 mL for 8 minutes and 45 seconds at 95°C to simulate UHT treatment) and a second Azocasein test was performed to measure the thermo-resistant protease activity. The culture was then incubated for two weeks at 37°C. After incubation, the TNBS test was performed as described in Materials and Methods section.

This preliminary experiment was performed with six reference strains, including a negative control (not inoculated milk) and the results are summarized in Table 2. The gene expression study demonstrated that, in the condition tested, the *aprX* gene is not expressed in *P. lundensis* and *P. marginalis*. The Azocasein assay gave OD<sub>366</sub> values ranging from 0.01 to 0.37. The TNBS assays gave values in mM Glycin from 0 (negative results for three strains) to 24.09 in micro method and from 0 to 3.69 in macro method. Proteolytic activity data is only partially in agreement with expression data.

### Proteolytic activity in industrial ricotta samples

The results of proteolytic activity measured on ricotta samples are reported in Table 2 and Figure 2. The data evidenced variable activity in the freshly produced ricotta samples despite the three samples belonged to the same lot of production.

## Discussion

The analysis of 87 *P. fluorescens* strains for the presence of the *aprX* gene demonstrated that the gene is widespread in this bacterial group. The *aprX* negative strains are not strongly genetically related (Andreani *et al.*, 2014) suggesting that the lack of *aprX* amplification, could be due to loss of the gene or mis-

matches in primer sites that occurred independently in each strain. However, the *aprX* gene expression study demonstrated strong variability across strains, which might explain the large variability in proteolytic activity reported in previous studies (Dufour *et al.*, 2008; Marchand *et al.*, 2009a, 2009b).

To evaluate the spoilage activity of AprX in dairy products, milk and ricotta cheese were used as template to inoculate strains positive to *aprX* gene. The experimental protocol for milk, reported in Figure 1, was designed to simulate UHT milk productive process, with milk samples incubated first at refrigeration temperature, and after heat treatment, incubated at 37°C. Similar experimental design to simulate UHT milk production was proposed and applied in previous studies (Marchand *et al.*, 2008, 2009a).

The proteolytic activity was measured with two different assays, the first, the Azocasein assay, measures directly the activity with a colorimetric reaction, the second, the TNBS assay, measures free amino acids (amino-groups), as products of the proteolysis. The two different assays are both easy and fast to be carried out, inexpensive and require a common spectrophotometer. In the present study, these assays were applied to compare their sensibility on dairy products inoculated with a bacterial culture. However, the results of the two assays are only partially comparable. The Azocasein assay results are in good agreement with *aprX* expression data, if considering value less to 0.05 OD<sub>366</sub> as negative. On the contrary, TNBS assay results are discordant with *aprX* expression and Azocasein assay data for *P. lundensis* and *P. libanensis*. On the basis of these results the Azocasein assay might be more reliable as a direct measure of the enzyme activity, however some concerns remain. First, the Azocasein assay had not a standard curve to be used to compare the results. Secondly, protease activity immediately after the heat

treatment might be slower as a consequence of the treatment itself and this effect might be different in the different strains. This effect could be due to differences in protein structure despite the nucleotide sequence was reported to be very conserved among *P. fluorescens* strains (Marchand *et al.*, 2009b) or to different resistance of strains to heat treatment. The gelation of UHT milk, if occurs, usually takes place after weeks from production. This long time might depend on the time required by proteases to reactivate after heat treatment or to some chemical modification that might occur in milk and activate proteases.

Anyway, this preliminary study strongly confirms strain variability in protease activity. *P. lundensis* and *P. fragi* were indicated as the species mostly involved in spoilage of milk (Marchand *et al.*, 2009a). For these two strains, contrasting results were obtained in the present study. Regarding *P. lundensis* type strain, the *aprX* expression was negative in milk as it was proteolytic activity in plate and at Azocasein assay, even if the TNBS assay yielded a positive result. On the contrary, *P. fragi* type strain expressed *aprX* and was positive for proteolytic assay both in plate and with Azocasein assay, but was negative with TNBS assay. Thanks to MLST molecular typing, the *P. fluorescens* group fields strains were accurately identified and the application of the experiment in milk using fields strains belonging to *P. fragi* subgroup (that include *P. lundensis*) is interesting to confirm if the spoilage phenotype is related to this taxonomic group.

Finally, with the aim to test the applicability of these assays to analyze dairy products, in which spoilage activity might be due to thermo-resistant proteases, industrial ricotta was chosen as a study case. In fact, industrial ricotta is produced at high temperature (90°C) and successively pasteurized (80°C) starting from milk whey that usually presents high bacterial loads (among these bacteria, *Pseudomonas* is

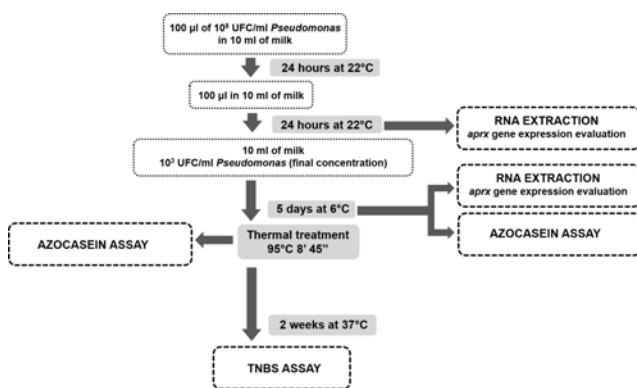


Figure 1. Schematic description of the experiment set-up simulating the conditions of ultra-high temperature milk production.

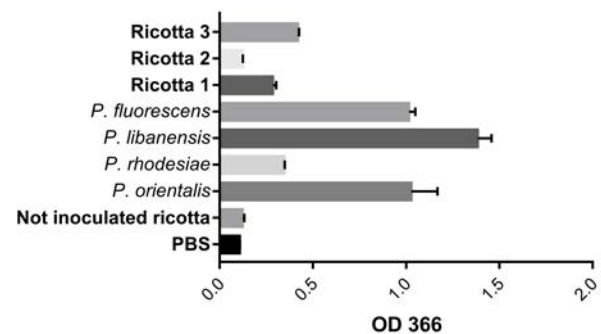


Figure 2. Proteolytic activity in ricotta samples.

often present). Industrial *ricotta* often shows premature spoilage that reduces product shelf-life. The application of Azocasein assay on freshly produced industrial *ricotta* samples showed protease activity that might be one of the responsible factors for premature spoilage. This result highlights the importance of good quality raw material to extend shelf-life also for heat-treated products.

## Conclusions

These preliminary data highlight the interest, but also the complexity of studying the proteolytic activity of the *aprX* gene in *P. fluorescens*. The availability of MLST-typed strains might help to identify the major *aprX* producing strains and evaluate if such spoilage activity is a phenotypic trait linked to specific lineages in the *P. fluorescens* group. Azocasein and TNBS assay should be improved, but might be suitable (in particular the Azocasein assay) to evaluate proteolytic activity in dairy products such as industrial *ricotta* or UHT milk to assess in advance the spoilage potential during shelf-life.

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