Original article

Purification and characterization of a proteinaceous compound from *Pseudomonas fluorescens* ATCC 948 with inhibitory activity against some Gram-positive and Gram-negative bacteria of dairy interest

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Summary — A proteinaceous antimicrobial compound from *Pseudomonas fluorescens* ATCC 948 was purified to homogeneity by chromatography steps and characterized. The compound showed a large spectrum of activity. Inhibition of thermophilic *Lactobacillus*, *Listeria monocytogenes*, *Enterobacteriaceae* and *P. fluorescens* strains was mainly detected. The antimicrobial compound produced in skim milk had an apparent molecular mass of 67 kDa, was quite heat-stable (80 °C for 15 min), insensitive to lipase and α -amylase, and completely inactivated by treatment with proteolytic enzymes. A bactericidal mode of action was detected against *Lactobacillus fermentum* D13 chosen as indicator strain. *P. fluorescens* ATCC 948 was not sensitive to the antimicrobial compound produced by itself. The production of the proteinaceous substance started in the early exponential phase of growth, and was highest after 72 h of incubation at 20 °C and pH 6.5. Some considerations about the nature of the antimicrobial compound are given.

Pseudomonas fluorescens / proteinaceous antimicrobial compound / thermophilic *Lactobacillus*

Résumé — Purification et caractérisation d'une substance protéinique par *Pseudomonas fluorescens* ATCC 948, avec activité d'inhibition contre certaines bactéries Gram positives et Gram négatives d'intérêt laitier. Une protéine présentant une activité antimicrobienne a été produite par *Pseudomonas fluorescens* ATCC 948. Elle a été purifiée jusqu'à homogénéité par chromatographie et caractérisée. Elle présente un large spectre d'activité et une inhibition contre certaines souches de *Lactobacillus* thermophiles, contre *Listeria monocytogenes*, *Enterobacteriaceae* et des souches de *P. fluorescens*. Le composé antimicrobien produit dans du lait écrémé présente un poids moléculaire apparent de 67 kDa, est suffisamment thermostable (80 °C pendant 15 minutes), pas sensible à la lipase et à l' α -amylase, mais est complètement inactivé par traitement aux enzymes protéolytiques. Son mode d'action bactéricide a été déterminé contre *Lactobacillus fermentum* D13, choisi comme souche-témoin. *P. fluorescens* ATCC 948 n'est pas sensible au composé antimicrobien produit par lui-même.

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La production de cette substance protéinique a commencé pendant la première phase de développement exponentiel, et a été la plus importante après 72 heures d'incubation à 20 °C et pH 6,5. Quelques considérations concernant la nature du composé protéinique ont été proposées.

Pseudomonas fluorescens / composé antimicrobien protéinique / *Lactobacillus thermophile*

INTRODUCTION

Psychrotrophic pseudomonads are the most common microorganisms that grow in raw milk during cold storage (Cousin, 1982). *Pseudomonas fluorescens* has been shown to secrete extracellular lipases, and especially proteinases, which are resistant to both pasteurization and UHT treatments (Griffiths et al, 1981). Proteinases cause bitterness in UHT milk (Gobetti, 1993) and contribute, together with plasmin, to age gelation of unconcentrated UHT milk (de Koning et al, 1985).

Several studies have been conducted on the inhibitory effect of lactic acid bacteria against *Pseudomonas* strains. Inhibition of psychrotrophic pseudomonads, either by lowering pH, producing lactic acid and hydrogen peroxide or by more complex microbial interactions, has been shown with *Leuconostoc mesenteroides* subsp *dextranicum* (Mans De Marion et al, 1995); commercial lactic cultures (Champagne et al, 1990) inoculated in raw milk stored at low temperature; lactic acid bacteria starters used in cultured milks (Balasubramanyam and Varadaraj, 1994); culture supernatants of strains of *Bifidobacterium* cultivated in skim milk (Mitsuhashi and Murata, 1991) and with neutralized extracellular culture filtrates of *Lactobacillus* and *Lactococcus* species (Varadaraj et al, 1993). On the contrary, psychrotrophic pseudomonads which are initially not antagonized can promote the lactic acid bacteria metabolism by their proteolytic products (Koburger and Claydon, 1961; Tayfour et al, 1981) and by urease activity, which produces a decrease in

the amount of native urea of milk and increases in the ammonia concentration (Jaspe et al, 1995).

To our knowledge, no reports have been made on the antagonistic activity of psychrotrophic pseudomonads towards lactic acid bacteria. Siderophores, pterines, indoles, phenazines, lipids, peptides and bacteriocins are produced by *Pseudomonas* spp strains and have been extensively studied for their antimicrobial activity in the biocontrol of plant diseases (Dowling and O' Gara, 1994). Bacteriocins from *P. glycinea*, *P. phaseolicola* (Vidaver et al, 1972), *P. syringae* pv *syringae* (Smidt and Vidaver, 1986), *P. syringae* subsp *savastanoi* (Iacobellis et al, 1995), *P. solanacearum* (Cuppels et al, 1978) and *P. fluorescens* (Reeves, 1965) have been isolated and characterized. A narrow spectrum of activity, in particular shown against important plant pathogens, is a common feature of pseudomonad bacteriocins: several studies (Cuppels et al, 1978; Iacobellis et al, 1995) have shown a species-specific inhibition. There are some strains, however, that have shown a wider spectrum of antagonism, including unrelated Gram-negative and Gram-positive species.

Analogous interactions have been reported in foods. Inhibition of foodborne bacteria by *Pseudomonas* spp has been observed and attributed to siderophores or bacteriocin-like substances (Freedman et al, 1989; Champomier-Vergès and Richard, 1994) but inhibition of unrelated *Bacillus cereus* and *Listeria* spp strains has only been detected in a few strains (Freedman et al, 1989).

In this study, a proteinaceous compound with inhibitory activity mainly towards lactic acid bacteria has been purified and characterized from *P. fluorescens* ATCC 948.

MATERIALS AND METHODS

Strains and culture conditions

Pseudomonas fluorescens ATCC 948 and the other pseudomonad strains isolated from raw milk (Gobbetti and Rossi, 1992), were aerobically cultivated in nutrient broth (BN) (Difco Laboratories, Detroit, MI, USA) at 28 °C for 24 h. Mesophilic and thermophilic *Lactobacillus* and *Enterococcus* strains were propagated in MRS (De Man et al, 1960), streptococci and lactococci in M17 (Difco) with 0.5% glucose (GM17); *Enterobacteriaceae* in McConkey medium (Difco) with 0.5% glucose; *Listeria monocytogenes* in TSY (Difco) and *Bacillus* strains in BN (Difco) media.

Production of culture supernatants and detection of antagonistic activity

Pseudomonas fluorescens ATCC 948 and 20 other strains were grown at 28 °C for 48 h in BN or skim milk, the cells were harvested by centrifugation (7000 g for 10 min), and the supernatants adjusted to pH 6.5 and filtered through a 0.22 µm pore size filter (Nucleopore; Costar Corp, Cambridge, MA, USA). Due to the proteolytic activity of *P. fluorescens* ATCC 948 during the 48-h growth period, a limpid whey was also harvested by the centrifugation of cells grown in skim milk. To detect antagonistic activity, a well-diffusion assay was used (Schillinger and Lücke, 1989). The assays were conducted in different agar media (specific for each indicator strain) overlaid with 7 mL of different soft agar media which contained 4% inoculum of an overnight culture of the indicator strain. Wells, 3 mm in diameter, were cut into these agar plates and filled with 300 µL of the culture supernatant of the potential producer strain. The plates were incubated for 24 h at 28 or 37 °C and subsequently examined for zones of inhibition. Inhibition was scored positive if the width of the clear zone around the wells was 1.0 mm or larger.

A quantitative determination based on a critical dilution assay (Barefoot and Klaenhammer, 1983) was used to express the arbitrary units (AU) of antimicrobial activity. An AU was defined as the maximum dilution of the antimicrobial compound preparation which inhibited *Lb. fermentum* D13 after 24 h of incubation at 37 °C.

Purification of the antimicrobial compound

Five L of the supernatant culture of *P. fluorescens* ATCC 948 in BN medium were concentrated 30-fold by freeze-drying (Edwards MOD E1PTB; Edwards, Milan, Italy), resuspended in 50 mmol/L phosphate buffer, pH 7.0 and crystalline ammonium sulphate was gradually added with slow stirring until 50% saturation was reached. The mixture was kept overnight at 4 °C for complete precipitation and then centrifuged at 15 000 g for 15 min. The precipitate was dissolved in 20 mmol/L Na-acetate buffer, pH 5.0, dialyzed for 24 h at 4 °C and applied to a S-Sepharose HR 16/50 column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with the same buffer. After washing, elution was carried out at a flow rate of 35 mL/h with a linear NaCl gradient, 0.1 to 0.5 mol/L. As for the other purification steps, the collected fractions (7 mL) were tested for inhibition against *Lb. fermentum* D13 by the well-diffusion method. This bacterium was chosen as indicator because, among the lactic acid bacteria, it had shown an intermediate sensitivity to the antimicrobial compound. Fractions with strong inhibitory activity were pooled, dialyzed for 24 h at 4 °C against 5 mmol/L phosphate buffer, pH 7.0 and concentrated 10-fold by freeze-drying.

Inhibitory fractions from the previous step were redissolved in a small volume of 50 mmol/L phosphate buffer, pH 7.0, and further purified by gel-filtration on Superose 12 HR 10/30 column (Pharmacia), equilibrated with 50 mmol/L phosphate buffer, pH 7.0, containing 0.15 mol/L of NaCl. Elution with the same buffer was at a flow rate of 40 mL/h and 1-mL fractions were collected and assayed for inhibition.

The pooled, dialyzed and 10-fold concentrated inhibitory fractions from gel-filtration were finally purified on a FPLC-Mono-Q HR 5/5 column (Pharmacia). Elution was made with 20 mmol/L Bis-Tris propane buffer, pH 6.5, by a linear NaCl

gradient (0 to 0.3 mol/L) at a flow rate of 45 mL/h; 0.5-mL fractions were collected.

Protein determination

The protein concentration in the solutions containing the antimicrobial compound was determined by the method of Bradford (1976), using bovine serum albumin as standard. The protein concentration during the purification steps was monitored by measuring absorbance at 280 nm.

Electrophoresis

Purity and molecular mass of the proteinaceous compound were estimated by SDS-PAGE. β -Galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa) and triosephosphate isomerase (26.6 kDa) were used as molecular mass markers (Boehringer Mannheim GmbH, Germany). The SDS-PAGE was conducted according to Laemmli's procedure (1970); gels contained 10% acrylamide (separation distance, 10 cm; gel thickness, 1 mm). The protein samples were mixed 1:1 with sample buffer (0.18 mol/L Tris-HCl, pH 6.8, 0.3% SDS, 8.6% glycerol, 1% mercaptoethanol and 0.07% bromophenol blue) and applied to the gels. Electrophoresis was performed at 200 V for approximately 45 min. Gels were stained with Coomassie brilliant blue R250 (Sigma Chemical Co, St Louis, MO, USA).

Characterization of the antimicrobial compound

Sensitivity to heat and hydrolytic enzymes

Solutions of the purified antimicrobial compound in 50 mmol/L phosphate buffer, pH 7.0, were heated at 80 to 100 °C for 5 to 20 min, and the remaining activity was determined by well-diffusion assay.

The following enzymes (Sigma Chemical Co, St Louis, MO, USA) were tested for their hydrolytic activity on the purified antimicrobial compound: proteinase K (2.6 U/mg), trypsin (22 U/mg), proteinase B500 (45 U/mg), lipase (50 U/mg) and α -amylase (15 U/mg). The assays

were performed at a final concentration of 0.5 mg/mL and at pH 6.5, except for pepsin (pH 3.0). Samples with and without enzymes were held at 35 °C for 6 h and the remaining activity was determined by well-diffusion assay.

Mode of action

To study the effect of the antimicrobial compound from *P. fluorescens* ATCC 948 on sensitive cells, 2 mL of a 30-fold concentrated (under vacuum), adjusted to pH 6.5 and filtered supernatant (specific activity, 37.5 AU/mg) were added to 10 mL of MRS broth containing *Lb. fermentum* D13 in a cell concentration of log 5.2 cfu/mL. For comparison, a concentrated supernatant from *P. fluorescens* M8, which is not a producer of antimicrobial compound, was used. At 3-h intervals, the number of viable cells was determined on MRS agar plates after incubation at 37 °C for 48 h.

To determine whether the compound was bacteriostatic or bactericidal, the *Lb. fermentum* D13 cells were cultivated for 9 h in MRS broth containing the lowest inhibitory concentration of the compound (5 AU/mg), the broth was then diluted 5-fold by the addition of MRS and the growth of cells was allowed to continue for 48 h at 37 °C before plating.

Phase of growth, pH and temperature optima

P. fluorescens ATCC 948 cells were cultivated on BN medium and skim milk at 28 °C for 144 h and samples were taken at different periods of time to determine the inhibitory activity on *Lb. fermentum* D13.

The influence of the pH and temperature on the production of the proteinaceous antimicrobial compound was assayed by growing *P. fluorescens* ATCC 948 in BN medium, pH 4.0–8.0, and in BN medium, pH 7.0, and at temperatures of 5 to 40 °C.

RESULTS

Inhibitory spectrum of the antimicrobial compound

The inhibitory spectrum of the antimicrobial compound contained in the extracellular supernatant of *P. fluorescens* ATCC 948 culture in BN broth is shown in table I. No

Table I. Inhibitory spectrum of the antimicrobial compound produced by *P fluorescens* ATCC 948.Spectre d'inhibition du composé antimicrobien produit par *P fluorescens* ATCC 948.

Indicator species	Strain no	Origin ^a	Inhibition ^b
Gram-positive bacteria			
<i>Bacillus subtilis</i>	S21	Our strain collection	—
<i>B cereus</i>	7464	NCTC	—
<i>Enterococcus faecalis</i>	E58	Our strain collection	—
<i>E faecalis</i>	E71	Our strain collection	—
<i>E faecium</i>	E34	Our strain collection	—
<i>Lactobacillus plantarum</i>	2741	UCC	(+)
<i>Lb plantarum</i>	2739	UCC	—
<i>Lb pseudoplatanturum</i>	2742	UCC	(+)
<i>Lb pseudoplatanturum</i>	2745	UCC	—
<i>Lb casei</i>	2752	UCC	—
<i>Lb casei</i>	2756	UCC	—
<i>Lb curvatus</i>	2081	UCC	—
<i>Lb curvatus</i>	2042	UCC	—
<i>Lb bulgaricus</i>	B397	Our strain collection	++
<i>Streptococcus thermophilus</i>	F142	Our strain collection	+
<i>Lb acidophilus</i>	A2	Our strain collection	(+)
<i>Lb acidophilus</i>	N2	Our strain collection	(+)
<i>Lb fermentum</i>	DT41	Our strain collection	(+)
<i>Lb fermentum</i>	D13	Our strain collection	+
<i>Lb helveticus</i>	PR4	Our strain collection	++
<i>Lb lactis</i>	DT42	Our strain collection	+
<i>Lb reuteri</i>	20016	Our strain collection	(+)
<i>Lactococcus lactis</i> ssp <i>lactis</i>	MG1363	Our strain collection	—
<i>Listeria monocytogenes</i>	X88	Our strain collection	+
<i>Staphylococcus aureus</i>	M35	Our strain collection	(+)
Gram-negative bacteria			
<i>Pseudomonas fluorescens</i>	948	ATCC	—
<i>P fluorescens</i>	M4	Our strain collection	+
<i>P fluorescens</i>	M8	Our strain collection	—
<i>P fluorescens</i>	M20	Our strain collection	—
<i>P fluorescens</i>	M22	Our strain collection	+
<i>P fluorescens</i>	M30	Our strain collection	—
<i>P fluorescens</i>	M31	Our strain collection	—
<i>P fluorescens</i>	M40	Our strain collection	—
<i>P fluorescens</i>	M44	Our strain collection	(+)
<i>P fluorescens</i>	M45	Our strain collection	—
<i>P fluorescens</i>	M47	Our strain collection	(+)
<i>Proteus vulgaris</i>	X31	Our strain collection	(+)
<i>Salmonella</i> ssp	X14	Our strain collection	(+)
<i>Serratia liquefaciens</i>	X53	Our strain collection	+
<i>Shigella</i>	X23	Our strain collection	—

^a Abbreviations: NCTC: National Collection of Type Cultures (London); ATCC: American Type Culture Collection (Rockville, MD); UCC: Culture Collection of the Department of Food Chemistry of University College Cork, Ireland; ^b Symbols: ++: very large inhibition zone (> 5.0 mm); +: large inhibition zone (> 2.0 mm); (+): small inhibition zone (2.0–1.0 mm); —: no inhibition zone.

differences were detected by cultivating the strain in skim milk. The large spectrum of inhibition included Gram-positive and Gram-negative species. Thermophilic *Lactobacillus*, *L monocytogenes*, some *P fluorescens* strains and *Enterobacteriaceae* were markedly inhibited. In particular, *Lb delbrueckii* subsp *bulgaricus* B397 and *Lb helveticus* PR4 showed a zone of inhibition larger than 5.0 mm. *P fluorescens* ATCC 948 was not sensitive to the antimicrobial compound produced by itself.

Similar results were obtained by using the culture supernatant of *P fluorescens* M22 and M40 strains, but only a narrow spectrum of inhibition (including a few *Pseudomonas* strains and *Proteus vulgaris*) was determined with the other 18 pseudomonad strains tested (data not shown).

Purification of the antimicrobial compound

The antimicrobial compound produced by *P fluorescens* ATCC 948 in BN broth was purified by precipitation with ammonium sulphate and subsequent chromatography steps (table II). On the S-Sepharose column, the most inhibitory fractions were eluted near the start of the salt gradient (fig 1a) and were separated from most of the protein contained in the ammonium sulphate precipitated. Chromatography on Superose 12 (fig 1b) resolved the fractions with inhibitory activity into one major peak corresponding to the lowest protein peak. The final purification was achieved on a Mono-Q anion exchange column (fig 1c); the peak of activity was superimposed on the second protein peak. The antimicrobial compound produced from *P fluorescens* ATCC 948 was purified 260-fold with a final yield of 6% and specific activity of 9750 AU/mg (table II).

SDS-PAGE showed that the enzyme preparation was homogeneous, with an apparent molecular mass of 67 kDa (fig 2).

Characterization of the antimicrobial compound

The purified antimicrobial compound produced by *P fluorescens* ATCC 948 was assayed for heat sensitivity and activity of hydrolytic enzymes. It was resistant to treatment at 80 °C for 15 min, but the inhibitory activity was completely abolished by treatment at 100 °C for 5 min. The antimicrobial compound was insensitive to α -amylase and lipase enzymes and was inactivated by all the proteolytic enzymes used. A representative plate with inactivation by trypsin, proteinase K and heat treatment at 100 °C for 5 min is shown in figure 3.

The addition of the concentrated culture supernatant of *P fluorescens* ATCC 948 to a culture of *Lb fermentum* D13 in MRS broth rapidly inactivated the sensitive cells (fig 4). After 6 h of incubation, the number of viable cells decreased from log 5.2 to log 2.6 cfu/mL and to log 1.2 cfu/mL after 9 h. No growth was observed after exposure (9 h) of *Lb fermentum* D13 to the lowest inhibitory concentration of the compound and subsequent cultivation (48 h) in MRS containing the 1:5 diluted antimicrobial substance. This result indicated that the compound produced by *P fluorescens* ATCC 948 seems to have a bactericidal action.

The production of the antimicrobial compound did not begin before the early exponential phase of growth ($\sim$ log 4.8 cfu/mL) and rapidly increased during the last stages of growth (fig 5). The highest production of the antimicrobial compound was detected after 72 h of incubation at 28 °C followed by a decrease, probably due to the partial hydrolysis of the proteinaceous compound by extracellular proteinases (data not

Table II. Purification of the proteinaceous compound from the culture supernatant of *P fluorescens* ATCC 948.

Purification du composé protéinique présent dans le surnageant de la culture de *P fluorescens* ATCC 948.

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification (-fold)
Culture supernatant	1652	6.2×10^4	37.5	100	1
(NH ₄) ₂ SO ₄ precipitation	617	4.7×10^4	76.2	76	2
S-Sepharose	52	1.9×10^4	365.4	30	10
Superose 12	4	8.8×10^3	2200	14	58
Mono-Q	0.4	3.9×10^3	9750	6	260

Total activity is expressed as arbitrary units (AU) of inhibitory activity (Barefoot and Klaenhammer, 1983). Specific activity was defined as AU per mg protein.

Fig 1. Purification of the proteinaceous antimicrobial compound from *P fluorescens* ATCC 948: protein concentration (□), inhibitory activity (▲) and NaCl gradient (---). **a.** Cation-exchange chromatography on S-Sepharose. **b.** Gel filtration on Superose 12. **c.** Anion-exchange chromatography on Mono-Q.

Purification du composé protéinique à activité antimicrobienne produit par *P fluorescens* ATCC 948, concentration protéique (□), activité inhibitrice (▲) et gradient de NaCl (---). **a.** Chromatographie à échange cationique sur S-Sepharose. **b.** Gel filtration sur Sephacryl 200. **c.** Chromatographie à échange anionique sur Mono-Q.

Fig 2. The SDS-PAGE of the purified proteinaceous antimicrobial compound from *P fluorescens* ATCC 948. Lane 1, reference proteins; lane 2, purified proteinaceous antimicrobial compound.

SDS-PAGE du composé protéinique à activité antimicrobienne produit par *P fluorescens* ATCC 948. Ligne 1, protéines de référence; ligne 2, composé protéinique purifié à activité antimicrobienne.

Fig 3. Inhibition of *Lb fermentum* D13 by *P fluorescens* ATCC 948 proteinaceous antimicrobial compound by the agar well-diffusion assay. Wells contained the filtered (0.22 µm) antimicrobial compound solution, which was treated by trypsin (0.5 mg/mL) (well 1), proteinase K (0.5 mg/mL) (well 2), α-amylase (0.5 mg/mL) (well 3), lipase (0.5 mg/mL) (well 4) and by treatment for 5 min at 100 °C (well 5).

Inhibition de Lb fermentum D13 par le composé protéinique à activité antimicrobienne produit par P fluorescens ATCC 948 mise en évidence par la technique de diffusion en puits. Les puits contenaient la solution du composé antimicrobien filtré (0,22 µm), traitée à la trypsine (0,5 mg/mL) (puits 1), à la protéinase K (0,5 mg/mL) (puits 2), à l'α-amylase (0,5 mg/mL) (puits 3), à la lipase (0,5 mg/mL) (puits 4) et par traitement pendant 5 min à 100 °C (puits 5).

Fig 4. Bactericidal effect of the proteinaceous antimicrobial compound produced by *P fluorescens* ATCC 948 on a sensitive strain (*Lb fermentum* D13). (▲), addition of a concentrated culture supernatant from *P fluorescens* ATCC 948 in MRS containing log 5.2 cfu/mL of *Lb fermentum* D13; (■), addition of a concentrated culture supernatant from *P fluorescens* M8 not a producer of proteinaceous antimicrobial compound.

Effet bactéricide du composé protéinique à activité antimicrobienne produit par P fluorescens ATCC 948 contre une souche sensible (Lb fermentum D13); (▲), adjonction d'un surnageant de culture concentré de *P fluorescens* ATCC 948 dans MRS contenant log 5,2 cfu/mL de *Lb fermentum* D13; (■), adjonction d'un surnageant de culture concentré produit par *P fluorescens* M8 non producteur de composé protéinique à activité antimicrobienne.

Fig 5. Production of the proteinaceous antimicrobial compound from *P fluorescens* ATCC 948 (■) during the growth period (□).

Production du composé protéinique à activité antimicrobienne par P fluorescens ATCC 948 (■) pendant la période de développement (□).

by extracellular proteinases (data not shown).

The optimum pH for producing the antimicrobial compound was 6.5 (fig 6a). The compound produced showed stability from pH 3.0 to 8.0 (data not shown).

Optimal production of the proteinaceous compound was at 20 °C (fig 6b), which did not coincide with the optimal growth of *P fluorescens* ATCC 948 (data not shown). A relatively high production was also observed at low temperatures (5 to 15 °C). The antimicrobial compound was stable after 45 days at ~ 15 °C, but a decrease in activity was observed on the freeze-dried compound after 15 days of storage at -20 °C.

Similar results concerning the characterization of the antimicrobial compound were obtained by cultivating *P fluorescens* ATCC 948 in BN medium or in skim milk.

DISCUSSION AND CONCLUSION

A 67-kDa proteinaceous compound with inhibitory activity against unrelated Gram-negative and Gram-positive species has been

Fig 6. Effect of pH (a) and temperature (b) on the production of the proteinaceous antimicrobial compound from *P fluorescens* ATCC 948.

Effet du pH (a) et de la température (b) sur la production du composé protéinique à activité antimicrobienne produit par P fluorescens ATCC 948.

extracellularly produced and purified from *P fluorescens* ATCC 948. The inhibition was shown to be particularly marked on thermophilic *Lactobacillus*, and the strain of *L monocytogenes* tested was also inhibited.

Psychrotrophic pseudomonads have been extensively studied for their production of antimicrobial compounds, which play an important role in the biocontrol of plant diseases (Dowling and O' Gara, 1994). Most of them (indoles, phenazines, lipids, antibiotics and pterines) are not of protein nature or represent complex substances in which the protein is not the only biologically active moiety. Siderophores, produced by pseudomonads, are high-affinity iron transport molecules which could sequester available

iron and reduce the activity of the electron transfer proteins, of the enzyme involved in hydrogen peroxide and oxygen metabolism, as well as the activity of the DNA biosynthetic enzymes (Freedman et al, 1989). Well-diffusion assays conducted in the presence of iron-supplemented media showed inhibitory activity similar to that determined without integration (data not shown), excluding the role of siderophores in the antimicrobial activity of *P. fluorescens* ATCC 948. An antagonism linked to bacterial viruses contained in the extracellular culture filtrate has been shown in pseudomonads (Vidaver, 1983; Smidt and Vidaver, 1986) and other microbial species (De Klerk and Coetzee 1961; Betz and Anderson, 1964; Hamada and Ooshima, 1975). Dilutions of the antimicrobial preparation from *P. fluorescens* ATCC 948 produced smaller zones of inhibition and did not increase plaque formation (data not shown), thus also excluding the presence of viruses in the culture supernatant. Antibiotics and complex toxins (syringomycin and syringotoxin) are also produced by *Pseudomonas* strains (Iacobellis et al, 1992; Dowling and O'Gara 1994). The proteinaceous nature of the antimicrobial compound purified in this study, together with the high molecular mass and the absence of carbohydrate and lipid moieties (treatments with acetone and n-butanol did not modify the inhibitory activity of *P. fluorescens* ATCC 948 supernatant) (Iacobellis et al, 1992) seem to exclude the compound from this category. Bacteriocins with features quite similar to those determined for the antimicrobial compound produced by *P. fluorescens* ATCC 948 (active protein moiety, molecular mass of ~ 60 kDa, optimal production at 20 °C and a certain degree of thermostability) have been shown in *P. syringae* pv *syringae* (Smidt and Vidaver, 1986), *P. syringae* subsp *savastanoi* (Iacobellis et al, 1995), *P. solanacearum* (Cuppels et al, 1978), *P. phaseolicola* and *P. glycinea* (Vidaver et al, 1972). By comparing these studies, the

main difference lies in a narrower spectrum of activity, in some cases species-specific, which characterizes the isolated bacteriocins. A bacteriocin-like compound recently characterized for *P. fluorescens* from fish (Champomier-Vergès and Richard, 1994), has shown inhibition against related pseudomonad pathogens. Nevertheless, inhibition against some unrelated Gram-negative (*Escherichia coli* and *Erwinia*), and Gram-positive (*Bacillus cereus* and *Listeria* spp) species and fungi has been shown by a few strains of *P. syringae*, *P. phaseolicola*, *P. glycinea* (Vidaver et al, 1972), *P. cepacia* (Abe and Nakazawa, 1994), *P. aeruginosa* (Rasool and Khan, 1993) and by fluocins from *P. fluorescens* (Hamon et al, 1961), not excluding the synthesis of broad range bacteriocins. Only three strains (ATCC 948, M22 and M40) among the 21 *P. fluorescens* strains tested in this study have shown a similar wide spectrum of activity.

Based on extensive studies of the colicins produced by Gram-negative bacteria, Tagg et al (1976), and subsequently Jack et al (1995) suggested six criteria for characterizing bacteriocins mainly produced by Gram-positive microorganisms, and proposed the definition 'bacteriocin-like inhibitory substance' for proteins which are not widely accepted or genetically characterized. Exceptions to these six criteria have been noted. Montville and Kaiser (1993) hold that there are only two requisites for bacteriocins: their proteinaceous nature and their lack of lethality to cells which produce them. The antimicrobial compound produced by *P. fluorescens* ATCC 948 is of protein nature, not lethal for the producing strain, with a bactericidal mode of action but which shows a broad spectrum of activity. As a consequence of these features we decided, before further characterization (mass spectrometry and genetic studies), not to include it in the bacteriocins but to simply define it as a 'proteinaceous compound with a broad range of inhibitory activity'.

Psychrotrophic pseudomonads have been extensively studied for their proteolytic enzymes which cause spoilage in dairy products (de Koning et al, 1985; Gobbetti, 1993). A correlation between cell growth during the cold storage of raw milk and the production of extracellular proteinases has been shown (Mottar, 1989). This study shows that *P fluorescens* ATCC 948 when grown in skim milk produces an antimicrobial compound with inhibitory activity against thermophilic *Lactobacillus* (especially one strain of *Lb delbrueckii* subsp *bulgaricus* B397 and of *Lb helveticus* PR4 were inhibited). Mesophilic *Lactobacillus* and other lactic acid bacteria strains have a limited sensitivity or are insensitive. The proteinaceous compound, optimally produced at pH 6.5, shows the highest rate of production within 72 h of incubation (the synthesis began during the early exponential phase of growth, log 4.8 cfu/mL), is resistant to pasteurization, is stable at 15 °C for 45 days and maintains more than 50% of the maximum inhibitory activity in the temperature range of 5 to 10 °C, which coincides with a limited growth of the psychrotrophic strain. Even though the microbial relationships have to be considered in a complex interactive dynamic, these features raise another problem linked to the excessive growth of psychrotrophic pseudomonads in raw milk.

Considering its importance in the dairy field and, in food microbiology, in general (inhibition of foodborne bacteria), mass spectrometry and genetic studies of this antimicrobial compound from *P fluorescens* ATCC 948 are warranted.

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