LOW MOLECULAR WEIGHT PROTEINS FROM PSYCHROTROPH *PSEUDOMONAS* ARE OVEREXPRESSED AT LOW TEMPERATURE.

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Pseudomonas fragi K1 is a psychrotrophic bacterium which has the ability to grow between 0 and 35°C, with an optimum at 30°C. Cells in steady-state growth at 4 and 30°C appeared to exhibit different patterns in the levels of certain intracellular proteins. Some of them are overexpressed or specifically expressed at the optimal growth temperature, others are overexpressed at low temperature. Among these latest, two low molecular weight proteins are in important quantity. The same results have been observed with eight other strains, isolated from different food products, meat, milk or cheese. Polyclonal antibodies against the two small proteins overexpressed in *P. fragi* K1 have been raised into rabbits. The sera were specific of each peptide and also recognized, in the eight other strains, proteins which presented similar pHi and molecular weight than those of K1. The two proteins have been partially copurified by gel filtration chromatographies from crude extracts of *P. fragi* K1 intracellular proteins. These are monomeric proteins of about 8,0 kDa.

INTRODUCTION

The ability of psychrotrophic bacteria to grow at a temperature near 0°C is a characteristic that differentiates them from the mesophilic bacteria. Psychrotrophic bacteria are common in food and the increased use of refrigeration, in recent years, for the foodstuffs conservation has greatly enlarged the importance of psychrotrophic bacteria in the food industries. The psychrotrophic population in meat and dairy products is mainly composed of Gram-negative bacteria. Among these micro-organisms, the most troublesome are the *Pseudomonas* which have the ability to produce thermally-resistant enzymes, lipases and proteases, responsible for undesirable odours and flavours. In spite of the impact of psychrotrophs in food, few studies concern the physiological and molecular basis of the growth at low temperature. Changes in membrane structure occured at low temperature and membrane fluidity is generally conserved by an increase in the proportion of insaturated fatty-acyl residues (HERBERT, 1986 ; RUSSELL, 1990 ; GOUNOT, 1991). Protein synthesis is also maintained at 0°C in psychrotrophic *Pseudomonas* (SZER, 1970) while an early initiation step in protein synthesis is blocked below 8°C in mesophilic bacteria (OSHIMA et al., 1980, 1987 ; BROEZE et al., 1978). JAENICKE (1990) suggested that conformational changes in the protein structures probably occurred at low temperature and permit to these proteins to be functional. However, there are no studies of the effects of temperature on the levels of individual proteins. Only the physiological events which took place after a shift up or a shift down in temperature are known, but the steady-state levels of most cellular proteins at different temperatures have not been examined. The results reported here indicate that the levels of several proteins of *P. fragi* K1 were significantly different at 4°C and at 30°C. Some of the K1 proteins which were overexpressed at low temperature presented the same characteristic in all the strains of *Pseudomonas* that we have tested.

MATERIALS AND METHODS

Bacteria and culture conditions. P. fragi K1 and *P. fragi* M2L4 were isolated at the laboratory from minced beef meat and sheep meat respectively. *P. fragi* 2177(NCFB 2177), *P. fluorescens* 787 (CNRZ 787) and 795 (CNRZ 795) were isolated from raw milk. *P. fluorescens* MF0 was a gift from Pr J. Guespin-Michel (Laboratoire de Microbiologie du froid, Evreux, France) and has been isolated from skim milk. *P. fluorescens* biotype I (ATCC 13525) has been isolated from a prefilter of a milk tank. *P. fragi* 2145 (NCFB 2145) is a cheese strain and *P. fragi* 752 (NCFB 752, ATCC 4973) is a type strain. All the strains were maintained in rich medium (pH 7.5) that contained 10 g protease peptone, 5 g bacto tryptone, 10 g meat extract powder, 10 g agar, and 5 g glucose per liter of distilled water. All the experiments were carried out in the M9 synthetic medium (pH 7.5) that contained 6 g Na_2HPO_4-2 H_2O, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 3 g glucose, 0.5 g Yeast Nitrogen base and 0.25 g $MgSO_4$ per liter of distilled water.

Preparation of cell-free extracts. For each experiment, cultures were inoculated with precultures carried out at the same temperature and in the same M9 liquid medium than the culture. Cells were grown to the mid log phase at either low temperature (4° or 10°C according to the strains) or optimal growth temperature of each strain (Table 1). Cells were harvested by centrifugation at 10 000 g for 20 min and resuspended in 10 mM Tris/HCl, pH 6.8, 0.1 mM EDTA, 5 mM $MgCl_2$ (buffer A) containing 2.5 U pancreatic RNase (buffer B). Cells were broken by sonication and cell walls were removed by centrifugation at 18 000 g for 10 min. Supernatants were stored at -20°C.

Radioactive labeling of cellular proteins. An aliquot of cells (2 ml) in exponential growth was incubated with the addition of 100 µCi (3.7 MBq) L-{^{35}S}-methionine (Amersham ; specific activity > 37 TBq/mmol). Growth was allowed to continue for one time of generation. Cells were harvested by centrifugation at 12 000 g for 10 min and were resuspended in 200 µl of buffer B. The bacteria were then disrupted as previously described and the unbroken cells were removed by centrifugation at 12 000 g for 5 min. Samples were stored at -20°C.

Determination of radioactivity and protein concentration. Acid-insoluble radioactivity was determined by spotting 5 µl of solubilized proteins onto glassfibre filters (Whatman GF/C), which were rinsed with 10 ml cold 25% TCA, 10 ml cold 10% TCA and 10 ml 96% (v/v)

ethanol. Filters were put into scintillation vials with 4 ml of BCS scintillation cocktail (Amersham) and precipitated radioactivity was counted in a liquid scintillation analizer (Packard).

Protein concentration in the samples was determined by the method of BRADFORD (1976), with bovine serum albumine as standard.

Table 1. Different low and optimal growth temperature for each strain of *Pseudomonas*.

STRAINS	GROWTH TEMPERATURE (°C) LOW	OPTIMAL
P. fragi K1	4	30
P. fragi M2L4	4	26
P. fragi 752	4	26
P. fragi 2145	4	26
P. fragi 2177	4	26
P. fluorescens MF0	10	26
P. fluorescens biotype I	10	30
P. fluorescens 787	10	26
P. fluorescens 795	10	26

Mono- and two-dimensional gel electrophoresis. The 2-D SDS-PAGE (two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis) was done by the method of O'FARRELL (1975). Isoelectric focusing (IEF) was done with 5% carrier ampholytes (LKB) ampholines (2 parts pH 5-7, 3 parts pH 3-10). Isoelectric focusing was conducted for a total of 15 000 V.h. Gels were incubated for 20 min in the denaturing buffer of LAEMMLI (1970) and placed on top of a slab gel. The standard second-dimension electrophoresis was performed in 15% T, 2.66% C polyacrylamide gels. Gels were stained with 0.1% Coomassie blue R 250 in 50% (v/v) methanol/10% (v/v) acetic acid and destained with 50% (v/v) methanol/10% (v/v) acetic acid. The mono-dimensional gel electrophoresis (1-D SDS-PAGE) were achieved as the second dimension of 2-D SDS-PAGE. Fluorographs were obtained by exposing dried gels to Amersham X-ray Hyperfilms-MP.

Preparation of polyclonal antibodies. Spots of the two LMW (low molecular weight) proteins C7 and D7 were cut out separately from four hundred 2-D SDS-PAGE, ground in a potter with PBS (phosphate buffer saline) and freeze-dried. The powder was resuspended in distilled water and mixed with an equal volume of complete freund's adjuvant for the initial injection and with incomplete freund's adjuvant for the next ones. Polyclonal antibodies against proteins C7 and D7 were raised separately in rabbits by subcutaneous and intradermic injections of the emulsions at 3-week intervals on seven occasions after the first inoculation. Last boosters were given 9 days before recovering the sera.

Western blot analysis. The intracellular proteins from the different *Pseudomonas* species were separated by 1-D or 2-D SDS-PAGE and electroblotted from gels to nitrocellulose membranes. Membranes were pretreated overnight at 4°C with 1% bovine serum albumine in (Tris Buffer Saline). They were washed in TBS-Tween 20 and treated firstly with antibodies and then with goat anti-rabbit IgG conjugated with horseradish peroxydase (Interchim). After an extensive wash, membranes were stained with 4-chloro-1-naphtol.

Prepurification of the two proteins C7 and D7. Intracellular proteins from *P. fragi* K1 grown at 4°C were precipitated with solid ammonium sulfate. The fraction 40-60% saturation of ammonium sulfate was dialyzed against buffer A containing 0.1 M NaCl (= buffer C) and loaded on an Ultrogel AcA34 (IBF) gel filtration column eluted with buffer C. The fractions containing the two LMW proteins were precipitated to 80% saturation of ammonium sulfate. The pellet was solubilized in the buffer C, dialyzed and then filtered with buffer C on a TSK G2000 SWG gel filtration column (Pharmacia). After each step, the two LMW proteins were identified by 1-D and 2-D SDS-PAGE.

RESULTS AND DISCUSSION

Protein patterns of P. fragi K1 at low and optimal growth temperature. Cells of strain K1 were grown at either 4 or 30°C and an aliquot of the cell culture was labelled with L-{^{35}S}-methionine for one time of generation. Samples of equal amount of proteins and radioactivity were subjected to 2-D SDS-PAGE. The resulting electrophoregrams are shown in Fig. 1. The pattern of the intracellular proteins from cells grown at 4°C differed qualitatively and quantitatively from that of cells grown at 30°C. The levels of 22 proteins (referenced in Fig. 1 A) were higher in cells grown at 4°C while 28 proteins (referenced in Fig. 1 B) were characteristic of cells grown at 30°C, including six polypeptides (indicated by circles) which were specifically expressed at the optimal growth temperature. The autoradiograms (Fig. 2) showed that higher quantities of proteins revealed by electrophoregrams were well correlated with an increased synthesis for the great majority of the proteins. Among the 4°C characteristic proteins, several low molecular weight (LMW) proteins (C7, D7, E6.1, F7) appeared in very important quantities (Fig. 1 A) and strongly labelled (Fig. 2 A) in comparison with the other overexpressed proteins (Fig. 2 A). The C7 and D7 proteins were particularly important at 4°C on the autoradiography whereas they were very few labelled at 30°C (Fig. 2 B). In the same way, at 30°C some LMW proteins (A7.1, A7.2, C6, E7) were much more labelled than the other overexpressed proteins at this temperature. It would seem that, according to the temperature, the synthesis of certain proteins is greatly increased because they probably play an important role in the physiology of the cell or their synthesis stay at a low constitutive level and they are replaced by other proteins.

ARAKI (1991) has also found, by 2-D SDS-PAGE, different patterns of intracellular proteins from a psychrophilic bacterium, *Vibrio* sp. strain ANT-300 which have the ability to grow efficiently between -2 and 13°C. The comparison, established between cells in steady-state growth at 0 and 13°C, exhibited qualitative differences with 28 polypeptides characteristic of cells grown at 0°C and 25 polypeptides very high in cell grown at 13°C. However, these results cannot be exactly compared with ours because they have been established between a low

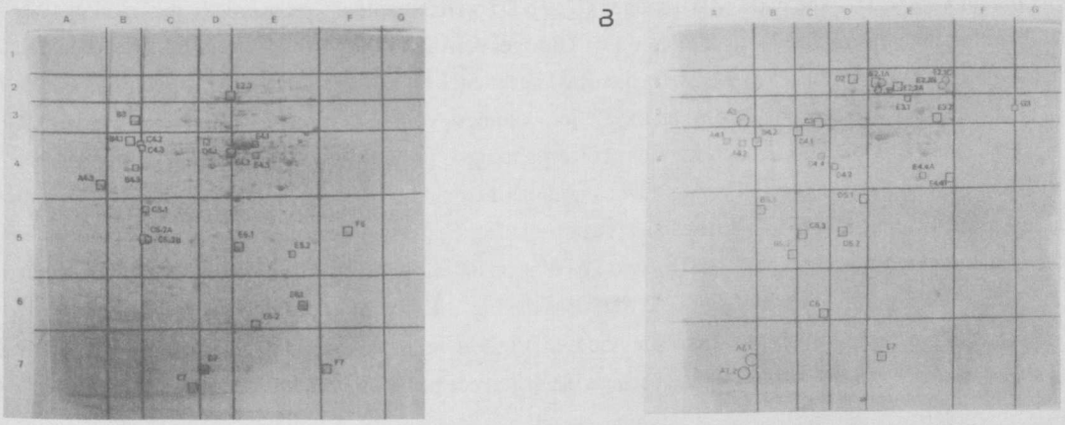

Figure 1. 2-D SDS-PAGE of intracellular proteins from *P. fragi* K1 grown at 4°C (**A**) or at 30°C (**B**). □ : overexpressed proteins ; ○ : proteins specifically expressed at 30°C.

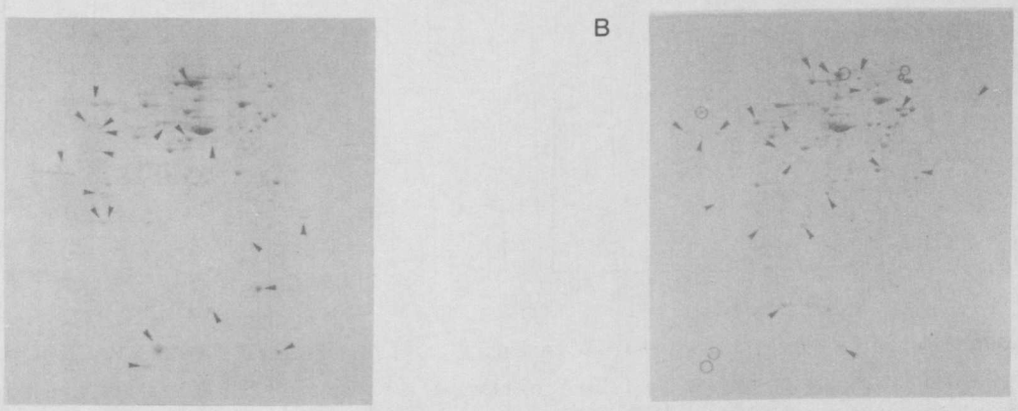

Figure 2. Autoradiographies after 2-D SDS-PAGE of intracellular proteins from *P. fragi* K1. **A** : growth at 4°C. **B**: growth at 30°C.

temperature and a suboptimal growth temperature (13°C) but not the optimal growth temperature (7°C) of *Vibrio*, and the physiology of a bacterium is undoubtedly different in these two last states.

Comparison with other strains of Pseudomonas. Intracellular proteins from eight other strains of *Pseudomonas* grown at low and optimal temperature (Table 1) were compared by 2-D SDS-PAGE. As for *P. fragi* K1, qualitative and quantitative differences in the protein patterns were also observed between the two temperatures. In all the strains, we have particularly observed two LMW proteins, with similar pHi and molecular mass to the C7 and D7 proteins of *P. fragi* K1, which were also overexpressed at low temperature.

Immunological analysis of proteins C7 and D7. Polyclonal antibodies against either protein C7 or protein D7 of *P. fragi* K1 have been raised from spots of about four hundred 2-D SDS-PAGE. Each serum recognized strongly the protein injected but also weakly cross-reacted with the other protein. This cross-reaction was due either to similar epitopes in the two proteins or to contaminants in the preparation used to provoke the antibody because, in the 2-D SDS-PAGE, the two proteins were close from each other. Few proteins in the gel were also weakly recognized by the sera. This phenomena is currently observed when antibodies are produced from proteins separated by SDS-PAGE. Indeed, proteins in the gel are denaturated and some polyclonal antibodies can be produced against primary structure sequences of the injected protein which exist in other proteins. So, these common epitopes are recognized by these antibodies.

The Western blot on proteins separated by 2-D SDS-PAGE gave the same results than the colorations or autoradiographies, i.e. a signal much more intense at low temperature than at optimal growth temperature, which confirmed the presence at 30°C of the two LMW proteins but at a low constitutive level whereas they were strongly expressed at 4°C.

The anti-C7 and anti-D7 sera have been tested on eight strains of *Pseudomonas* grown at low or optimal growth temperature. Intracellular proteins from each physiological state were separated by 1-D SDS-PAGE and electroblotted on a membrane of nitrocellulose which were incubated either with the anti-C7 or anti-D7 sera. Each serum recognized strongly a protein with the same molecular weight than C7 or D7 (data not shown). As for *P. fragi* K1, the stains were more intense at low temperature than at optimal growth temperature, which was well correlated with the results obtained by the Coomassie blue colorations.

All the samples of intracellular proteins from the eight strains have been separated by 2-D SDS-PAGE and treated with the polyclonal antibodies. So, we were able to confirm that, in all cases, the proteins labelled presented the same molecular mass and pHi than the C7 and D7 proteins of *P. fragi* K1. These two LMW proteins were overexpressed at low temperature in the nine strains of *Pseudomonas* isolated from foodstuffs ; however we cannot conclude that they are ubiquitous in the genus.

Partial purification of C7 and D7. The two LMW proteins C7 and D7 were completely recovered in the fraction 40-60% saturation of ammonium sulfate. This fraction was dialized and filtered on an Ultrogel AcA 34 with buffer C ; C7 and D7 were coeluted in the same fractions (data not shown). The molecular mass of the two proteins was estimated by calibrating the column with standard proteins of known molecular masses. A linear relationship was obtained when the elution volumes (Ve/Vo) of the standard proteins were plotted against the log of the molecular mass. C7 and D7 were recovered in fractions corresponding to proteins of molecular mass comprised between 12.5 and 5 kDa. These fractions were then pooled and precipitated to 80% saturation of ammonium sulfate and the pellet of centrifugation (10 000 g, 15 min) was dissolved in a small volume of buffer C. After dialysis against buffer C, the sample was loaded on a TSK G2000 SWG gel filtration column equilibrated and eluted with the same buffer. The two LMW proteins were afresh collected in the same fractions (Fig. 3 A). The calibration of the column, as precedently described, and 1-D SDS-PAGE (Fig. 3 B) indicated that C7 and D7 were monomeric proteins with molecular mass of 7.2 and 8 kDa respecrtively. At this stage, the two proteins were pure at about 95%. Infortunately, it was impossible to separate the two proteins from each other with this method because the difference between their molecular mass was too narrow.

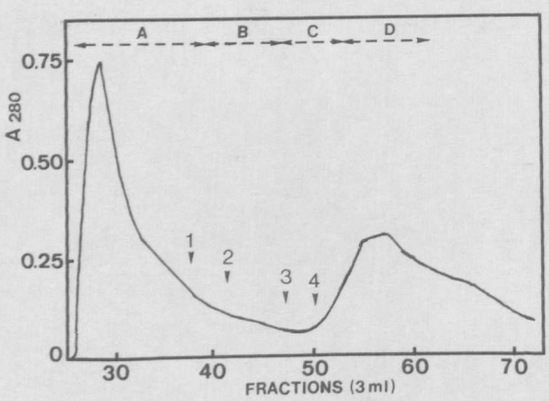

Figure 3 A. Gel filtration on TSK G2000 SWG. The two LMW proteins C7 and D7 were recovered in the fractions 54-61 (pool D). **1** : BSA (67 kDa) ; **2** : ovalbumin (42.7 kDa) ; **3** : inhibitor T (22 kDa) ; **4** : myoglobin (17 kDa).

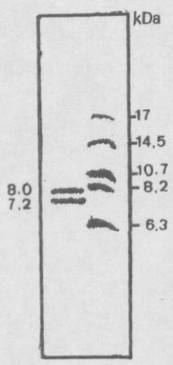

Figure 3 B. 1-D SDS-PAGE of the pool D (line A) from the TSK G2000 SWG gel filtration column. Line B : molecular weight standards (Fluka).

The great majority of the intracellular proteins from the *Pseudomonas* tested are expressed at a constant level at low or optimal growth temperature. However, some proteins are overexpressed in one physiological state in comparison with the other. It seems that, according to the temperature, some functions in the cells are ensured by different proteins or are replaced by others. We have been more particularly interested by two LMW proteins which appeared in great quantity and strongly synthetized at low temperature in all the strains. Polyclonal antibodies against these two proteins have confirmed their overexpression at low temperature and their low constitutive level at optimal growth temperature for the nine *Pseudomonas.* These are two monomeric proteins of approximately 8 kDa. Now, the future objectives are to know if these two LMW proteins are ubiquitous in the genus *Pseudomonas* and to determine their nature and the conditions of their overexpression.

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