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Compositional Changes in Cold Raw Milk Supporting Growth of *Pseudomonas fluorescens* NCDO 2085 before Production of Extracellular Proteinase

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ABSTRACT

Changes in milk native content of several carbon and nitrogen sources were studied, along with growth at 7°C of a *Pseudomonas fluorescens* strain. The aim was to characterize the particular compositional environment in milk in which psychrotrophic bacteria produce their extracellular proteinases. Glucose and lactate were depleted from milk, pyruvate and gluconate were significantly diminished, but citrate was mostly unused when proteinase was first detected by the Hide Powder Azure assay, the psychrotrophic count being around 10^{10} CFU/ml. At that stage, levels of ammonia, amino acids and short peptides had just started to rise and only about 20% of the original urea had been consumed. A procedure to anticipate, in cold stored raw milk batches, the time for production of extracellular proteinase, on the basis of sensitive lactate and ammonia determination, is suggested.

Production of extracellular proteinases by gram-negative psychrotrophic bacteria is one of the main factors involved in quality defects in dairy products made from raw milk held at 7°C or below for several days *(5,16)*. The amount of proteinase attainable in cold raw milk samples depends on the bacterial contamination quantity and quality and on conditions and length of the storage.

Available evidence on the patterns of regulation for production of these enzymes, seems to favor models controlled by nutritional factors. Many apparent inductors (proteins, peptides or amino acids) and repressors (sugars, organic acids, urea, ammonia, high concentrations of amino acids or other growth-limiting components of the media) have been observed; an excellent review on this topic has recently been published *(7)*. Most of the data on this subject, however, have been obtained by growing psychrotrophic strains (some of them isolated from raw milk) on artificial media, with a rich supply of single carbon and nitrogen sources, and at temperatures often above 20°C. It is difficult therefore to extend the conclusions of such studies to the conditions prevailing in cold raw milk as a growth medium. Apart from lactose (generally not utilized by the psychrotrophs) and protein, milk has many C- and N-sources, but in much lower concentrations than what are usually provided in artificial media *(31)*. Besides, temperature has been shown to have an influence on the route for C-source dissimilation *(20)* and on the effect of C-sources on proteinase production *(15)*.

In the present study, we have tried to identify the sequence in use of the different substrates present in milk, during growth at 7°C of a typical psychrotrophic strain, always in reference to production of its extracellular proteinase. Since sudden onset of this activity causes considerable spoilage in rather a short time, we have attempted to find clues in milk composition that could warn of its impending occurrence.

MATERIALS AND METHODS

Single herd raw milk, kept below 7°C after its milking, less than 24 h before, was collected from a local dairy farm; its psychrotrophic count was between 10^3 and 10^4 CFU/ml. Bacteriological media were from Difco Laboratory (Detroit, MI). Fluorescamin (Fluram® Roche) was purchased from Fluka AG (Buchs, Switzerland) and Hyde Powder from Sigma.

Pseudomonas fluorescens NCDO 2085, previously known as AR 11, was isolated from raw milk reference flora *(19)*. Many features of this strain and its extracellular proteinase have previously been studied *(1,8,17,19)*. Trypticase-soya broth was inoculated from a Standard Plate Count Agar slant and incubated statically at 24°C for 24 h. One-L Erlenmeyer flasks containing 500 ml of raw milk were inoculated with four drops of this culture. The flasks were shaken at 95 rpm and 7 ± 0.5°C. Samples were aseptically drawn at indicated times and separate portions were taken for psychrotrophic plate count, proteinase assay, tyrosine equivalent determination and enzymatic assays. All but the first received 0.002% sodium azide and were kept frozen at -30°C until used. Psychotrophic counts in milk were estimated by deep culture in Yeastrel Milk Agar plates, incubated at 7 ± 0.5°C for 10 d *(4)*.

A modification of the Hide Powder Azure (HPA) method *(4)* was used, to assay proteinase activity. Milk samples were defatted and decaseinated by centrifugation after adjusting pH to 4.6 with HCl; the supernatant liquid was brought to pH 8.0 and was centrifuged once more to eliminate as much fat as possible.

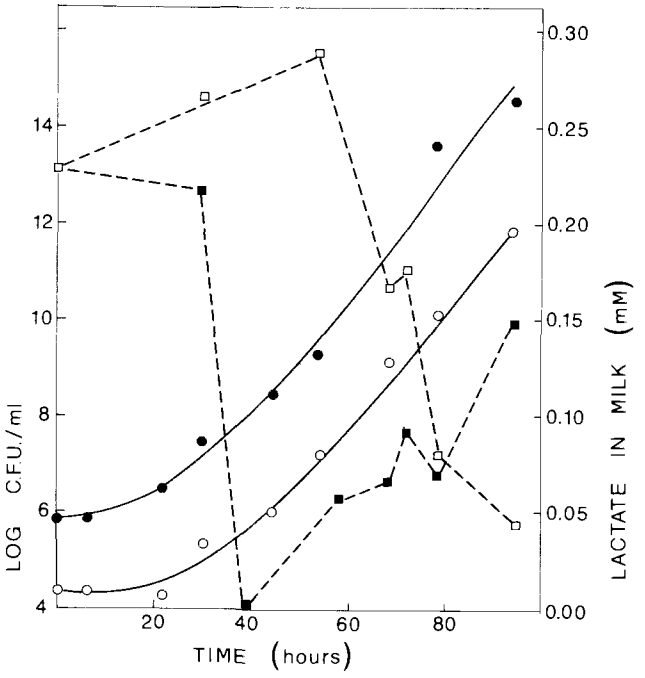

Figure 1. *Psychrotrophic growth (—) and changes in lactate concentration (--) in raw whole milk not inoculated (open symbols) and inoculated (closed symbols) with* P. fluorescens *NCDO 2085, shaken at 7°C.*

The assay was performed in a 3 ml mixture, with 0.2 *M* Tris-HCl buffer pH 8.3 and 10 mg HPA/ml. After 1 h of incubation at 37°C, while shaking at 160 rpm, the assay mixture was centrifuged at 800 × *g* for 5 min; the supernatant liquid was clarified by adding 4% EDTA and 1% Triton X-100 and incubating at 37°C for 30 min, as described by *(8),* before measuring absorbance at 595 nm.

A fluorometric method, based on reaction with fluorescamin of the primary amines present in the milk fraction soluble in 10% TCA, was used for "Tyrosine Equivalent" determination, as it has been described *(26),* but omitting incubation at 40°C. Relative fluorescence (λ excitation = 395 nm, λ emission = 480 nm) was determined using a double-beam Kontron SFM spectrofluorimeter, with a xenon lamp. "Tyrosine Equivalent" figures were calculated from a standard curve. Ammonia and urea do not interfere with the assay.

Glucose, gluconate, L-lactate, pyruvate, citrate, ammonia and urea were assayed at semi-micro scale using Boehringer enzymatic kits. To deproteinize milk samples, they were added one volume of ice-cold 1 *N* perchloric acid and then centrifuged for 15 min at 4500 × *g*. To convert glucono-δ-lactone to gluconate, the samples were treated according to instructions of the kit. Use of solution 1 in pyruvate determination kit, was omitted.

RESULTS AND DISCUSSION

Just after inoculation with *P. fluorescens* NCDO 2085, those cells accounted for 95-99% of the psychrotrophic flora present in the milk sample (Fig. 1). Assuming this proportion was kept during growth at 7°C, this strain could be presumed to be responsible for the bulk of the compositional changes taking place in the milk. Mean generation time for the NCDO 2085 strain under our conditions and during the exponential growth phase was around 4 hours. Growth of the natural mixed psychrotrophic flora present as a background in the raw milk samples took place at a similar pace than the inoculated strain (Fig. 1); it is presumed that the pattern of C- and N-source usage followed by this mixed flora (usually dominated by *Pseudomonas* strains), is similar to that observed for the NCDO 2085 strain. Lactate, for instance, was depleted from both uninoculated and inoculated samples, when population levels reached between 10^7 and 10^8 CFU/ml (Fig. 1).

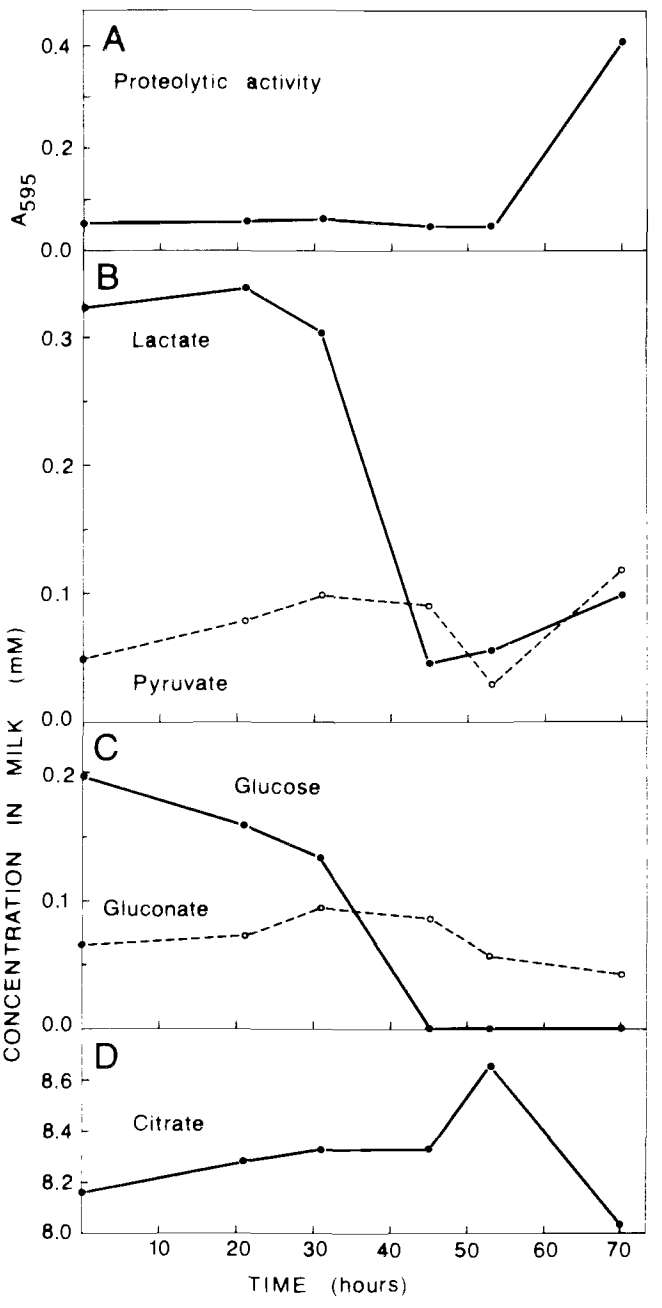

Figure 2. *Changes in extracellular proteolytic activity (A_{595} of hydrolyzed HPA) and concentration of various C-sources in raw whole milk inoculated with* P. fluorescens *NCDO 2085, shaken at 7°C.*

Aside from lactose, most of the low molecular weight C-source material found in milk is citrate (1300-2100 mg/

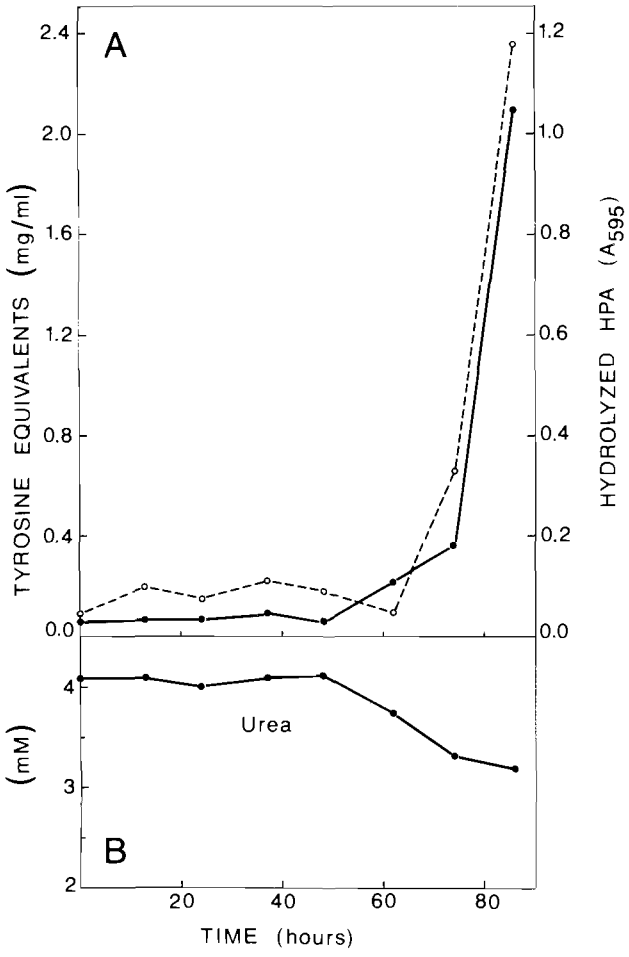

Figure 3. *Evolution in extracellular proteolytic activity (A) in terms of "Tyrosine Equivalents" (●) and hydrolyzed HPA (○), and urea concentration (B) in raw whole milk inoculated with* P. fluorescens *NCDO 2085 and shaken at 7°C.*

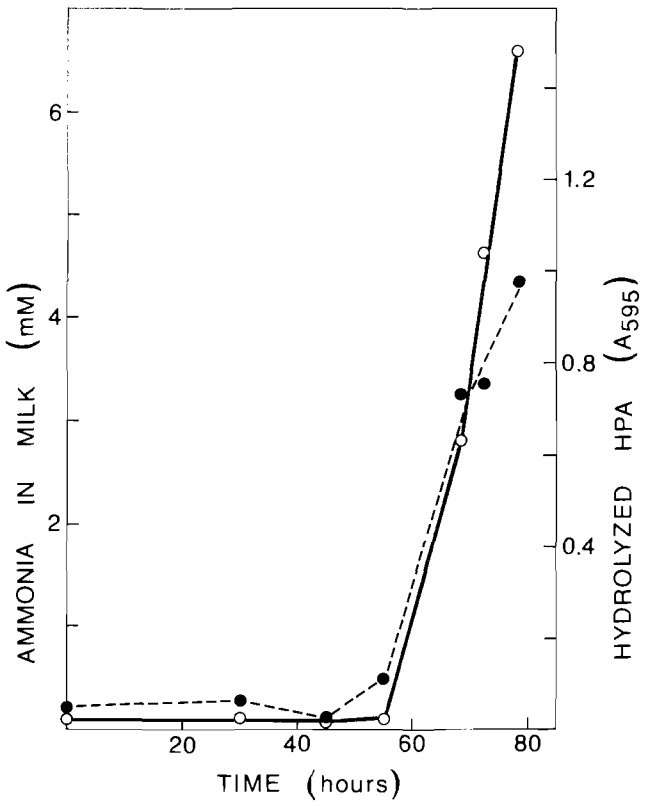

Figure 4. *Extracellular proteolytic activity (hydrolyzed HPA) (○) and ammonia concentration (●) in raw whole milk inoculated with* P. fluorescens *NCDO 2085 and shaken at 7°C.*

kg milk) *(9,31)*. Known to be a good substrate for *Pseudomonas* growth in artificial media, citrate, however, was not consumed from cold raw milk supporting the growth of NCDO 2085 by the time extracellular proteolytic activity was first detected (Fig. 2); that is when psychrotrophic count was between 10^{10} and 10^{11} CFU/ml.

Other C-sources measured in milk, however, showed different degrees of exhaustion when extracellular proteolytic activity started to be detected. Glucose and lactate were used up practically at the same time, starting at 10^7 CFU/ml, and shortly afterwards, pyruvate and gluconate went down too (Fig. 2). Levels of lactate and pyruvate went up once again, following the onset of proteolysis, as it could be expected from intermediary metabolites.

Among the low molecular weight N-sources in milk, the most important, molarity-wise, is urea (4-5 m*M*). During growth of NCDO 2085 under our conditions, urea concentration in milk started dwindling as glucose and lactate were being exhausted, but it remained about 80% of its initial level before detection of proteolytic activity (Fig. 3). Free ammonia, originally around 0.25 m*M*, decreased after some time and increased later on, shortly before the HPA assay could detect proteolytic activity (Fig. 4). After that point, there was a brisk build-up of ammonia, presumably resulting from use of newly released amino acids as C-source. The level of free amino acids (included along with short peptides under the heading of "Tyrosine equivalents"), initially under 0.5 m*M*, and that of free ammonia, began to increase just before proteolytic activity (as measured by the HPA assay) was first detected (Fig. 3).

There was thus no exhaustion of the low molecular weight C- or N-sources (most of the citrate and urea were still unused) when extracellular proteolytic activity started to appear. It is difficult to quantify the entire array of low molecular weight C-sources available for psychrotrophic growth in milk, but a rough estimate could fix them at about 3 g/kg milk (excluding lactose) *(31)*. Assuming 10^{12} cells to have a dry weight of 1 g, and oxidation of carbon substrates to give a maximum yield of 50%, those 3 g could only sustain growth up to 1.5×10^9 cells/ml. Even assuming that some intermediate molecular weight materials are put to use before proteinase production, it is still remarkable that most of the citrate is spared. Maybe its dissimilation in impaired by some deficiency in the oxidative metabolism. Air injection, however, has been reported not to cause radical changes in citrate concentration in cold raw milk whose natural mixed psychrotrophic population had reached 10^8

CFU/ml *(11)*. Iron supply in milk may also play a role in this context. Pyoverdine, the yellow-green fluorescent pigment of *P. fluorescens,* is excreted by iron-deficient cells and has a high specific affinity for Fe^{+3}, increasing the rate of entry of this cation into the cell *(25)*. When *P. fluorescens* NCDO 2085 grew in milk at 7°C, detection of pyoverdine and that of extracellular proteolytic activity occurred almost simultaneously (results not shown). Fe^{+3} content of mineral media has been observed to determine the stage of growth at which *P. fluorescens* B52 produced its extracellular proteinase *(24)*.

Catabolite repression has for long been assumed to play an essential part in regulation of extracellular proteinase production *(3,7,33),* but the role that milk's native citrate, glucose or lactate could play, is not yet clear. There are several studies dealing with the effect of citrate in artificial media, reporting repression at least with concentrations about 3-fold those in milk *(6,18,32)*. Other citrate cycle intermediate compounds, particularly ketoglutarate, have the same type of effect *(3)*. As for glucose and lactate, concentrations about 100-fold those present in milk, cause repression of proteinase production in *Pseudomonas* and *Vibrio* strains in artificial media, but there are also reports on good *Pseudomonas* proteinase activity in media with even higher glucose contents *(14,27)*. Even addition of glucose to milk has given rise to differing results *(15)*. Incubation temperatures *(15),* aeration *(11),* or even fat content *(13)* with milk and, in artificial media, availability of iron *(33)* and/or N-sources, could possibly account for the reported differences. Control of various enzymes involved in assimilation of organic N-sources is thought to be accomplished by a combination of catabolite repression, induction and so-called nitrogen control *(12,21)*. In those instances, catabolite repression seems to be effective when, in spite of the presence of an inducer, a preferred carbon source and ammonia are available, the repression losing its hold when ammonia becomes deficient *(12,29)*. Maybe this regulatory mechanism is also valid for extracellular proteinases of psychrotrophic bacteria.

Although ammonia as only N-source can sustain proteinase production *(23),* enzyme productivity is higher in the presence of organic N-sources, especially when they are also used as C-sources *(2,22,32)*. Amino acids differ in their stimulating capacity *(22,30,33)* and a soluble fraction of milk, with molecular weight lower than 5000, has shown to be particularly active as an inducer *(22)*. The level at which this inducer was found to be active, is not very different from that of our "Tyrosine equivalents" just before the rise in proteolytic activity (Fig. 3), and that coincides in our samples with a temporary drop in ammonia concentration and with exhaustion of some C-sources. Whether the combination of those factors can account for proteinase derepression in milk, remains to be ascertained. As we mentioned above, there might be another factor, maybe another nutrient limitation, involved. In any event, the association in time of proteinase onset and some compositional changes in the milk, could be of some practical value, serving to estimate the time for initiation of this spoilage process in raw milk batches arriving at a dairy plant.

TABLE 1. *Outline for a procedure to assess the risk for proteinase-caused defects in cold raw milk samples.*

Concentration in milk (m*M*)		Extracellular proteo-
Lactate	Ammonia	lytic activity is:
≥0.20	<0.50	Not to be detected immediately
≤0.15	≤0.25	To be detected immediately
≤0.15	<1.0	Still low, starting
>0.15	≥1.0	High

Since native milk's glucose levels can be drained away by somatic and non-psychrotrophic bacterial cells, lactate concentration seems to be a better indicator candidate. Lactate may be somewhat increased by early non-psychrotrophic cell growth, but it would be later used by psychrotrophic bacteria; proteinase activity would cause it to rise again. That decrease in lactate concentration, lasting about 24 h with NCDO 2085 growing in milk at 7°C, immediately precedes detection of extracellular proteolytic activity, and could therefore be used as an indicator to anticipate this moment. Determination of ammonia could complete the appraisal, as it is suggested in Table 1. The possibility of using depletion of substrates utilized by the bacteria, or accumulation of products of protein degradation, to anticipate the onset of spoilage in meat, has been previously discussed *(10)*. The ability, in milk, for solutes to diffuse and easily reach homogeneous distribution, choice of lactate as an indicator, and use of enzymatic analytical methods, providing good discrimination at low metabolite concentrations, are advantages supporting the chances of this method to prove its potential in prediction of the onset of spoilage by psychrotrophic proteinases in cold stored raw milk.

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