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Original article

Autolysis of 57 strains of dairy propionibacteria

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Summary — Dairy propionibacteria contribute to proteolysis and lipolysis of the curd in Swiss-type cheese technology by the release of intracellular enzymes. Autolysis of 57 strains of dairy propionibacteria has been compared in 0.1 mol/l potassium phosphate buffer (pH 6.2) and the rate and extent of autolysis appeared to be strain-dependent. Two distinct clusters were observed: cluster A containing 8 strains highly prone to lysis and cluster B containing all the other strains. Genomic fingerprinting of the 8 strains constituting cluster A revealed 7 distinct profiles. Growth of 4 of these 7 strains showed a spontaneous autolysis in the growth medium just after the maximal growth, without any stationary phase.

autolysis / *Propionibacterium*

Résumé — Activité autolytique de 57 souches de bactéries propioniques laitières. *Dans les fromages à pâte pressée cuite, les bactéries propioniques participeraient à la protéolyse et à la lipolyse de la pâte fromagère par la libération d'enzymes intracellulaires. Dans cette étude, l'autolyse de 57 souches de bactéries propioniques laitières a été comparée en tampon phosphate de potassium (0,1 mol/l, pH 6,2) et les résultats apparaissent clairement souche-dépendants. Deux groupes ont été définis : le groupe A contenant 8 souches fortement autolytiques et le groupe B contenant toutes les autres. L'analyse du profil de restriction du chromosome des 8 souches du groupe A (par electrophorèse en champs pulsés) révèle 7 profils différents. Ces 7 souches différentes pourront être utilisées pour des essais fromagers et/ou pour la caractérisation plus fine du système autolytique chez ce genre bactérien. De plus, la croissance de 4 souches du groupe A* (P freudenreichii *CNRZ 725, CNRZ 726, CNRZ 727, CNRZ 435) a été suivie en milieu de culture YEL et révèle l'absence de phase stationnaire: la croissance maximale est aussitôt suivie par une phase de lyse spontanée, ce qui confirme la forte activité autolytique de ces souches.*

***autolyse* / Propionibacterium**

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INTRODUCTION

The dairy propionibacteria (mainly *Propionibacterium freudenreichii)* are essential for ripening of Swiss-type cheese (Langsrud and Reinbold, 1973a,b; Bergère and Accolas, 1986), where they transform lactate into propionate and acetate with the concomitant production of CO_2 which is essential for the formation of the expected opening of the cheese.

Propionibacteria contribute to proteolysis and lipolysis of the curd by the release of intracellular enzymes. Indeed, peptidase activity of *P freudenreichii* subsp *shermanii* is thought to be the source of proline, giving the cheese its characteristic flavour (Langsrud, 1974; Langsrud *et al,* 1978). An intracellular esterase has also recently been characterized (Dupuis and Boyaval, 1993; Dupuis *et al,* 1993). Thus the ability of a strain to release cytoplasmic enzymes by autolysis could be a new characteristic to take into consideration when selecting starters for rapid and convenient ripening of cheese.

The autolytic process is carried out by endogenous autolysins hydrolysing covalent bonds of the peptidoglycan, which is the main cell wall component (Rogers *et al,* 1980; Shockman and Höltje, 1994). A detailed analysis of the peptidoglycan of dairy propionibacteria has been undertaken in only a few instances (Allsop and Work, 1963; Schleifer *et al,* 1968; Kamisango *et al,* 1983). To our knowledge, the other cell wall polymers as well as the cell wall located autolysins have never been described.

The autolytic activity of 57 strains from our collection, including industrial strains, was checked. Eight strains were shown to possess high autolytic activity *in vitro.* They could be used for technological assays or for further enzymatic characterization of the autolytic system in this genus.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Most of the strains were obtained from the CNRZ collection (INRA, Jouy–en–Josas) as indicated in table I. Some strains (designated by letters) are of commercial origin. The strains were all reisolated before being stored at –70°C in YEL (Malik *et al,* 1968) containing 15% (v/v) glycerol. They were grown statically in YEL broth at 30°C for 2 days from a 1% (v/v) inoculum of 2-day culture. Viability was determined on YEL agar plates after incubation for 5 days at 30°C under anaerobic conditions. Growth was followed by optical density at 650 nm (OD_{650}) using a spectrophotometer (Beckman DU7000). For OD_{650} >1 the samples were diluted with distilled water. In order to maintain constant conditions (without stirring), separate tubes were used to measure OD_{650} and viability at different times of the growth. The generation time (g) was determined as follows:

$g = t \log 2 / (\log N_{t2} - \log N_{t1})$ with $t = t_2 - t_1$ (t_1 and t_2 belong to the exponential growth phase); N_{t1}= viable cell count at time t_1; N_{t2}= viable cell count at time t_2.

Induction and measurement of autolysis

Whole cells were harvested (10 000 *g*, 15 min, 4°C) in the exponential growth phase (cultures of 50 h; dry weight from 0.2 to 0.6 mg/ml) and washed once in cold distilled water. The cells were resuspended in 0.1 mol/l potassium phosphate (pH 6.2) previously autoclaved at 121°C, 15 min. This buffer is known to be convenient for the detection of lysozyme activity (Difco) and was thus arbitrarily chosen for the screening. The suspensions were then incubated at 37°C without stirring. Autolysis (%) was expressed as the percent decrease of the OD_{650} after 2 and 24 h of incubation. Both parameters roughly characterize the initial rate (2 h) and the final extent (24 h) of autolysis.

For some strains, autolysis was continuously followed by suspending cells in stirred and thermostated (37°C) cuvettes in the spectrophotometer. In this case, the time course of autolysis was characterized again by two parameters: the

Table I. Origin of the strains used in this study
Origine des souches utilisées.

Species	*Source*	*Strains*
P freudenreichii	CNRZ	81, 82, 89, 277, 433, 435, 722, 724, 725, 726, 727, 728, 729
	CIP	103026, 103027, 5932
	Glatz	P93, P103, P113
	Orsay	6207, 13673
	Industry	A, B, C, D, E, F, G, H, I, L, M, N, O, P, Q, R, S
P jensenii	CNRZ	79, 83, 87, 288, 434, 730
	CIP	103028
	Glatz	P63
	Industry	J
P acidipropionici	CNRZ	80, 86, 287, 721
	DSM	4900, 20273
	Industry	K
P thoenii	CNRZ	84, 85, 732
	CIP	103029, 6434

CNRZ, INRA, Jouy-en-Josas, France; CIP, Collection of the Institut Pasteur, Paris, France; Glatz, strains obtained by the courtesy of B Glatz, Iowa State University; Orsay, strains obtained by the courtesy of J Legault-Demare; University Paris Sud, 91405 Orsay Cedex, France; DSM, Deutsche Sammlung von Mikroorganismem, Braunschweig, Germany; Industry, confidential origin.

rate of autolysis (Vi) expressed as the decrease of OD_{650} per minute over the initlal 30-min period (unit of $OD_{650} \times min^{-1}$) and the extent of autolysis (%) expressed as the percent decrease of OD_{650} during 24 h.

Preparation of intact genomic DNA in agarose blocks

Genomic DNA of each strain was prepared by the method of Gautier *et al* (1992), modified as follows: the dairy propionibacteria were grown in YEL medium supplemented with 0.5 mol/l sucrose and 0.5 – 4.5% (w/v) of glycine (depending on the strains) in order to fragilize the cell wall (Hammes *et al*, 1973). 9 ml of the culture at an OD_{650} of about 0.3 was centrifuged (7000 *g*, 10 min) and the cells were washed once in 5 ml of TES buffer (Maniatis *et al*, 1982) and suspended in 400 µl of 50 mmol/l EDTA. The suspension was warmed to 45°C and mixed with 700 µl of 1% (w/v) agarose (ultrapur BRL-Gibco) containing 125 mmol/l of EDTA. Samples were then held at 4°C for 15 min to allow solidification of the agarose. The blocks were washed in autoclaved TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8). The agarose blocks were then suspended in 5 ml of lysis buffer (20 mg/ml of lysozyme (Afilact), 0.05% Sarkosyl in TE) at 37°C for 4 h and washed twice in TE buffer and incubated overnight at 50°C in 4 ml of proteinase K buffer (100 mmol/l EDTA, 1% SDS, 0.25 mg/ml of proteinase K, in 10 mmol/l Tris, pH 8). The blocks were washed three times in TE buffer and finally resuspended in 5 ml of TE buffer. After 30 min at room temperature, 50 µl of phenylmethylsulfonyl fluoride (17.5 mg/ml dissolved in propanol-2) were added and the incubation continued for another 30 min. The blocks containing the purified DNA were then washed three times in TE buffer (20 min at room temperature).

Restriction enzyme digestion of DNA in agarose blocks and pulsed field gel electrophoresis

The agarose blocks were equilibrated overnight in the restriction endonuclease digestion buffer at 4°C, after which they were transferred to 250 µl fresh digestion buffer containing 72 units/ml of *Xba*I endonuclease (Boehringer, Mannheim) and incubated for 8 h at 37°C. Pulsed field electrophoresis was performed on a Biorad CHEF DRII electrophoresis cell. Samples were electrophoresed through 1% (w/v) agarose gels (ultrapur BRL-Gibco), in a running buffer (45 mmol/l Tris, 45 mmol/l boric acid, 125 mmol/l EDTA, pH 8) for 20 h at 200 V at 14°C with pulsed times increasing from 3 to 20 s. The size standard (TL) used is an internal standard of our laboratory.

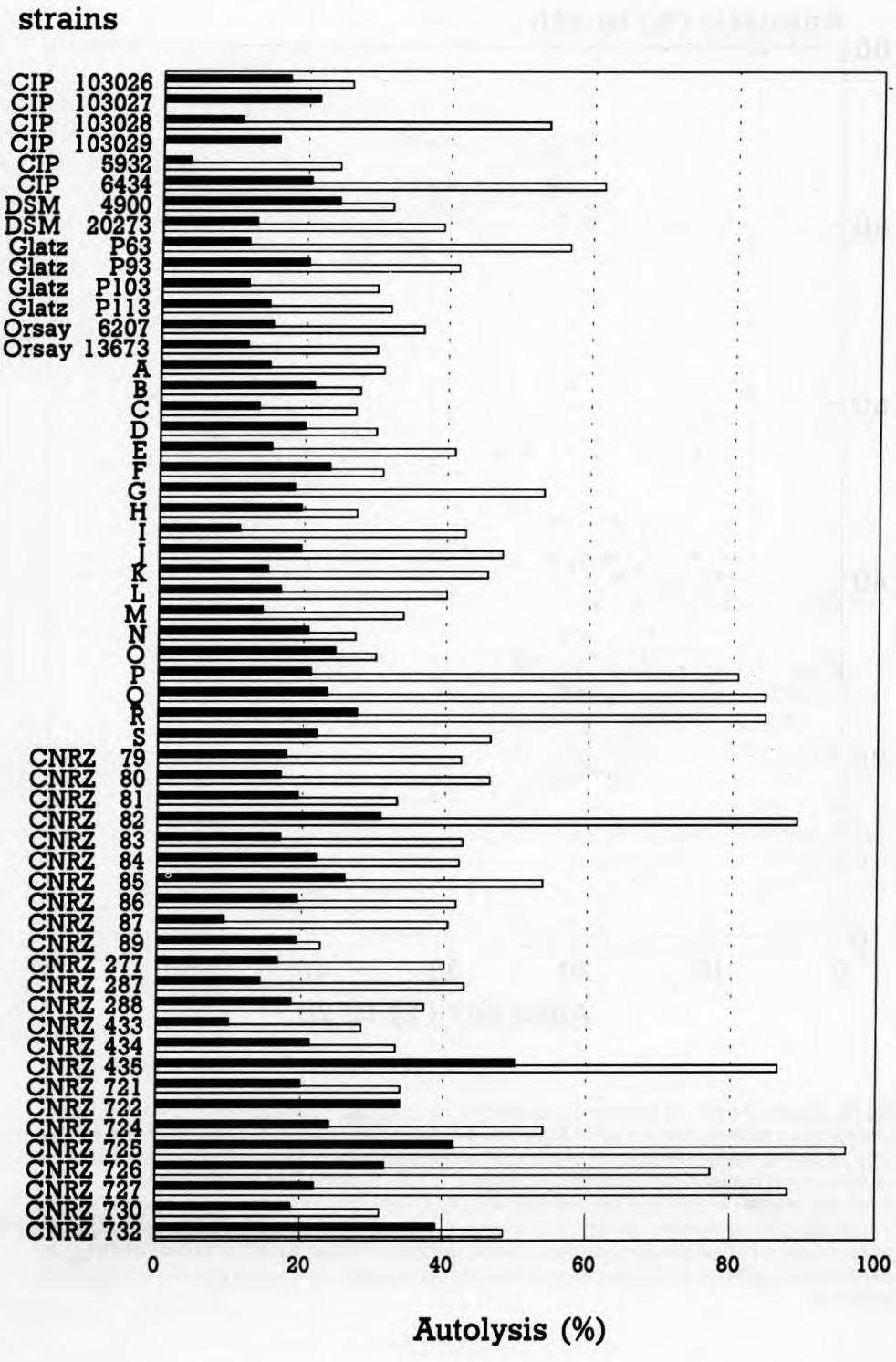
RESULTS

Autolysis in potassium phosphate buffer

The resuspension of whole cells under nutrient starvation (buffered solutions) is a widely used method to induce autolysis (Neujahr and Logardt, 1973; Leduc and van Heijenoort, 1980; Lortal *et al*, 1991). Under these conditions, the hydrolytic action of the cell wall autolysins towards the peptidoglycan provokes the bursting of the cells, which is easily followed by the reduction of optical density at 650 nm (Ohmiya and Sato, 1975). As described in numerous papers, the autolytic activity depends on the state of growth of the cells. Thus, as far as possible, the 57 strains of dairy propionibacteria used in the present study were all harvested during the exponential growth phase. The autolyses (%) observed after 2 h and 24 h of incubation are shown in figure 1a. It is important to note that after 24 h no significant variations in OD_{650} were observed (data not shown). The data in figure 1a indicate that the autolysis was a strain dependent phenomenon. The data presented in an alternative format (fig 1b) demonstrated that the strains examined could be divided into two distinct clusters with respect to their autolytic properties. Cluster A contained eight strains highly prone to autolysis (P, Q, R, CNRZ 726, CNRZ 727, CNRZ 82, CNRZ 725, CNRZ 435) with an average loss of OD_{650} about 31 ± 10% in 2 h and 86 ± 5% in 24 h. These eight strains were strains of the species *P freudenreichii*. Amongst them, two strains showed in addition a large autolysis in 2 h (CNRZ 725, CNRZ 435). The complete autolysis curves of these two strains are shown in figure 2. Cluster B contained the other 49 strains. For this cluster, the average loss of OD_{650} was 18.1 ± 5.8% in 2 h and 38.3 ± 9.4% in 24 h.

In order to determine how closely related the strains in cluster A are, fingerprints of the chromosomes were compared by pulsed field electrophoresis after digestion with the restriction enzyme *Xba*I. The results (fig 3) revealed two identical patterns for industrial strains P and Q. This last result has been confirmed by a second restriction enzyme (*Ssp*I) (data not shown). The other patterns were different indicating that the other isolates were distinct strains. For the seven remaining cluster A strains a second screening was carried out using a continuous mea-

Fig 1a. Comparison of the autolytic activity of 57 strains of dairy propionibacteria. The cells were harvested during the exponential growth phase, washed once in distilled water and suspended in 0.1 mol/l potassium phosphate buffer pH 6.2. The autolysis was estimated by the decrease of OD_{650} after 2 h (■) and 24 h (❑) of incubation at 37°C.
Comparaison de l'activité autolytique de 57 souches de bactéries propioniques laitières. Les bactéries sont récoltées pendant la phase exponentielle de croissance, lavées dans de l'eau distillée puis reprises dans une solution tamponée de phosphate de potassium à 0,1 mol/l, pH 6,2. L'autolyse est estimée par la réduction de DO_{650}(%) après 2 h (■) et après 24 h (❑) d'incubation à 37°C.


strains
CIP 103026
CIP 103027
CIP 103028
CIP 103029
CIP 5932
CIP 6434
DSM 4900
DSM 20273
Glatz P63
Glatz P93
Glatz P103
Glatz P113
Orsay 6207
Orsay 13673
A
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CNRZ 85
CNRZ 86
CNRZ 87
CNRZ 89
CNRZ 277
CNRZ 287
CNRZ 288
CNRZ 433
CNRZ 434
CNRZ 435
CNRZ 721
CNRZ 722
CNRZ 724
CNRZ 725
CNRZ 726
CNRZ 727
CNRZ 730
CNRZ 732
0
20
40
60
80
100
Autolysis (%)

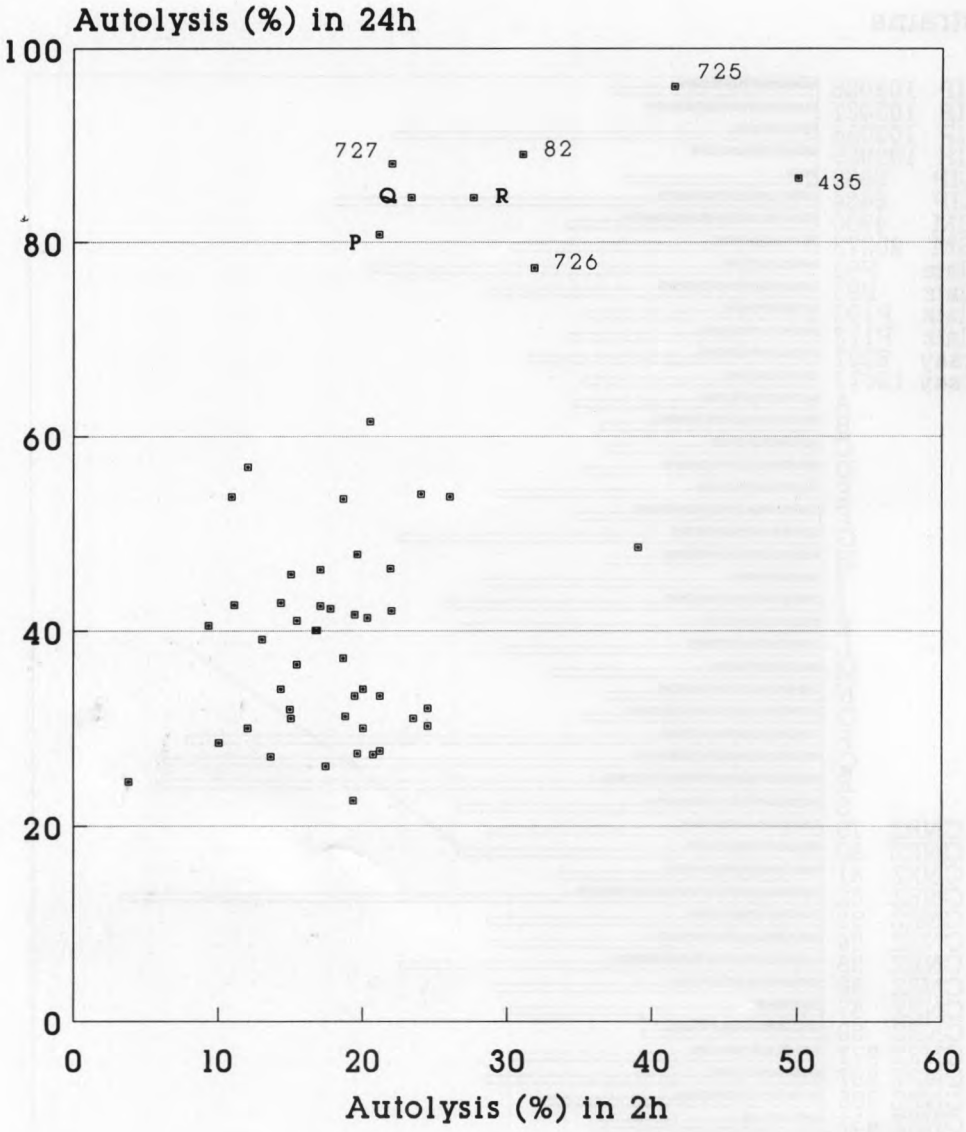

Fig 1b. Results of figure 1a presented in another format (each point corresponds to one strain) clearly identifying two clusters of strains according to their autolytic activity. Cluster A contains eight strains showing the largest extent of autolysis in 24 h (more than 80% decrease of OD_{650}). The number of the eight strains forming cluster A are indicated on the figure. Cluster B contains all the other strains.
Autre représentation graphique des résultats de la fig 1a (un point correspond ici à une souche). Les 57 souches testées forment clairement 2 groupes distincts au regard de leur activité autolytique. Le groupe A est constitué des 8 meilleures souches autolytiques (plus de 80% de chute de DO_{650} en 24 h). Les numéros de ces 8 souches sont indiqués sur le graphe. Les autres souches constituent le groupe B.

surement of OD_{650} directly in thermostated and stirred cuvettes of the spectrophotometer. For these experiments the strains were harvested at the beginning of the exponential growth phase (OD_{650} < 1). The results (table II) confirmed the high extent of autolysis for strains CNRZ 725, CNRZ 727, CNRZ 435, P and R. The extent of autolysis was not as high as expected for strains CNRZ 82 and CNRZ 726. The comparison between the two experiments was limited by the fact that the two screenings were carried out in the same buffer but not exactly under the same conditions; the stages of growth of the cells at harvesting and stirring were different.

The strain CNRZ 725 showed the highest initial rate of autolysis (Vi=5.4 10^{-3} uOD_{650}/min), and may constitute a model strain for further investigation of the autolytic system. In addition, it was noticed that strain 725, when cured of its plasmids, exhibited the same autolytic activity (data not shown).

Autolysis in the growth medium

The growth of four strains (CNRZ 725, CNRZ 726, CNRZ 727, CNRZ 435) belonging to cluster A was followed in YEL broth

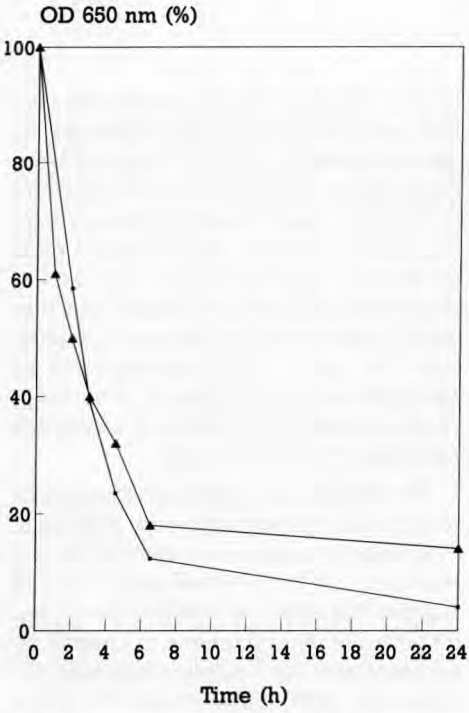

Fig 2. Autolysis of two strains of *P freudenreichii* (• CNRZ 725; ▲ CNRZ 435) belonging to cluster A, in 0.1 mol/l potassium phosphate (pH 6.2), at 37°C.
Autolyse en milieu tamponé (phosphate de potassium 0,1 mol/l, pH 6,2) de 2 souches de l'espèce P freudenreichii *(• , CNRZ 725; ▲ , CNRZ 435) appartenant au groupe A.*

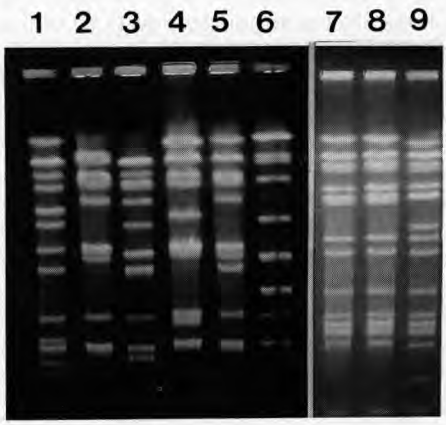

Fig 3. Genomic fingerprints of the eight strains of *P freudenreichii* showing the largest extent of autolysis (cluster A) observed by PFGE after digestion of the DNA by the restriction enzyme *Xba*I. Lanes: 1, CNRZ 82; 2, CNRZ 435; 3, CNRZ 725; 4, CNRZ 726; 5, CNRZ 727; 6, TL standard (18 to 550 kb); 7, P; 8, Q; 9, R.
Profil de restriction des 8 meilleures souches autolytiques de l'espèce P freudenreichii *(groupe A) obtenu par digestion de l'ADN chromosomique par l'enzyme de restriction Xba I, et observé par électrophorèse en champs pulsés.*
Puits: 1 CNRZ 82; 2 CNRZ 435; 3 CNRZ 725; 4 CNRZ 726; 5 CNRZ 727; 6 TL standard (18 à 550 kb); 7 P; 8 Q; 9 R.

Table II. Comparison of the initial rate (Vi) and the final extent (24 h) of autolysis for the seven strains forming the cluster A.
Comparaison entre la vitesse initiale (Vi) et l'étendue maximale en 24 h de l'autolyse des 7 souches formant le groupe A.

Strain	Time (h) of growth/OD_{650} before harvesting	Autolysis	
		Vi (10^{-3}) uOD/min)	Extent (%)
CNRZ 725	40 / 0.93	5.4	92
CNRZ 435	16 / 0.50	2.1	93
CNRZ 80	16 / 0.80	4	53.5
CNRZ 726	16 / 0.38	3.2	52
CNRZ 727	40 / 0.50	2.4	93
P	17 / 0.67	1.5	90
R	17 / 1	1	93.5

(fig 4). The absence of a typical stationary phase was a remarkable feature of the growth curves. Maximal growth is immediately followed by a drastic reduction of OD_{650} corresponding to a spontaneous lysis in the growth medium, with a constant rate of respectively 3.2 10^{-4}, 2.35 10^{-4}, 2 10^{-4}, 2.4 10^{-4} (uOD_{650}/min). The starting point of the lysis corresponded to the exhaustion of lactate in the growth medium (data not shown). Growth was also followed by viability measurements (data not shown) from which generation times were calculated as being 7, 4, 9 and 5 h for strains CNRZ 725, CNRZ 726, CNRZ 727 and CNRZ 435 respectively. At 400 h of growth, a loss of viability of 1 to 1.3 log cfu (corresponding to a reduction of viable cells of 90 to 95%) depending on the strain was observed from the maximal growth concomitantly with 80% decrease of OD_{650}.

DISCUSSION

Autolysis appeared as a strain-dependent phenomenon in the genus *Propionibacterium*. Among 57 strains of dairy propionibacteria, seven strains were highly prone to lysis. Their high autolytic activity was verified by a second screening. Moreover, as demonstrated by genomic fingerprinting, these seven strains were truly different strains and could be used for further *in vitro* studies or in cheese. All the other strains constituted a second cluster. The strain-dependent character of autolysis was previously observed in the literature, in particular for some lactic acid bacteria as *Lactococcus lactis* (Vegarud *et al*, 1983; Chapot-Chartier *et al*, 1994) or *Lactobacillus helveticus* (Premi *et al*,1973).

The method of screening (measurement of autolysis in buffer) was easy and rapid, the maximal extent of autolysis being reached at 24 h. Moreover, some authors suggest that autolysis in buffers are a good reflection of what happens in cheese as demonstrated for *Lactobacillus casei* (El Soda *et al*, 1993). Furthermore, the impact of 2 strains of *L lactis* on the ripening process depending on their autolytic activity has recently been described by Chapot-Chartier *et al* (1994). The development of a similar assay for autolysis of propionibacteria in cheese would thus be a useful test in the characterization of propionibacterium starters.

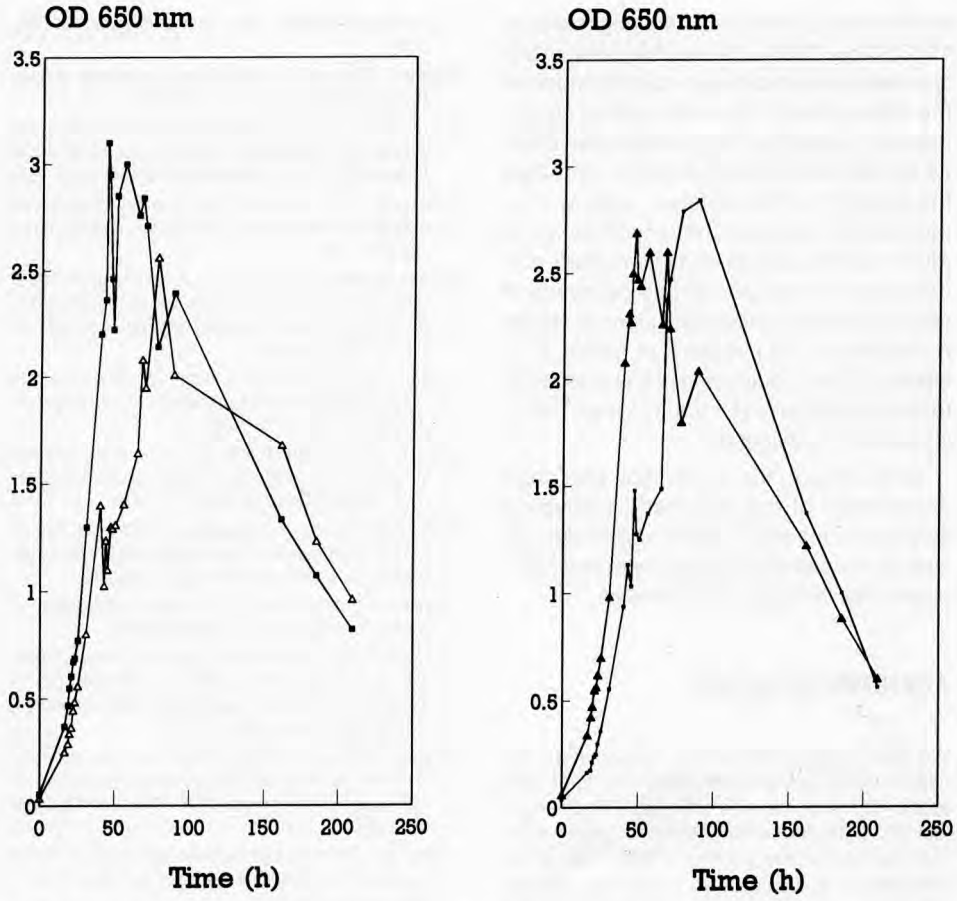

Fig 4. Growth of four strains of dairy propionibacteria belonging to cluster A followed in YEL medium at 30°C by OD_{650}: ■, CNRZ 726; ▲, CNRZ 435; Δ, CNRZ 727; •, CNRZ 725.
Croissance en milieu de culture YEL à 30°C de 4 souches P freudenreichii a*ppartenant au groupe A: DO_{650}:* ■ *, CNRZ 726;* ▲ *, CNRZ 435;* Δ *, CNRZ 727;* • *, CNRZ 725.*

Nevertheless, some limits have to be underlined about the screening procedure employed in this work. To compare strains they must be harvested at the same stage of growth, ideally during the exponential growth phase. Moreover, the conditions arbitrarily chosen for screening (type of buffer, pH, temperature, molarity) are not necessarily the best for the expression of autolysis. This method of screening remains very useful to detect lytic strains; it cannot guarantee that the other strains are all poorly autolytic strains.

The growth of four strains belonging to cluster A was followed by OD_{650} and viability measurements. Maximal growth was immediately followed by a spontaneous lytic phase (with a constant rate of about 2.5 10^{-4} uOD_{650}/min). The absence of any stationary phase was particularly obvious for these four strains compared to other growth curves of dairy propionibacteria described in

the literature (Lee *et al*, 1974; Langsrud *et al*, 1977; Crow, 1986). The starting point of this spontaneous lysis phase corresponded to the exhaustion of the main carbon source (lactate) suggesting that nutrient starvation, as for the experiments in buffer, provokes the induction of the autolytic system. Further work to establish the growth curves of all 57 strains needs to be conducted to determine if the lysis phase observed is specifically associated with strains of cluster A. Moreover, the influence of growth conditions (carbon source and pH) on this spontaneous autolysis (Hsu and Yang, 1991) should be investigated.

In the future, the purification and characterization of the enzymes (autolysins) involved in autolysis will be undertaken as well as cheese making trials with dairy propionibacteria highly prone to lysis.

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