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SUMMARY

Lactobacilli have been used as industrial starters for a long time, but in many cases their phenotypic identification is still neither easy nor reliable. Previously we observed that the cell wall peptidoglycan hydrolases of *Lactobacillus helveticus* were highly conserved enzymes; the aim of the present work was to determine whether peptidoglycan hydrolase patterns obtained by renaturing SDS-PAGE could be of interest in the identification of lactobacilli species. For that purpose, the peptidoglycan hydrolase patterns of 94 strains of lactobacilli belonging to 10 different species were determined; most of the species studied are used either in dairy, meat, bakery or vegetable fermentations: *L. helveticus, L. acidophilus, L. delbrueckii, L. brevis, L. fermentum, L. jensenii, L. plantarum, L. sake, L. curvatus* and *L. reuteri.* Within a species, the strains exhibited highly similar patterns: the apparent molecular weights of the lytic bands were identical, with only slight variations of intensity. Moreover, each species, including phylogenetically close species such as *L. sake* and *L. curvatus,* or *L. acidophilus* and *L. helveticus,* gave a different pattern. Interestingly, the closer the species were phylogenetically, the more related were their patterns. The sensitivity of the method was checked using various quantities of *L. acidophilus* cells: a peptidoglycan hydrolase extract of 5×10^6 cells was sufficient to obtain an informative pattern, as was a single colony. Finally, the method was also successfully applied to distinguish two *Carnobacterium* species. In conclusion, the electrophoretic pattern of peptidoglycan hydrolases is proposed as a new tool for lactobacilli identification: it is rapid, sensitive and effective even for phylogenetically close species. Furthermore, this work provides the first evidence of the potential overall taxonomic value of bacterial peptidoglycan hydrolases.

Key-words: Autolysis, Peptidoglycan hydrolase, *Lactobacillus, Carnobacterium;* Identification, Renaturing SDS-PAGE.

INTRODUCTION

Over 50 species are currently recognized in the genus *Lactobacillus,* which is very heterogeneous. Few of them are actually used as industrial starters in dairy, meat, fish, wine, bakery or vegetable fermentations (*L. helveticus, L. acidophilus, L. delbrueckii, L. paracasei, L. rhamnosus, L. plan-*

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tarum, L. brevis, L. sake, L. curvatus, etc.). Some lactobacilli species are also recognized as valuable members of the normal flora of the mouth and the intestinal and urogenital tracts of humans and animals. The phylogenetic analysis of this genus by reverse transcriptase (RT) sequencing of 16S rRNA revealed three clusters (Collins *et al.*, 1991) which did not correspond to the three physiological groups of Bergey's manual (obligately homofermentative, facultatively heterofermentative and obligately heterofermentative). On the basis of DNA-DNA hybridization data, several changes in the taxonomy of this genus have been made, and new species have been defined (Johnson *et al.*, 1980; Lauer *et al.*, 1980; Weiss *et al.*, 1983; Fujisawa *et al.*, Dicks *et al.*, 1996; Curk *et al.*, 1996). In many cases, the determination of the species by biochemical assays or on the basis of growth temperature and fermentation end-products is ambiguous. For that reason, numerous identification methods at the species level have been proposed (for review see: Gasser *et al.*, 1994; Curk *et al.*, 1994a): for example, the electrophoretic mobilities of lactic dehydrogenases (Gasser, 1970; Uemura *et al.*, 1994), the total soluble cell protein patterns (Dicks and Van Nuuren, 1987; Hertel *et al.*, 1993), the analysis of cellular fatty acids and neutral monosaccharides (Rizzo *et al.*, 1987; Decallone *et al.*, 1991; Gilarova *et al.*, 1994), Fourier-transform infrared spectroscopy (Curk *et al.*, 1994b), the restriction endonuclease pattern associated with multivariate analysis (Stahl *et al.*, 1990), M13 DNA fingerprinting (Miteva *et al.*, 1992) and species-specific probes (Hertel *et al.*, 1993; Schleifer *et al.*, 1995). Each of these methods has specific applications and advantages, but most of them involve an extraction step of the DNA or require particular equipment or have been developed for a small number of species.

Peptidoglycan (PG) hydrolases are endogenous cell wall bacterial enzymes that hydrolyse bonds in the protective and shape-maintaining peptidoglycan (Shockman and Höltje, 1994). Depending on their specificity (glycosidase, amidase, peptidase), they hydrolyse various bonds in this three-dimensional network. Most often, several PG hydrolases of various specificities coexist in the cell wall. Numerous potential roles in bacterial growth and division have been proposed for these enzymes (Rogers *et al.*, 1980) but most of them, as well as their regulation, are still not clearly established. Some of these PG hydrolases, named autolysins, are able to provoke bacterial autolysis in particular conditions and for that reason, must be well regulated. Recently, a powerful method was described to visualize lytic ensymes, including bacterial PG hydrolases, after SDS-PAGE (Audy *et al*, 1988; Leclerc et Asselin, 1989). In the framework of our study of the autolytic system of *L. helveticus* (Valence and Lortal, 1995), this method was applied to several strains. Using whole cells of *Micrococcus luteus* as substrate included in the gel, several PG hydrolases were detected at 42 and 30 kDa. We noticed that for all the strains the pattern was the same regarding the molecular weight of the PG hydrolases; only the intensity of the lytic bands was in some cases variable. As the strains came from various geographical origins and were not genomically closely related as shown recently by genomic DNA macrorestriction analysis (Lortal *et al*, 1997), this observation suggested that PG hydrolases are highly conserved enzymes and could eventually be a useful taxonomic marker for species determination.

The aim of this work was to determine whether PG hydrolase patterns can be a reliable new phenotypic tool for the identification of a large number of lactobacilli species.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The origin of the lactobacilli strains is given in table I. They were stored at −70°C in MRS medium (de Man *et al.*, 1960) containing 15% (v/v) glycerol. The cultures were performed at 30 or 37°C (as indi-

MRS = Man-Rogosa-Sharpe (medium).
PG = peptidoglycan.
SDS-PAGE = sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Table I. Origin of the strains.

Strain	Source	Other strain designation(s)
L. acidophilus (37°C)		
CNRZ 204^T	Human	ATCC 4356, CIP 76.13, DSM 20079, LMG 7943/8150, NCDO 1748, NCIMB 8690
CNRZ 55	Faeces, rat	NCDO 1, NCIMB 1723, ATCC 832
CNRZ 216	Digestive flora, conventional rat; France	
CNRZ 217◆	Digestive flora, conventional rat; France	
CNRZ 251◆	Comté artisanal lactic starter; France	
CNRZ 462◆	Lactic starter; Germany	
CNRZ 1081◆		
CNRZ 1295		
CIP 103595	Human isolate	ATCC 4357, NCDO 1697, NCIMB 8607
CIP 103600	Sweet acidophilus milk	
CIP 103601		
SC 41	Argentina	
L. brevis (30°C)		
CNRZ 215^T	Human faeces	ATCC 14869, DSM 20054, CIP 102806, LMG 7944, NCDO 1749
CNRZ 234	Dutch-type cheese; France	
CNRZ 423	Bakery starter; France	
CNRZ 734◆	France	
L. curvatus (30°C)		
LMG 9198^T	Milk	ATCC 25601, CIP 102992, DSM 20019, NCDO 2739, NCIMB 9710
L. delbrueckii* subsp. *bulgaricus (37°C)		
CIP 101027	Bulgarian yoghurt; Bulgaria	ATCC 11842, NCDO 1489, NCIMB 11748, DSM 20081, CNRZ 208, LMG 6901
CIP 71.36		
CNRZ 208		
IL 29		
IL 1207		CNRZ 61, NCDO 1373
IL 1215	Sugared yoghurt	CNRZ 384
IL 1220	Yoghurt lactic starter; France	CNRZ 396
IL 1221	Yoghurt lactic starter; France	CNRZ 397
IL 1222	Yoghurt lactic starter; France	CNRZ 398
IL 1224	Yoghurt lactic starter; France	CNRZ 400
IL 1225	Commercial lactic starter; France	CNRZ 401
IL 1227	Yoghurt lactic starter; France	CNRZ 417
IL 1232	Yoghurt lactic starter; France	CNRZ 422
L. delbrueckii* subsp. *delbrueckii (37°C)		
CIP 57.8^T	Sour grain mash	ATCC 9649, NCDO 213, DSM 20074
CNRZ 231	Must; UK	NCDO 1744
L. delbrueckii* subsp. *lactis (37°C)		
CNRZ 207^T	Emmental cheese	ATCC 12315, NCDO 1438, DSM 20072, CIP 101028, LMG 7942
CIP 5361		ATCC 7830, NCDO 302, NCIMB 8118, DSM 20355, LMG 6401
CIP 54.3		ATCC 4797, NCDO 299, NCIMB 8117, DSM 20076

Strain	Source	Other strain designation (s)
CIP 57.5		ATCC 8000, NCDO 270, NCIMB 7278
ITG[9] 11		
ITG12		
ITG 14		
ITG 42		
ITG 57		
ITG 69		
ITG 75		
L. fermentum (37°C)		
CNRZ 209^T	Fermented beet	ATCC 14931, NCDO 1750, DSM 20052, CIP 102980, LMG 6902
CNRZ 17	Comté artisanal lactic starter;	
CNRZ 64	Mouth	NCDO 335
CNRZ 229◆	Saliva	ATCC 14933
CNRZ 233	Emmental; France	
CNRZ 431◆	Bakery starter	
ATCC 9338		CIP 53.163, DSM 20391, NCDO 215, NCIMB 8028
L. helveticus (37°C)		
CIP103146^T	Emmental swiss cheese	ATCC15009, CNZ 223, DSM 20075, LMG 6413, NCDO 2233, NCIMB 11971
BS8	Grana cheese starter; Italy	
CIP 57.15		ATCC12046, NCDO 1829, NCIMB 8733
CNRZ 32	Comté artisanal lactic starter; France	
CNRZ 58	Emmental cheese lactic starter	
CNRZ 67	Comté artisanal lactic starter; France	
CNRZ 241	Comté artisanal lactic starter; France	
CNRZ 303	Comté artisanal lactic starter; France	
CNRZ 414	Cow milk koumis; Russia	
CNRZ 493	Emmental cheese lactic starter; Finland	
CNRZ 834	Emmental cheese; Switzerland	
CNRZ 1094	Emmental cheese lactic starter; Finland	
CNRZ 1102	Grana cheese artisanal starter; Italy	
CNRZ 1110	Commercial lactic starter; France	
CP 615	Japan	
CSL	Industrial starter; Italy	
ISLC5	Parmesan cheese; Italy	
IL 430	Industrial lactic starter isolate	
IL 590	Industrial lactic starter isolate	
IL 735	Industrial lactic starter isolate	
IL 1235	Industrial lactic starter isolate	
IL 1236	Industrial lactic starter isolate	
ITG 1		
ITG 43		
ITG 56		
ITG 58◆		
ITG 68		
ITG 77		
ITG 155		
NCDO 262		ATCC 8018, NCIMB 8025
NCDO 384	Calf rennet	
R. 5088	Canada (Rosell Institut)	
R. 5089	Canada (Rosell Institut)	
SL1 40	Beaufort cheese artisanal rennet; France	

Strain	Source	Other strain designation (s)
L. jensenii (37°C)		
CIP 6917^T	Vaginal discharge, human	ATCC 25528, NCDO 2165, DSM 20557, LMG 6414
L. plantarum (30°C)		
LMG 6907^T	Cabbage seasoned with vinegar	ATCC 14917, NCDO 1752, DSM 20174, CIP 103151, CNRZ 211, LMG 7945
CNRZ 73	Sauerkraut	ATCC 10241, NCDO 343, LMG 9208
CNRZ 184	France	
CNRZ 424	Bakery starter; France	
CNRZ 1008		
CIP A159		ATCC 8014, NCDO 82, DSM 20205, LMG 1284
NCFB 1988	Cheese	
L. reuteri (37°C)		
DSM 20016^T	Faeces	ATCC 23272, NCDO 2589, NCIMB 11951, CIP 101887, LMG 9213
L. sake (30°C)		
ATCC 15521^T	Sake starter	NCDO 2714, CIP 103139, LMG 9468

CNRZ = Centre National de Recherche Zootechnique, Jouy-en-Josas, France.
ATCC = American Type Culture Collection, Rockville, MD USA;
CIP = Collection des Bactéries de l'Institut Pasteur, Paris, France.
DSM = Deutche Sammlung von Microorganismen und Zelkulturen GmbH, Braunschweig, Germany.
LMG = Laboratorium Microbiology Gent Culture Collection, Rijksuniversiteit Gent, Belgium.
NCDO = National Collection of Dairy Organism, Shinfield, Reading Berkshire, UK.
NCIMB = National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK.
IL = Institut National de Recherche Agronomique, Collection du Laboratoire de Technologie laitière, Rennes, France.
ITG = Institut Technique du Gruyère, La Roche-sur-Foron, France.
NCFB = National Collection of Food Bacteria, Shinfield, Reading, Berkshire, UK.
The sign ◆ indicates a potential misclassification of the strain as detected by the method of identification described in this work.

cated in table I) in MRS broth. The growth was monitored at 650 nm using a spectrophotometer (Beckman, DU 7400). The cultures of *Carnobacterium piscicola* 2762 and *C. divergens* 41 (kindly provided by P. Boyaval) were performed on MRS broth at 30°C.

Renaturing gel electrophoresis

Renaturing SDS-PAGE (zymogram) was performed according to the method of Leclerc and Asselin (1989) using a "Bio-rad" mini-system under the following conditions: SDS/polyacrylamide separating gel (14 or 16% polyacrylamide as indicated on the figures, pH 8.8) containing 0.2 % (w/v) (freeze-dried *M. luteus* cells (Sigma) was used to detect the lytic activities). After electrophoresis (1 h for the 14% separating gel and 1 h 30 min for the 16%, both at 180 V, constant voltage at room temperature), the gels were soaked for 30 min in distilled water at room temperature. Then they were transferred into a 50-mM Tris-HCl buffer at pH 8.0 containing 1 % (v/v) Triton X-100 and gently shaken for 2 h at 37°C to enable renaturation of the enzymes. The lytic activities appeared as translucent bands on the opaque background. The contrast was enhanced by staining the gels in 0.1 % (w/v) methylene blue in 0.01 % (w/v) potassium hydroxide, destaining in distilled water under stirring and keeping the gels at least one night at 4°C.

Extraction of the PG hydrolases

Eight ml of an exponential culture (OD_{650} of about 1) were harvested by centrifugation (10,000 *g*, 15 min, 4°C); the pellet was washed once in cold distilled water. If the analysis could not be performed extemporaneously, the 8-ml culture was kept at 4°C for one night or the washed cell pellets were frozen at −20°C. The pellet was directly suspended in 80 µl of Laemmli buffer (62.5 mM Tris-HCl pH 6.8, contain-

ing 10 % glycerol, 2 % SDS and 5 % 2-mercaptoethanol) (Laemmli, 1970). The suspension was gently mixed, heated for 2 min at 100°C (this heating step is facultative) and centrifuged (10,000 *g*, 10 min); 15 µl of the supernatant i.e., the whole-cell SDS extract which contains the PG hydrolases, was loaded on the gel. In some cases, the extraction can be improved by grinding the pellet with alumina powder for 3 min before adding the Laemmli buffer.

Molecular mass determination of the PG hydrolases

Ten µl of a prestained standard (Biorad) were loaded on each gel; it contained phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. Depending on the batch provided by Biorad, the molecular weights of these six prestained proteins varied slightly as follows: standard 1: 112, 84, 53.2, 34.9, 28.7 and 20.5; standard 2: 105, 82, 49, 33.3, 28.6 and 19.4; standard 3: 104, 80, 46.9, 33.5, 28.3 and 19.8. If necessary, the standard used was indicated on the figure. The prestained standard was still visible after staining the gels by methylene blue. In order to determine the apparent molecular mass of the lytic bands, the gels were scanned and analysed using a "Bioimage" system (Pharmacia).

RESULTS

PG hydrolase patterns of various lactobacilli: stability within a species and species specificity

The PG-hydrolase patterns were determined for all the strains indicated in table I, including the type strain of each species. The patterns of several strains of *L. acidophilus, L. helveticus, L. plantarum* and *L. delbrueckii* are presented in figure 1. An obvious similarity of the profiles was noted within species and in particular a high stability of the apparent molecular weight of the lytic band(s) (fig. 1a,c,d). The species *L. delbrueckii* was the only one showing a certain extent of variation in the number of the lytic bands detected, as figure 1b illustrates (from 3 to more that 10 lytic bands). Nevertheless, at least two bands were systematically present (lane 2 represents the profile most frequently encountered for that species), and the stability in the molecular weights of the lytic activities was also true for *L. delbrueckii.* It can also be highlighted that the lowest band (at 29 kDa) systematically had a diffuse aspect (like a smear, fig. 1b). Regarding the other species, *L. sake, L. curvatus, L. reuteri, L. fermentum, L. brevis* and *L. jensenii,* for all of which a smaller number of strains was analysed, the PG hydrolase patterns are presented in figure 2. For most of the strains, the analysis was performed at least twice, and the resuls were highly reproducible. In the case of *L. plantarum* and *L. fermentum,* the lytic bands had low intensity under the conditions used, but we noted that this intensity was enhanced by keeping the gel in the cold after staining (at 4°C, one night to one week). With few exceptions which will be considered in the next paragraph, all the strains exhibited the same pattern as their respective type strain.

The characteristics of the PG hydrolase pattern (apparent molecular weight, intensity and aspect of the lytic bands) deduced from the observation of all the strains of the ten species considered are summarized in table II. It was obvious that each species had a specific pattern (figs. 1 and 2, table II). Interestingly, phylogenetically close species (Collins *et al.,* 1991), such as *L. acidophilus* and *L. helveticus,* or *L. sake* and *L. curvatus,* or *L. brevis* and *L. fermentum,* exhibited close but distinct PG hydrolase patterns. For example, the presence of two additionnal lytic activities at 27 and 28 kDa (as indicated in table II) allowed easy differentiation between *L. acidophilus* and *L. helveticus.* It can be highlighted that apparently, as far as the molecular weight of the lytic bands was concerned, the closer the species were phylogenetically, the closer were the patterns. To the best of our knowledge, this is the first report in the literature of the potential taxonomic value of PG hydrolases.

Detection of potentially misclassified strains and confirmation by other techniques

A small number of the 94 strains exhibited a PG hydrolase pattern different from the pattern of their type strain (they have been indicated by a sign (♦) in table 1): *L. fermentum* CNRZ 229, CNRZ 431, *L. brevis* CNRZ 734, *L. helveticus*

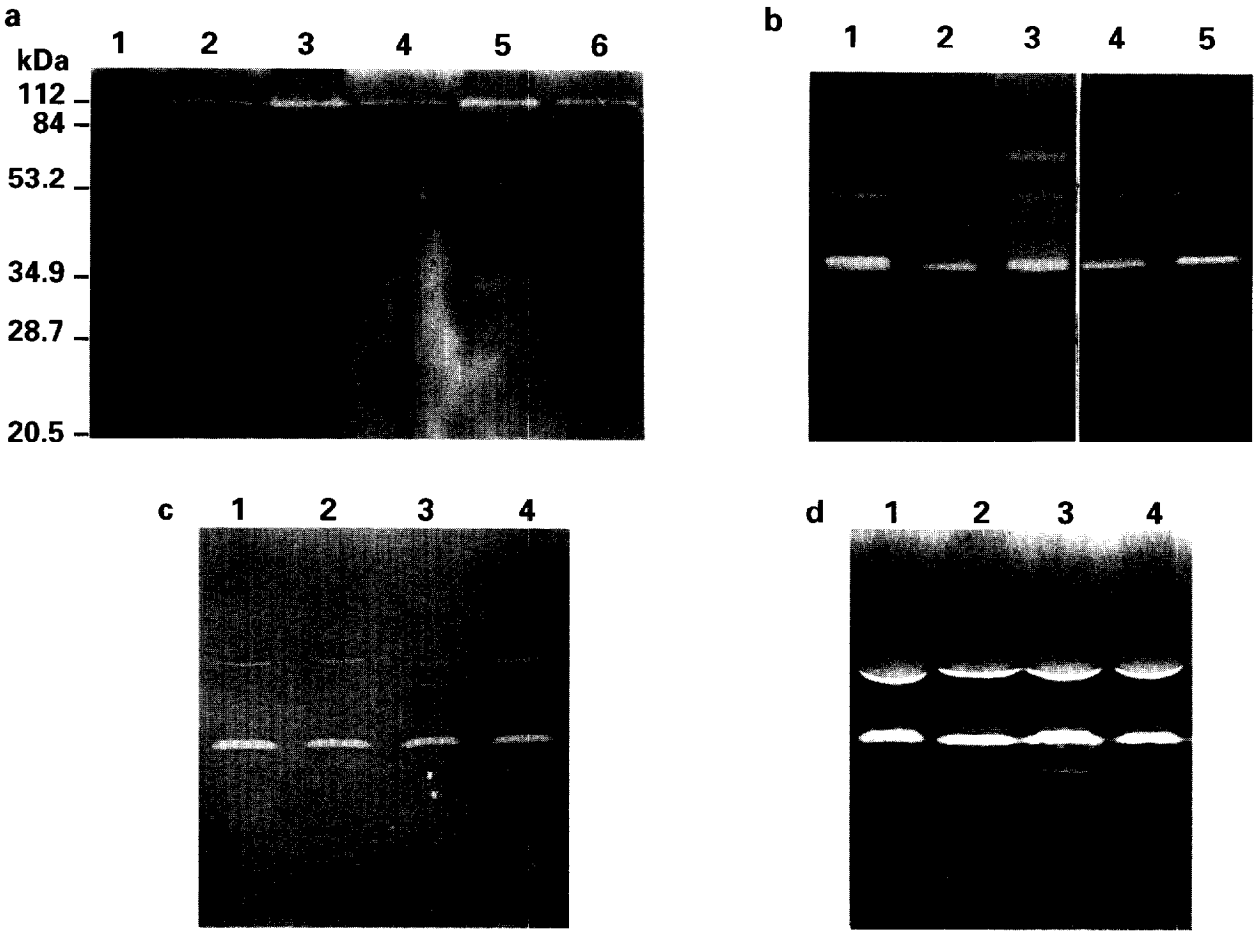

Fig. 1. PG hydrolase patterns of several strains illustrating the stability of the pattern within a species.

a) *L. plantarum*: lane 1 = prestained standard no. 1, lane 2 = LMG 6907^T, lane 3 = NCFB 1988, lane 4 = CNRZ 73, lane 5 = CNRZ 184, lane 6 = CNRZ 1008; b) *L. delbrueckii*: lane 1 = IL1207, lane 2 = IL1222, lane 3 = IL1227, lane 4 = IL1221, lane 5 = IL1220; c) *L. helveticus*: lane 1 = IL 590, lane 2 = IL 735, lane 3 = IL 1235, lane 4 = IL 1236; d) *L. acidophilus*: lane 1 = CIP 103601, lane 2 = CIP 103595, lane 3 = CIP 103600, lane 4 = CNRZ 204^T. The apparent masses of the lytic bands detected are given in table II.

ITG 58, *L. acidophilus* CNRZ 217, CNRZ 251, CNRZ 462 and CNRZ 1081. Taking into account the observations made in the previous paragraph, misclassification of these strains could be suspected. The pattern exhibited by strain ITG 58 was similar to the type strain of *L. delbrueckii*. Carbohydrate fermentation profiles (API 50 CH strips) and electrophoretic protein profile (data not shown) indicated that this strain effectively belongs to the species *L. delbrueckii* subsp. *lactis*.

Regarding the strains *L. fermentum* CNRZ 229 and CNRZ 431 and *L. brevis* CNRZ 734, carbohydrate fermentation profiles (API strips) were performed. The results indicated that strain 229, which is supposed to belong to *L. fermentum*, had a profile better matching that of *L. brevis*. Nevertheless, two sugars should not be fermented (D-mannose, D-mannitol), and the aesculin should not be hydrolysed. The sugar fermentation profile of strain 431 matched well with the species *L. fermentum*. Strain 734 showed a fermentation

Table II. PG hydrolase patterns of the ten lactobacilli species.

Species[a]	Pattern[b]	Characteristics of the lytic bands[c] (MW, intensity, aspect)
L. helveticus		42 kDa / 1-2 / slightly incurved, thin 30 kDa / 1-3 / thick
L. acidophilus		41 kDa / 3 / slightly incurved, thick 30 kDa / 3 / thick 28 kDa / 1-2 / thin 27 kDa / 1-2 / thin
L. delbrueckii		41 kDa / 1-2 / thin 29 kDa / 1-2 / diffuse
L. plantarum		110 kDa / 1 / thin
L. fermentum		79 kDa / 1 52 kDa / 1-2 30 kDa / 1
L. brevis		85 kDa / 1 55 kDa / 1
L. sake		90 kDa / 1 / thin 79 kDa / 3 / thick
L. curvatus		74 kDa / 3 / thick 63 kDa / 1 / thin 60 kDa / 1 / thin 58 kDa / 1 thin
L. reuteri		67.5 kDa / 3 / thick 51 kDa / 1 / thin
L. jensenii		75 kDa / 1 29 kDa / 1

[a] Obtained in the conditions described in "Materials and Methods"; [b] schematized patterns; dotted lines correspond to minor lytic bands which were not considered because of excessive weakness, absence of reproducibility and/or bad resolution; [c] MW: apparent molecular weight; intensity: 1 = very weak to weak; 2 = middle; 3 = strong; aspect: if they are typical, incurvation and/or thickness were indicated.

profile of *L. brevis*, with the exception of two sugars which should not be fermented: D-mannose and 2-ceto-D-gluconate. In order to have a clearer answer regarding these three strains, their total protein contents were analysed by SDS-PAGE and compared with profiles of strains in a data bank, and their PG type was determined. The results (C. Barreau, Institut Pasteur, personal

communication) indicated that at least two of the three strains were effectively misclassified. Strains 229 and 431 cannot be classified as *L. fermentum,* since their PG type was not of the Orn-D-Asp type as expected for that species, but belongs to the more classical Lys-D-Asp type. Moreover, the protein profile of strain CNRZ 229 was similar to that of *L. brevis* (confirming the indication of the API strips), and the protein profile of strain 431 did not match with anything. Strain CNRZ 734 might belong to *L. brevis,* but its protein profile matched better with *L. brevis* CIP 103474 than with the type strain, CIP 71.35. This observation could be explained by the heterogeneity of the *L. brevis* group, which has already been detected (Dicks and Van Vuuren, 1987).

The *L. acidophilus* group is also highly heterogenous; because of the absence of any simple phenotypic way to distinguish the six DNA homology groups described (A1, A2, A3, A4, B1 and B2) (Johnson *et al.,* 1980; Lauer *et al.,* 1980), the potential misclassification of strains CNRZ 217, CNRZ 251, CNRZ 462 and CNRZ 1081 was not verified in that work. Nevertheless, the protein profiles of these four strains were analysed by SDS-PAGE (data not shown), and it was obvious that CNRZ 462 and CNRZ 251 were devoid of the predominant S-layer-forming protein which was described as a typical feature of the group A1, A2, A3 and A4 (Johnson *et al.,* 1987; Lortal, 1993), confirming their misclassification.

Sensitivity of the method and direct use from a colony

All the aforementioned results were obtained with less than 10 ml of broth of an exponential culture. We determined that about 10^8 cells were present in the pellet to which 80 µl of Laemmli buffer was added; 15 µl of the SDS-whole cells extract were loaded into the polyacrylamide gel. From these values, it can be assessed that a quantity of PG hydrolases extracted from 10^7 cells is required to obtain a satisfying pattern (figs. 1 and 2). In order to verify whether it would be possible

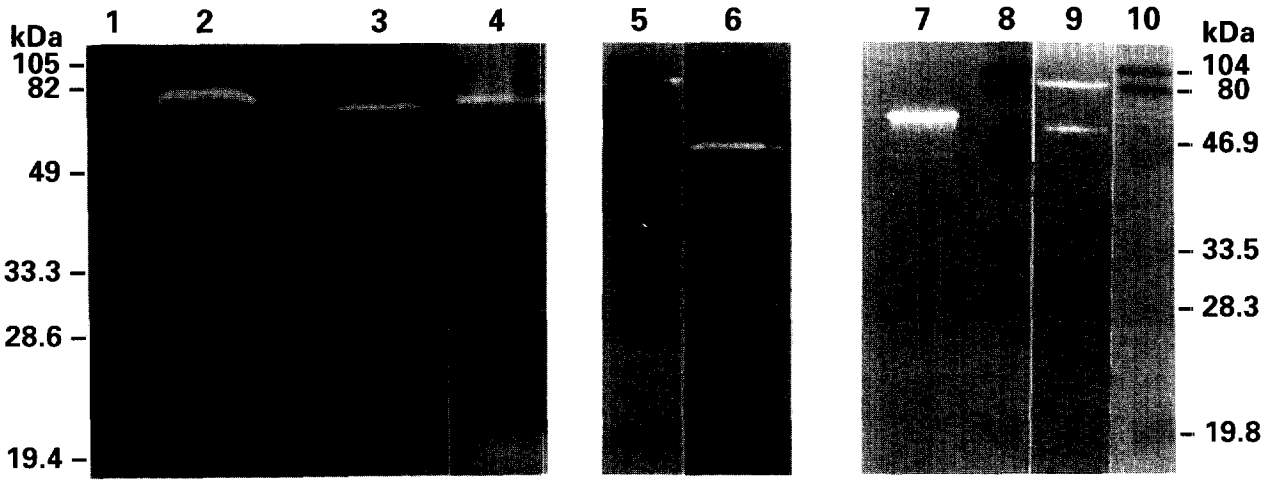

Fig. 2. PG hydrolase patterns of the six other species studied in this work, further illustrating the species specificity.

Lane 1 = prestained standard no. 2, lane 2 = *L. sake* ATCC 15521^T, lane 3 = *L. curvatus* LMG 9198^T, lane 4 = *L. jensenii* CIP 6917^T, lane 5 = prestained standard no. 2, lane 6 = *L. fermentum* CNRZ 209^T, lane 7 = *L. reuteri* DSM 20016^T, lane 8 = prestained standard no. 3, lane 9 = *L. brevis,* CNRZ 215^T, lane 10 = prestained standard no. 3. The apparent masses of the lytic bands for all the species shown here are given in table II.

to reduce this quantity of cells, successive dilutions of an exponential culture of *L. acidophilus* CIP103600 were performed. Each of seven pellets containing 4×10^6 to 4×10^9 cells was treated with 80 µl of Laemmli buffer in order to extract the PG hydrolases. As 10 µl were loaded per lane, it can be estimated that the quantity of PG hydrolases loaded was extracted from a number of cells ranging from 5×10^5 to 5×10^8 cells. The samples were loaded on an *M. luteus*-containing gel and in parallel on a gel devoid of substrate in order to obtain the protein profiles of the extracts. For that species at least, the method can be applied with 5×10^6 cells only (fig. 3). It

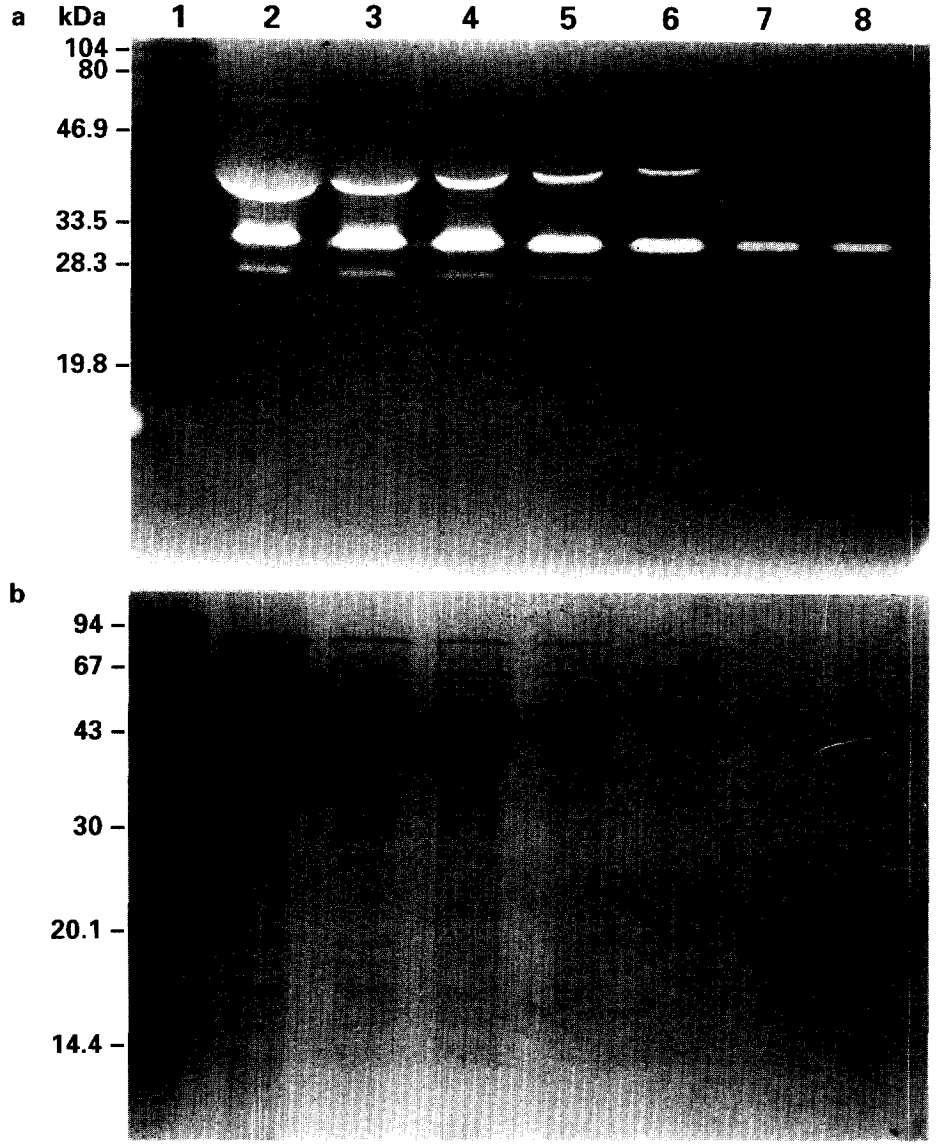

Fig. 3. Evaluation of the sensitivity of the method.

a) PG hydrolase patterns and b) SDS-PAGE patterns obtained from various quantities of *L. acidophilus* CIP 103600 whole cells: lane 1 = standards, lane 2 = 5×10^8, lane 3 = 10^8, lane 4 = 5×10^7, lane 5 = 10^7, lane 6 = 5×10^6, lane 7 = 10^6, lane 8 = 5×10^5.

should be underscored that within a rather large range of cells studied, the characteristics of the pattern were not modified. Nevertheless, below a certain number of cells, the intensity of the 40-kDa lytic band was significantly reduced; in a similar manner, when an excessive quantity of cells were treated, the interpretation of the pattern could be altered. Thus, it was concluded that ideally a pellet containing 5×10^6 to 5×10^7 bacterial cells should be used in order to obtain satisfactory PG hydrolase patterns. With the same objective in mind, single colonies of *L. acidophilus* and of *L. sake* with a size between 1 and 2 mm were sampled and directly suspended in 50 µl of Laemmli buffer for PG hydrolase extraction. For these two species, informative patterns identical to those obtained from a liquid culture were obtained (data not shown), indicating that growth on a solid medium did not alter them. Finally, comparison of figures 3a and 3b illustrated the sensitivity of the PG hydrolase detection, since lytic bands (in particular at 30 kDa) were still obtained when the proteins in the extracts were almost not visible (when using Coomassie blue staining).

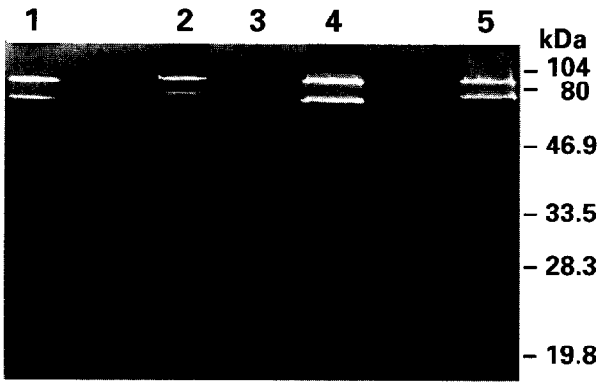

Fig. 4. Applicability of the method to other Gram$^+$ species.

PG hydrolase pattern of *C. divergens* 41 (lane 1 = 3 µl and lane 4 = 15 µl) and PG hydrolase pattern of *C. piscicola* 2762 (lane 2 = 3 µl and lane 5 = 15 µl); lane 3 = prestained standard no. 3.

Application to other Gram-positive species

In order to assay if the method could be applied to non-lactobacilli species, it was tested for *C. piscicola* and *C. divergens*. Again, related but distinct patterns were obtained (fig. 4). Two intense lytic bands were seen with an apparent, molecular weight of 89 and 72.7 kDa for *C. divergens* and of 92 and 76 kDa for *C. piscicola*. When a slightly larger quantity of PG hydrolase extract was loaded (fig. 4), it was obvious that they also differed in less intense lytic bands: two minor bands of 65 and 57.6 kDa for *C. divergens* and two minor bands of 65 and 38.5 kDa for *C. piscicola*.

DISCUSSION

The stability of the electrophoretic PG hydrolase pattern within a species, which was noted previously for *L. helveticus* (Valence and Lortal, 1995), was confirmed in this work for many other lactobacilli species. In addition, the PG hydrolase patterns of lactobacilli were shown to be species-specific, providing the first evidence of the taxonomic value of these enzymes or at least of their electrophoretic pattern. Indeed, the exact number of distinct PG hydrolases for a given species cannot be determined from this work; it depends on their resistance to denaturing conditions, on their ability to degrade *M. luteus* and on the eventual presence of proteolytically derived forms of the same enzyme. This last hypothesis is rather realistic, since it has been shown for *L. lactis* that the deletion of one gene (autolysin, *acmA*) led to the disappearance of all the lytic activities in the pattern (Buist *et al.*, 1995). Nevertheless, independently of the precise number of distinct enzymes, the PG hydrolase patterns obtained in standardized conditions as described here can be used as an identification tool as in our patent (Lortal *et al.*, 1995). Indeed, in addition to the stability and specificity of the patterns, some observations presented here strongly supported the taxonomical value of the proposed method. First, the closer the species were phylogenetically, the closer were their patterns. This was particularly obvious for *L. helveticus* and *L. acidophilus* and for the group *L. sake, L. curvatus* and *L. reuteri*.Secondly, misclassification, sus-

pected for some strains because of a PG hydrolase pattern different from that of the type strain, was effectively confirmed by other analyses.

To our mind, the total number of lytic bands as well as their apparent molecular weight constitute the objective criteria to consider in order to identify a species, but the aspect of the bands (incurvation, thickness, intensity) may also be useful to take into account. For example, *L. reuteri* and *L. sake* led to one thick and intense band compared with *L. curvatus,* which in the same molecular weight area, led to one thin intense band just above several weak lytic activities. Furthermore, the presence in some species like *L. helveticus* and *L. acidophilus* of an S-layer-forming protein (Lortal, 1993) just above the 40-kDa lytic activities, deformed them, causing an incurved aspect. In order to use the method as a new taxonomical tool, a bank of patterns which would take into account all the aforementioned characteristics (molecular weight and aspect of the bands) should be developed.

Compared with other identification tools developed for lactobacilli, the proposed method exhibits numerous advantages: it is easy, cheap and rapid (like protein electrophoresis), sensitive (it can be applied to a single colony) and effective, as it was able to distinguish closely related lactobacilli species. Further investigations are, however, required to see if it can be effective enough to distinguish the 6 DNA-homology clusters constituting the so-called *L. acidophilus* group (Johnson *et al.,* 1980; Lauer *et al.,* 1980). In addition, some limitations still exist. For example, the patterns obtained for 10 different lactobacilli species were obtained, but under the conditions used, it was easier to obtain informative patterns (intense lytic bands) for some species (*L. acidophilus, L. helveticus, L. sake, L. curvatus* and *L. reuteri*) than for the others (*L. fermentum, L. plantarum, L. brevis, L. jensenii* and *L. delbrueckii*). For these last species an optimization of the procedure has to be performed, concerning in particular the PG hydrolase extraction method and the renaturing conditions (pH, temperature and nature of the buffer).

As PG hydrolases are supposed to be ubiquitous enzymes, this method could be used for non-lactobacilli species. In this work distinct PG hydrolase patterns were effectively observed for two *Carnobacterium* species. Preliminary data were also obtained for dairy propionibacteria (Lortal *et al.,* 1995), enabling the differentiation of the three phylogenetically close species *Propionibacterium thoenii, P. acidipropionici* and *P. jensenii,* but the conditions used were different from those described in the present work. PG hydrolase patterns of numerous Gram$^+$ and Gram$^-$ species have been described in the literature (Foster, 1992; Bernadsky *et al.,* 1994). Unfortunately, most often only one strain per species was described, and thus the stability within a species cannot be assessed and *a fortiori*, the species specificity. Nevertheless, it was observed that the PG hydrolase patterns of several *L. lactis* strains were highly similar (Chapot-Chartier, 1996; Buist *et al.,* 1995), and 10 different clinical strains of *Staphylococcus aureus* also gave identical patterns (Sugai *et al.,* 1990). These two examples, at least, supported the hypothesis that the pattern stability between strains of a same species is a general property. In conclusion, from the survey of the literature as well as from the data of this study, the electrophoretic PG hydrolase pattern can constitute a new phenotypic tool in bacterial identification.

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Profil électrophorétique des hydrolases du peptidoglycane, une nouvelle méthode d'identification des espèces bactériennes: application à 10 espèces de lactobacilles

Les lactobaciles sont utilisés depuis longtemps dans de nombreuses fermentations, mais leur identification phénotypique est parfois encore difficile ou imprécise. Dans un précédent article, nous avions observé que les hydrolases du peptidoglycane de *Lactobacillus helveticus* étaient des enzymes haute-

ment conservées. Le but du présent travail est de déterminer si les profils électrophorétiques des hydrolases de peptidoglycane, obtenus après électrophorèse renaturante, selon Leclerc et Asselin, peuvent constituer un nouvel outil d'identification. Le profil des hydrolases de peptidoglycane a été déterminé pour 94 souches de lactobacilles appartenant à 10 espèces différentes; la plupart de ces espèces sont utilisées industriellement dans divers procédés fermentaires (laitiers ou non): *L. helveticus, L. acidophilus, L. delbrueckii, L. brevis, L. fermentum, L. jensenii, L. plantarum, L. sake, L. curvatus* et *L. reuteri*. Les souches d'une même espèce présentent un profil hautement similaire: le poids moléculaire apparent des activités lytiques est identique et seule leur intensité varie légèrement. De plus, il est apparu que chaque espèce présente un profil différent. Des espèces très proches telles que *L. sake* et *L. reuteri* ou encore *L. helveticus* et *L. acidophilus* peuvent être aisément différenciées. Il est à noter que plus les espèces sont proches phylogénétiquement, plus leur profils sont semblables. La sensibilité de la méthode a été testée par l'extraction des hydrolases de peptidoglycane à partir de quantités croissantes de cellules de *L. acidophilus*, et 5×10^6 cellules (ainsi qu'une colonie de 2 mm de diamètre) se sont avérées suffisantes pour obtenir un profil satisfaisant. Enfin, la méthode a également été appliquée avec succès pour la différenciation de deux espèces de *Carnobacterium*. En conclusion, le profil électrophorétique des hydrolases du peptidoglycane est proposé comme une nouvelle méthode d'identification des lactobacilles: c'est une méthode sensible, rapide et efficace, même pour des espèces proches. De plus, ce travail représente la première mise en évidence de l'intérêt taxonomique des hydrolases du peptidoglycane.

Mots-clés: Autolyse, Peptidoglycane hydrolase, *Lactobacillus, Carnobacterium;* Identification, Electrophorèse renaturante.

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