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Zymogram and Preliminary Characterization of *Lactobacillus helveticus* Autolysins

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The autolysins of *Lactobacillus helveticus* ISLC5 were detected and partially characterized by renaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with substrate-containing gels (zymogram). By using lyophilized *Micrococcus luteus* cells or heated whole cells of *L. helveticus* ISLC5 (0.2% [wt/vol]) as a substrate, several lytic activities were detected in the whole-cell SDS extract of strain ISLC5 (i) one activity at 42.4 kDa, which was named autolysin A, and (ii) six other activities having very similar molecular weights (29.1, 29.6, 30, 30.8, 31.7, and 32.8 kDa), which were named autolysins B (B1 through B6, respectively). As regards the temporal distribution of the enzymes, autolysins A and B were detected in the cells harvested from the beginning of the exponential growth phase. Autolysin A appeared to be associated only with viable cells, whereas the autolysins B remained associated with the cell envelope several days after the complete loss of culture viability. When SDS-treated walls of *L. helveticus* ISLC5 were used as a substrate, a supplementary lytic activity appeared at 37.5 kDa; it was considered a peptidoglycan hydrolase, since it was not able to induce lysis of whole-cell substrate. The autolysins of 30 other strains of *L. helveticus* from various geographical origins were also analyzed by zymogram; all the activity profiles obtained were similar to that of strain ISLC5 in terms of the number of lytic bands and their apparent molecular weights. Only the relative intensities of the lytic bands corresponding to autolysins A and B were variable depending on the strains. This observation suggested that autolysins are highly conserved enzymes. A concentrated crude lysate of the virulent bacteriophage 832-B1 infecting *L. helveticus* was also analyzed by zymogram; one lytic activity with an apparent molecular weight of 31.7 kDa, very close to the weights of the autolysins B, was observed. Finally, the autolysins of *L. helveticus* ISLC5 were successfully extracted from whole cells by using a 1 M lithium chloride solution; they were partially purified by precipitation, selective resolubilization, and gel filtration chromatography, which led to a 20-fold increase in specific activity.

Lactobacillus helveticus plays an important role in cheese technology, particularly for Swiss-type cheese (1, 49). By producing lactic acid, this bacterium contributes to the essential curd acidification step. In addition, cell wall-associated proteases (30, 56) and cytoplasmic peptidases and esterases (12, 18, 35, 37) of *L. helveticus* are involved in cheese maturation. Autolysis which results in opening of the cell allows these enzymes to reach their substrates and participate in the ripening of the cheese. Autolysis in cheese substrates (5, 6, 23, 34, 50) as well as in buffered solutions (27, 28, 36, 41) has been described for *L. helveticus*. A better understanding of the autolysis of cheese-related starters such as *L. helveticus* could offer an opportunity to influence, and even accelerate, the very complex ripening process (10, 53, 54).

Autolysis is the result of the action of endogenous enzymes (autolysins) that hydrolyze specific bonds in the protective and shape-maintaining cell wall peptidoglycan (45). On the basis of their cleavage specificities, autolysins are classified as β-1,4-*N*-acetylmuramidases (i.e., lysozymes), β-1,4-*N*-acetylglucosidases, *N*-acetylmuramyl-L-alanine amidases, and peptidases. Several autolysins showing similar or distinct specificities may exist together in the cell and constitute its autolytic system. Some peptidoglycan hydrolases are unable to induce whole-cell lysis, and thus they are not considered autolysins (45).

The cell walls of *L. helveticus* contain an L-Lys-D-Asp peptidoglycan type (21, 44), glycerol-type teichoic acids, and neutral sugars (3). The presence of a proteinaceous paracrystalline surface layer (S-layer) constituting the outermost layer of the cell wall and about 50% of its dry weight was also demonstrated (26, 29, 31–33).

Until now, the autolysins of *L. helveticus* have neither been isolated from the cell wall nor purified. Only a predominant β-1,4-*N*-acetylmuramidase activity in the autolytic system of *L. helveticus* ATCC 12046 was previously described (28).

In the present work, autolysins of *L. helveticus* ISLC5 were analyzed by renaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with polyacrylamide gels containing a convenient substrate for the autolysins. After electrophoresis, the polyacrylamide gels were incubated in a buffer solution containing Triton X-100 which allows the renaturation of the enzymes; the lytic enzymes then appeared as translucent bands on an opaque background. This method was first described by Potvin et al. (40), and since then it has proved to be very powerful for the study of bacterial autolysins (4, 15, 22, 25, 47, 52). The ability of the autolysins to renature after SDS-PAGE meant that their activity in crude extract as well as in partially purified extract could be studied. Because of the denaturing conditions, the zymogram method can determine the molecular weights of autolysins. An efficient extraction procedure as well as the partial purification of the autolysins of *L. helveticus* ISLC5 is also presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The origins of the strains are given in Table 1. The strains were stored at −18°C in MRS medium (11) provided by

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TABLE 1. Origins of the strains and results of the zymogram assay

Strain	Origin (source)	Designation(s) in other collection(s)	Intensity level of lytic band(s)[a]	
			Autolysin A	Autolysins B
CIP 5715[b]		ATCC 12046,[c] NCDO 1829,[d] NCIB 8333[e]	(+)	+++
NCDO 262		ATCC 8018	+++	+++
NCDO 384			+	(+)
ROSELL 5088	Canada		+	+
ROSELL 5089	Canada		+	+++
CNRZ 32[f]	France[g]		+	+
CNRZ 58		NCDO 99	+	++
CNRZ 67	France[g]	NCDO 384	+	++
CNRZ 223[T]	Switzerland	ATCC 15009, CIP 103146, NCDO 2712	+	+++
CNRZ 241	Switzerland		+	+
CNRZ 303	Switzerland		(+)	+++
CNRZ 414	Russia[h]		+	++
CNRZ 493	Finland[i]		+	++
CNRZ 834	Switzerland[j]		+	+
CNRZ 1094	Finland[i]	NCDO 766	+	+++
CNRZ 1102	Italy[k]		+++	(+)
CNRZ 1110	France[l]	IL 590[m]	+	+++
IL 430	France		+	+++
IL 735	France		+	+++
IL 1235	France		+	+++
IL 1236	France		++	+++
A[n]	France		(+)	+
C[n]	France		+	+
B[n]	France		(+)	+++
D[n]	France		+	+
E[n]	France		(+)	++
F[n]	France		++	+++
ISLC 5	Italy (Instituto Sperimentale Latiero-caesario)[k]		+++	++
BS 8	Italy (Chemiferm)[k]		(+)	+
CP 615	Japan		+	+++
CSL	Italy (Centre Sperimentale del Late)		+	+++

[a] (+), not clearly detected; +, low intensity; ++, middle intensity; +++, high intensity.
[b] CIP, Pasteur Institut collection, France.
[c] ATCC, American Type Culture Collection, Rockville, Md.
[d] NCDO, National Collection of Dairy Organisms.
[e] NCIB, National Collection of Marine Bacteria, Aberdeen, United Kingdom.
[f] CNRZ, Centre National de Recherche Zootechnique collection, France.
[g] Artisanal lactic starter of Gruyère de Comté.
[h] Milk cow koumiss.
[i] Commercial lactic starter of Emmental.
[j] Emmental.
[k] Artisanal lactic starter of Grana Padano.
[l] Commercial lactic starter.
[m] IL, Institut National de Recherche Agronomique, Technologie Laitière Laboratory, France.
[n] Industrial strain; confidential origin.

Difco (Detroit, Mich.) which contained 15% (vol/vol) glycerol. The cultures were grown in MRS medium at 37°C. Growth was monitored at 650 nm with a spectrophotometer (DU7400; Beckman). The cells were harvested from the growth medium by centrifugation at 7,000 × *g* for 20 min. The pellet was washed in cold sterile distilled water, and until use it was normally stored at 4°C except for the experiment concerning the temporal distribution of the autolysins (storage of all pellets at −18°C). Parallelly, cells were observed with a light phase-contrast microscope (Optiphot; Nikon). MRS medium containing 1.5% agar type E (Biokar, Beauvais, France) was used for the enumeration of *L. helveticus* ISLC5 cells. Petri dishes were incubated in anaerobic jars (with anaerocult A [Merck, Nogent-sur-Marne, France]) for 48 h at 37°C.

Preparation and concentration of crude bacteriophage lysate. Bacteriophage 832-B1 and its host strain *L. helveticus* CNRZ 223 were kindly provided in 1990 by Laurent Séchaud (Institut National de la Recherche Agronomique, Jouy-en-Josas, France). The phage was propagated by infecting a growing bacterial strain (optical density at 650 nm [OD_{650}] = 0.3) in MRS-Ca^{2+} broth at 37°C. Lysis of the culture occurred within 5 to 6 h. Cell debris was removed by centrifugation at 24,000 × *g* for 20 min. The supernatant was filtered through a 0.45-μm-pore-size filter (membrane HA; Millipore). The filtrate corresponding to the crude lysate was recovered and concentrated 10-fold by using an Amicon ultrafiltration cell (DIAFLO; YM10 membrane; 40 lb/in^2 at 4°C). The retentate was recovered and named concentrated crude lysate. For zymogram analysis, concentrated crude lysate was prepared as described for the autolysin-containing samples.

Preparation of crude cell envelopes and SDS-treated walls. Cells of *L. helveticus* ISLC5 were harvested during the exponential growth phase (OD_{650} = 1) and washed once in cold distilled sterile water (7,000 × *g*, 15 min). The pellet was resuspended in a volume of cold distilled sterile water 15 times smaller than the initial volume of the culture. This suspension was submitted to the disrupting effect of a French press (16) by use of a refrigerated French pressure cell at 138 MPa for 5 min (one run). The unbroken cells were discarded by centrifugation (3,000 × *g*, 40 min). The supernatant was centrifuged at 40,000 × *g* for 40 min, and the pellet containing the crude cell envelopes was washed twice in cold sterile distilled water. The final crude cell envelopes were stored at −18°C.

In order to prepare SDS-treated walls, a frozen or fresh crude cell envelope pellet (20 mg [dry weight]) was resuspended in 100 μl of distilled water and the suspension was added to 2 ml of boiling SDS (4.5% [wt/vol]). The resulting suspension was maintained at 100°C for 1 h, left for one night at room temperature, and then subjected to centrifugation at 50,000 × *g* for 1 h. The pellet corresponding to the SDS-treated walls was washed four times in cold distilled water and stored at −18°C.

Quantitative amino acid and hexosamine (muramic acid) analyses were performed with a Beckman 119 CL analyzer after hydrolysis of samples in sealed tubes with 5.7 M HCl at 95°C for 16 h (these conditions were necessary to avoid degradation of muramic acid) (46). The ratio of muramic acid to total dry weight allows the estimation of the purity of the SDS-treated wall preparation.

Induction of whole-cell autolysis in buffered solution. Whole cells were har-

vested by centrifugation (7,000 × *g*, 20 min) (growth phases are indicated below), washed once in sterile cold distilled water (7,000 × *g*, 20 min), and resuspended (0.38 mg [dry weight] · ml^{-1}) in 0.1 M potassium phosphate (pH 5.8) at 40°C. Autolysis was monitored by the decrease in OD_{650}. The extent of autolysis was expressed as the percent decrease in OD_{650} after 2 h.

Partial purification of the autolysins. (i) Extraction from whole cells with lithium chloride. Exponential-phase whole cells (OD_{650} between 1 and 2 corresponding to 0.4 to 0.8 mg [dry weight] · ml^{-1}) of *L. helveticus* ILSC5 were washed once and suspended in 1 M LiCl (except when otherwise indicated in the text below) (4 mg [cellular dry weight] · ml^{-1} of LiCl) and stirred gently for 30 min at 4°C. The suspension was centrifuged at 7,000 × *g* for 20 min; the supernatant was recovered and named crude extract.

(ii) Preparation of S/P1M. The crude extract was dialyzed overnight at 4°C against distilled water (Spectrapor molecular porous membrane tubing; 6,000- to 8,000-molecular-weight cutoff). A white precipitate which was recovered by centrifugation (40,000 × *g*, 45 min) appeared during this dialysis. The pellet was named P1M, and the supernatant was named S1M. P1M was stirred gently in 1 M LiCl again (volume 2.5 times smaller than the first volume of LiCl used for the extraction from whole cells) for 15 min at room temperature. The remaining insoluble pellet was recovered by centrifugation (40,000 × *g*, 45 min) and named P/P1M; the supernatant was named S/P1M.

(iii) Preparation of the E fraction. Gel filtration of S/P1M (120 μg of total protein) was carried out with a Superose HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated with 1 M LiCl–50 mM Tris-HCl (pH 8.6) at a flow rate of 0.5 ml · min^{-1}. Fractions were collected and then concentrated to dry state by speed vacuum centrifugation before the assay for lytic enzyme activity and zymogram analysis.

Assay for lytic enzyme activity against *Micrococcus luteus* cells. Portions (100 μl) of the autolysin-containing extracts (crude extract, S/P1M, and E) were added to 1.4 ml of an *M. luteus* cell suspension (0.2 mg [dry weight] of lyophilized *M. luteus* provided by Sigma · ml^{-1} in 0.1 M potassium phosphate at pH 5.8) in thermostated (40°C) and stirred cuvettes of the spectrophotometer. Autolysis was monitored for 2 h. One unit of enzyme activity is defined as the amount of enzyme necessary to allow a decrease in the OD_{650} of the *M. luteus* suspension of 0.001 U/min at 40°C (1 U of enzyme activity equals 0.001 U of OD_{650} min^{-1}).

SDS-PAGE and renaturing SDS-PAGE (Zymogram). SDS-PAGE was carried out as described by Lortal et al. (25) with an SDS–14% polyacrylamide separating gel (pH 8.8) and a constant voltage (180 V) at room temperature. Renaturing SDS-PAGE was performed according to the methods of Potvin et al. (40) and Foster (15), with some modifications. SDS-polyacrylamide separating gel (14% acrylamide, pH 8.8) containing 0.2% (wt/vol) lyophilized *M. luteus* cells (except when otherwise indicated in the text below) was used to detect the lytic activities. After electrophoresis (1 h, 180 V, constant voltage at room temperature), the gels were soaked for 30 min in distilled water at room temperature. The gels were then transferred into the renaturing buffer (50 mM Tris-HCl [pH 8.0] containing 1% [vol/vol] Triton X-100) and shaken gently for 2 h at 40°C to allow renaturation.

The renatured autolysins appeared as clear translucent bands on the opaque background. The contrast was enhanced by staining the gels in 0.1% (wt/vol) methylene blue in 0.01% (wt/vol) potassium hydroxide.

For SDS-PAGE or zymogram assay, the samples were prepared as follows. For whole-cell SDS extract and crude cell envelope SDS extract, the wet pellet (corresponding to 4 mg [dry weight]) of washed whole cells (or cell walls) was resuspended in 100 μl of Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol) (24). The suspension was mixed gently, heated for 2 min at 100°C, and centrifuged (7,000 × *g*, 10 min). The supernatant, i.e., the whole-cell SDS extract or the crude cell envelope SDS extract, was analyzed by SDS-PAGE for protein content and by zymogram assay to detect proteins showing lytic activity. For autolysin-containing extracts (CE, P/1M, S/P1M, and fraction E), the liquid extracts (CE, S/P1M, and E) were mixed, 1:1 (vol/vol), with Laemmli buffer and heated for 2 min at 100°C. The solid extracts (P1M) were directly resuspended in Laemmli buffer, heated (2 min, 100°C), and centrifuged before being loaded on the gels.

In order to determine the apparent molecular weight, the standards provided by Pharmacia were used (phosphorylase *b* [94 kDa], bovine serum albumin [67 kDa], ovalbumin [43 kDa], carbonic anhydrase [30 kDa], soybean trypsin inhibitor [20.1 kDa], and α-lactalbumin [14.4 kDa]). One lane was systematically loaded with the standards in all the gels for SDS-PAGE as for renaturing SDS-PAGE; in the latter case, the protein standards (except the soybean trypsin inhibitor and the α-lactalbumin) precipitated spontaneously in the gels during the renaturation step and were therefore visible. No difference in the migration of the standards due to the presence of cells of *M. luteus* in the gels was noted.

Photographs of the gels were taken by using a luminous table; the translucent bands appeared as white bands on the dark background. The results shown in the figures are representative, and all the observations were confirmed in at least two separate experiments.

Protein assay. The protein content in the autolysin-containing samples was determined by the Bradford procedure (7) using the Bio-Rad protein assay kit, with bovine serum albumin (Sigma) as a standard.

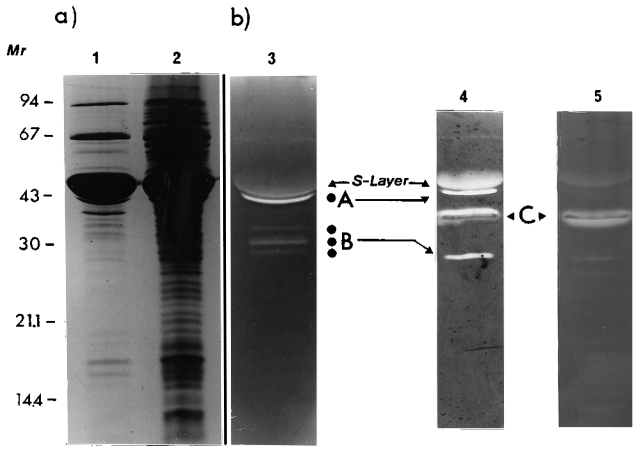

FIG. 1. (a) Protein profile of whole-cell SDS extract (lane 1) and crude cell envelope SDS extract (lane 2). (b) Activity profile of whole-cell SDS extract visualized by using gel containing 0.2% (wt/vol) lyophilized cells of *M. luteus* (lane 3) or SDS-treated walls of *L. helveticus* ISLC5 prepared from frozen crude cell envelope material (lane 4) or from fresh crude cell envelope material (lane 5) as a substrate. The apparent molecular masses (*Mr*; in kilodaltons) of standards are indicated on the left. Autolysins (black points) formed translucent areas in the gels and appear as white bands in the photographs. Some proteins (for example, the S-layer-forming protein) precipitated in the gel and thus appear as white bands in the photographs, but they are not at all translucent and cannot be confused with autolysins.

RESULTS

Zymogram of the autolysins of *L. helveticus* ISLC5. In order to visualize the lytic enzymes after denaturing polyacrylamide gel electrophoresis as previously described (40), several parameters had to be optimized: (i) the substrate included in the acrylamide network (*M. luteus*, whole cells, cell walls, or peptidoglycan of the species considered); (ii) the nature of the sample loaded, which should contain autolysins; and (iii) the electrophoretic and renaturing conditions.

Portions (5 μl) of whole-cell SDS extract and of crude cell envelope SDS extract of *L. helveticus* ISLC5 were analyzed by SDS-PAGE and by zymogram assay. The zymogram, made with *M. luteus* as the substrate, revealed two translucent zones, corresponding to the lytic activities, after 1 h of incubation at 40°C in the renaturing buffer (Fig. 1). The first translucent zone, labelled A, was narrow and completely transparent; its apparent molecular mass was in the range of 40 kDa. The other was composed of several lytic bands of 29 to 34 kDa. Since the exact number of bands was not easy to determine at this stage, all these activities were labelled B. These B bands were more diffuse than the band of activity A and were not completely transparent, revealing partial lysis of the substrate *M. luteus*. In addition, the aspect of this second lytic zone, zone B, can be slightly variable from one gel to another (with regard to intensity, width, and apparent number of lytic bands). It may be worth noting that the S-layer-forming protein, which constitutes the outermost proteinaceous layer of the cell wall (26, 29), was also extracted by the treatment and that it formed a white precipitate in the gel during the renaturation step and thus was visible in the gel photographs. At this stage, an accurate determination of the apparent molecular weight of the lytic activity A was not possible, because of the presence of the predominant S-layer protein activity just above it.

No differences between the crude cell envelopes and the whole-cell extracted samples as regards the profile of lytic activities were noted (data not shown). The protein profile of the crude cell envelope was more complex than those obtained

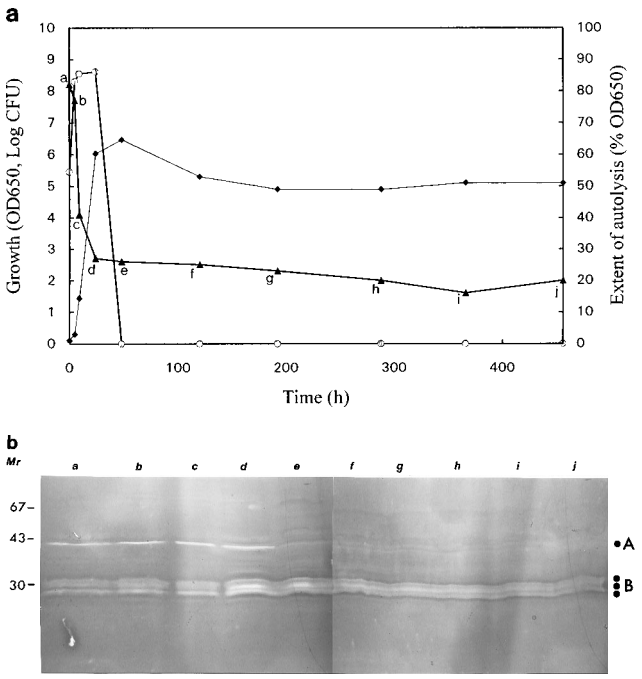

FIG. 2. Variation of the autolytic activity in buffered solution (a) and of the activity profiles in zymogram assays (b) of cells harvested at different times (a to j) of a 450-h growth of *L. helveticus* ISLC5. (a) Growth was monitored by OD_{650} (◆) and by viability (log CFU [◎]). Samples a to j were harvested and suspended in a potassium phosphate buffer (0.1 M, pH 5.8, 40°C) to induce their autolysis; the extent of autolysis was estimated as the percent decrease in the OD_{650} of the suspension after 2 h (▲). (b) Parallelly, identical quantities of cells for each lane were submitted to zymogram assay. Black points, autolysins.

with whole cells, suggesting that the cells are not lysed by the treatment and that perhaps only surface proteins were removed (Fig. 1a).

In order to test whether another substrate would reveal additional lytic enzymes, heated whole cells (100°C, 30 min) of *L. helveticus* ISLC5 as well as SDS-treated walls of this strain were incorporated into the gel (2 mg [dry weight] per gel). The profile obtained with whole cells of ISLC5 as the substrate was the same as that obtained with *M. luteus* as the substrate (data not shown), and this result puts forward the idea that activities A and B corresponded to autolysins of ISLC5. When SDS-treated walls prepared from frozen crude cell envelope material (ratio of muramic acid to total dry weight = 266 nmol/mg) were used, a third lytic zone at 37.5 kDa (named C) was clearly observed (Fig. 1b, lane 4). As this additional activity was not able to provoke the lysis of the cells of *L. helveticus* ISLC5, it was concluded that it is a peptidoglycan hydrolase and not an autolysin (45). Moreover, when these SDS-treated walls were used as a substrate, lytic zone B appeared as a single transparent and relatively narrow band at 29 kDa. When the SDS-treated walls were prepared from fresh crude cell envelope material, the ratio of muramic acid to total dry weight was higher (630 nmol/mg), indicating better purity of the preparation. Interestingly, when these SDS-treated walls were used, autolysins A and B were not detected and only the 37.5-kDa peptidoglycan hydrolase was observed (Fig. 1b, lane 5). As *M. luteus* was a convenient and standardizable substrate for the detection of autolysins A and B, it was used for all subsequent zymograms.

Temporal distribution of the autolysins. A growth curve of *L. helveticus* ISLC5 was established by both OD_{650} and viability measurements (Fig. 2a). Samples were removed at various times (a to j as indicated in Fig. 2a), and the cells were harvested by centrifugation, washed once in distilled water, frozen, and used later for the zymogram assay of autolysins (Fig. 2b). Equivalent quantities of cells were used for all lanes, and clear differences between the profiles depending on the age of the culture were observed. In addition, samples of cells (a to j) were suspended in potassium phosphate (0.1 M; pH 5.8) at 40°C, which was previously described as being efficiently able to induce autolysis of *L. helveticus* (27). The induced autolysis was estimated by the decrease (percent) in OD_{650} after 2 h (Fig. 2a).

Autolysin A appeared to be associated with the viable cells, but as soon as the cells died, the autolysin A disappeared. In contrast, the autolysin(s) B remained associated with the envelope of dead cells but was no longer able to provoke extensive autolysis as demonstrated (i) by the small extent of induced autolysis when the cells of samples e to j were transferred into buffer and (ii) by the absence of a significant decrease in OD_{650} between 100 and 450 h of growth. Nevertheless, the light microscopic observations of the culture (samples d to j) clearly revealed "ghost cells," indicating a progressive loss of cytoplasm without a bursting of the cells. As the cell wall fraction is partly responsible for the optical density, this kind of autolysis does not lead to a significant decrease in OD_{650}.

The temporal distribution of the autolysins was analyzed more precisely during the exponential growth phase. Again the cells were harvested at different times (samples 1 to 8 in Fig. 3a). After being kept overnight at 4°C, the cell pellets were subjected to zymogram assay. They were parallelly suspended in phosphate buffer to measure the extent of induced autolysis. The largest extent was reached with cells harvested at the beginning of the exponential growth phase (sample 1). Nevertheless, a second optimum (samples 4 to 6), corresponding to the transition between the exponential and the stationary phases, was detected. From the zymogram assays (Fig. 3b), it could be noted that this second optimum corresponded to an increase in the intensity of some of the autolysins B.

Extraction of the autolysins with lithium chloride. Lithium chloride has been previously used to extract cell wall-associated proteins in *L. helveticus* and in particular the S-layer-forming protein (29). In addition, this chemical agent has been successfully used to extract autolysins of various species such as *Clostridium perfringens* (55), *Bacillus subtilis* (8, 42), and *Streptococcus faecium* (9, 38).

Whole cells of *L. helveticus* ISLC5 (OD_{650} = 1.7) were suspended in various molarities (0.5, 1, and 5 M) of lithium chloride. The residual autolysins of treated cells were quantified by induction of whole-cell autolysis in buffered solution (Fig. 4a) and were analyzed by zymogram assay (Fig. 4b).

The results revealed that a 0.5 M LiCl solution was unable to extract the autolysins of *L. helveticus*, and 0.5 M LiCl-treated and untreated cells showed identical autolysin profiles (Fig. 4b) as well as similar whole-cell induced autolysis in buffer (Fig. 4a). In addition, no lytic bands appeared in the zymogram assay for the 0.5 M crude extract (data not shown). In contrast, 1 and 5 M LiCl efficiently extracted autolysins directly from the cells. Nevertheless, as shown by Fig. 4, this extraction was never complete. The level of residual autolytic activity decreased concomitantly with the increase in LiCl molarity, but the difference between 1 and 5 M was not as high as expected. Moreover, a 5 M LiCl solution extracted many more proteins from the cell envelope, giving a more contaminated crude extract (data not shown). For this reason, the extraction of

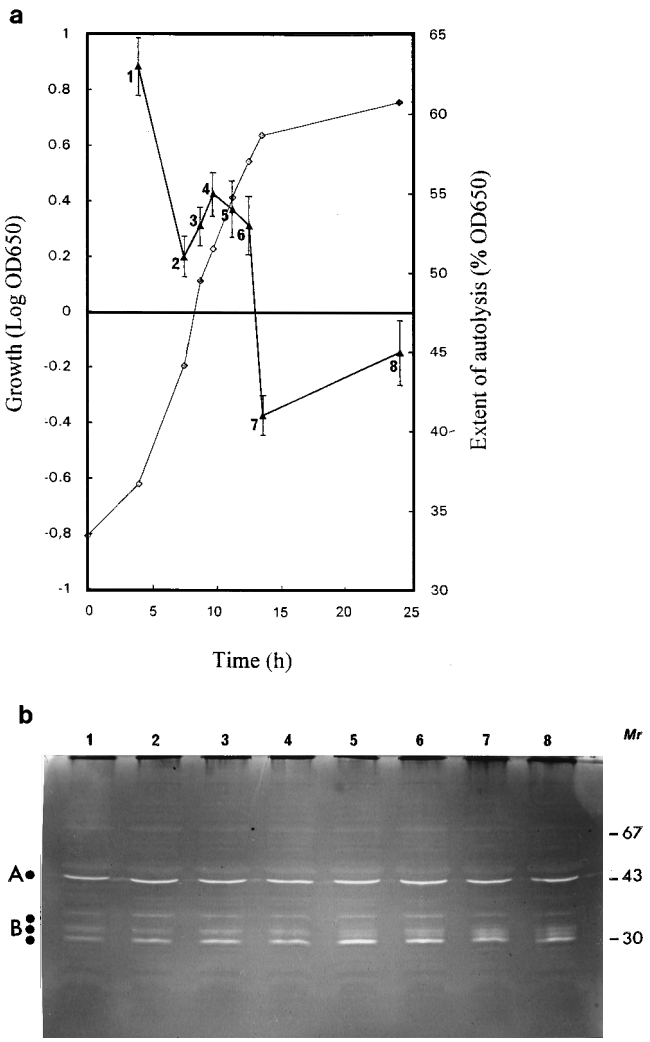

FIG. 3. (a) Exponential growth phase of *L. helveticus* ISLC5 (◆) and concomitant evolution of the autolytic activity (▲) of cells harvested at time points 1 to 8. For autolytic activity determinations, each experiment was performed three times; vertical bars represent the mean variation observed for each time point. (b) Activity profiles of samples 1 to 8 visualized by using whole-cell SDS extracts of identical quantities of cells for all lanes. Black points, autolysins.

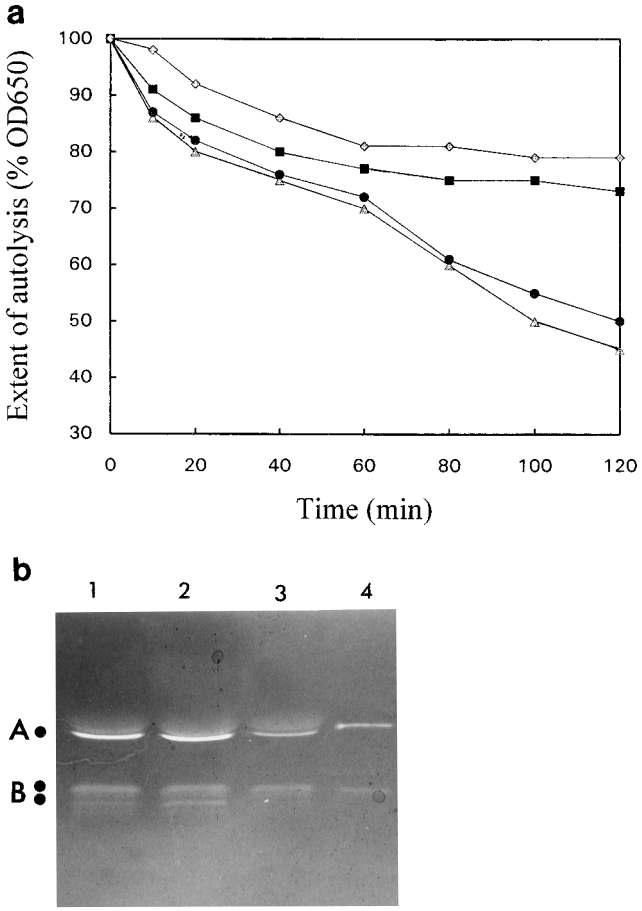

FIG. 4. Extraction of the autolysins of *L. helveticus* ISLC5 (OD_{650} = 1.7) by LiCl treatment. The residual autolytic activity of the treated cells was detected by induced autolysis at 40°C in 0.1 M potassium phosphate buffer (pH 5.8) (a) and by zymogram assay (b). Results for untreated cells (△; lane 1), 0.5 M LiCl-treated cells (●; lane 2), 1 M LiCl-treated cells (■; lane 3), and 5 M LiCl-treated cells (◆; lane 4) are shown.

autolysins was carried out with 1 M LiCl for all subsequent steps.

Considering previous work (29), the presence of some S-layer-forming protein in this 1 M LiCl crude extract was expected, and this presence was confirmed by the extract's protein profile (data not shown).

Partial purification of the autolysins and molecular mass determination. The 1 M crude extract was dialyzed overnight against distilled water. The white precipitate (P1M) observed was harvested by centrifugation. The supernatant (S1M) was dried under vacuum. The autolysins, as well as the S-layer-forming protein, were mainly recovered from the P1M sample (data not shown). At this stage, the diffuse activity B appeared to be more clearly composed of at least four similar bands of lytic enzymes. The presence of the structural S-layer protein just above the autolysin A limited an accurate determination of its apparent molecular weight. The precipitated S-layer-forming proteins are usually hard to resolubilize. Taking this property into account a second attempt to selectively resolubilize the autolysins was made by once more suspending the P1M in 1 M LiCl. After 30 min, the remaining insoluble fraction was recovered by centrifugation. The pellet (P/P1M) contained the S-layer-forming protein, some autolysin A, and a low level of autolysin B. The supernatant (S/P1M) contained autolysins A and B and very little S-layer-forming protein (Fig. 5). The apparent molecular masses of the autolysins were determined by using an S/P1M sample; the values obtained were 42.4 kDa for autolysin A and 29.1, 29.6, 30, 30.8, 31.7, and 32.8 kDa for autolysins B1 to B6, respectively.

Molecular masses were determined directly on the gel containing *M. luteus* cells. An attempt to correlate the lytic bands with the protein bands was made (Fig. 5), and this seemingly showed that the autolysins corresponded quantitatively to minor proteins of the S/P1M profile.

S/P1M, which can be easily obtained, was further used to determine the influence of some parameters (preparation of the sample and pH of the renaturing buffer) on the autolysin profiles obtained. The profile was not modified by heating (2 min, 100°C) of the S/P1M in Laemmli buffer without SDS and without 2-mercaptoethanol or by the absence of heating in complete Laemmli buffer (data not shown). In addition, several lanes were loaded with equivalent quantities of S/P1M; after electrophoresis, the lanes were cut and incubated in var-

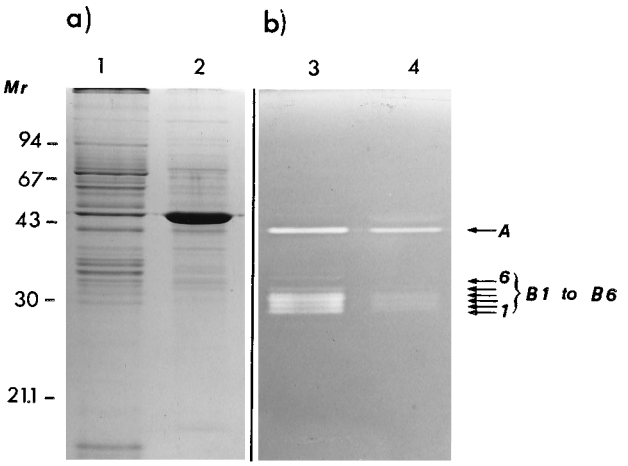

FIG. 5. Protein profiles (a) and zymogram (b) showing the selective resolubilization of the autolysins from the P1M precipitate which was obtained upon dialysis of the crude extract. P1M was shaken gently in 1 M LiCl, and the remaining insoluble pellet (P/P1M; lanes 2 and 4) contained the S-layer protein and some autolysin A; the supernatant fraction obtained (S/P1M; lanes 1 and 3) contained autolysins A and B (B1 to B6) and very little S-layer protein.

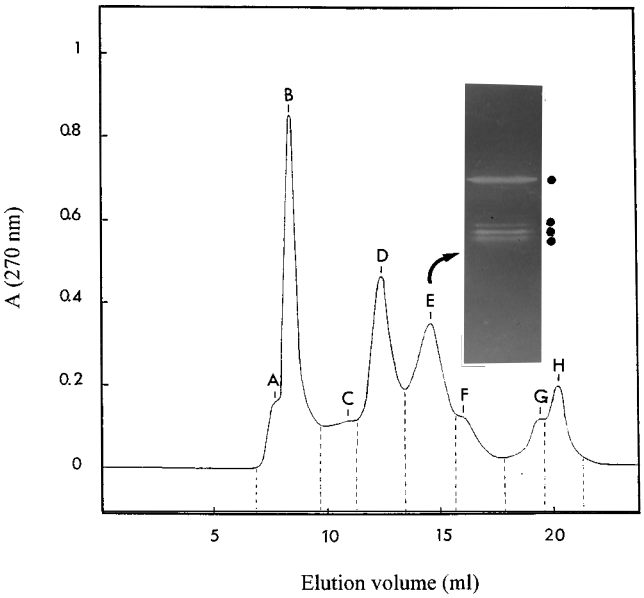

FIG. 6. Gel filtration of S/P1M. Five hundred microliters (120 μg of total protein) was loaded on a Superose HR10/30 column equilibrated with 1 M LiCl–50 mM Tris-HCl buffer (pH 8.6); the elution rate was 0.5 ml/min. The elution profile and zymogram assay results for fraction E are shown; the other fractions (A, B, C, D, G, and H) were devoid of lytic activity. Black points, autolysins.

ious 50 mM renaturing buffers (pH 4.0 to 9.0). The results are summarized in Table 2. Autolysin A can be renatured at pH 4.0 to 9.0, with an apparent preference for basic buffers. By contrast, the autolysins B were able to renature efficiently only at pH 6.0 to 9.0, with a clear preference for basic buffers. At pH 6.0, an inhibitory effect of citrate on the renaturation of the autolysins B was noted.

Finally, serial dilutions of S/P1M were analyzed by zymogram assay to quantify the high sensitivity of the method; a total quantity of protein loaded as low as 40 ng was sufficient to detect autolysins A and B (data not shown). Since the autolysins B represented much less than 10% of this protein content (percentage value estimated from Fig. 5), it was concluded that less than 100 pmol of autolysin can be detected in this way.

In order to further purify the autolysins, the S/P1M was submitted to gel filtration on a column equilibrated with 1 M LiCl–50 mM Tris-HCl (pH 8.6). Autolysin A and some of the autolysins B were recovered from one fraction (E) (Fig. 6). A 20-fold increase in specific activity was obtained, as summarized in Table 3.

Heat resistance of the extracted autolysins. S/P1M samples were heated at 100°C for 15, 30, or 60 min and analyzed by zymogram assay. Surprisingly, 60 min at 100°C was not sufficient to completely and irreversibly denature all the autolysins, although a clear reduction in intensity was perceptible. The autolysin A and one of the autolysins B (30 kDa) showed exceptional heat resistance, since they were present on the zymogram after a 60-min treatment of the S/P1M at 100°C (data not shown). The lytic activity of the S/P1M, quantified against *M. luteus* cell suspension, was reduced two- and threefold by heating of the S/P1M at 100°C for 30 and 60 min, respectively (data not shown).

Autolysin zymograms of 30 other strains of *L. helveticus*. In order to test whether the autolysin profile of *L. helveticus* ISLC5 was representative of the species as a whole, the cells of 30 other strains were harvested in the exponential growth phase (OD_{650} between 1.0 and 1.5), washed once in distilled water, and submitted to zymogram assay (Fig. 7; Table 1). Interestingly, the profiles obtained were similar to those obtained for *L. helveticus* ISLC5 in terms of the apparent molecular weights of lytic activities A and B. The fact that the strains were isolated in different countries (Table 1) suggests that autolysins are well-conserved enzymes. Some variations between strains regarding the respective intensities of the A and B bands were noted.

Comparison of the autolysin(s) with the lysin of bacteriophage 832-B1 infecting *L. helveticus*. Concentrated crude lysate of bacteriophage 832-B1 propagated in *L. helveticus* CNRZ 223 was obtained as described in Materials and Methods. A 10-μl sample was analyzed by the zymogram assay concomitantly with 10 μl of an S/P1M sample. One band of activity was

TABLE 2. Effects of various buffers on the renaturation of autolysins A and B of *L. helveticus* ISLC5[a]

Nature (pHs) of the renaturing buffer	Intensity level of lytic bands[b]	
	Autolysin A	Autolysins B
Sodium citrate (4.5, 5, 5.5, 6)	++	(+)
Potassium phosphate (6, 6.5, 7, 7.5)	++	+
Tris-HCl (7.5, 8, 9)	+++	+++

[a] All buffers contained 1% (vol/vol) Triton X-100.
[b] (+), not clearly detected; +, low intensity; ++, middle intensity; +++, high intensity.

TABLE 3. Purification of the autolysins of *L. helveticus* ISLC5

Purification step	Activity (U)[a]	Amt of protein (mg)	Sp act	Purification (fold)
Crude extract[b]	74	1.485	50	1
S/P1M	14	0.139	100	2
E	29	0.029	1,000	20

[a] U, units of enzyme activity.
[b] Crude extract was obtained from 54 mg (dry weight) of cells.

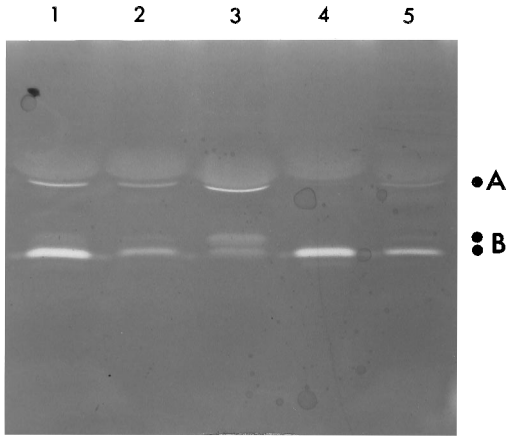

FIG. 7. Representative zymograms of whole-cell SDS extracts of several strains of *L. helveticus* (Table 1). Lanes: 1, CNRZ 223^T; 2, CNRZ 834; 3, CNRZ 1102; 4, CNRZ 303; 5, CNRZ 493. Black points, autolysins.

observed; its molecular mass (31.2 kDa) was very close to those of the autolysins B of *L. helveticus* ISLC5 (Fig. 8).

DISCUSSION

By use of a zymogram assay (renaturing SDS-PAGE), the autolysins of *L. helveticus* were analyzed for the first time in this work. The autolytic system of *L. helveticus* ISLC5 is apparently composed of several enzymes; seven reproducible bands were observed, one at 42.4 kDa named autolysin A and six (B1 to B6) at 29.1 to 32.8 kDa named autolysin(s) B. Nevertheless, the exact number of autolysins is difficult to assess, because (i) the zymogram assay revealed only SDS-stable autolysins and (ii) the four autolysins B1 to B4 have very similar apparent molecular masses and similar properties (with regard to renaturing pH and temporal distribution), leading to the hypothesis that they could be isoenzymes or several proteolytically processed forms of the same autolysin. Indeed, proteolysis of autolysins has been described in previous works as a way of regulation (39). However, since autolysin A and autolysin(s) B have different temporal distributions, different molecular masses, and different behavior regarding the pH of renaturation, it can be reasonably assumed that they are distinct enzymes. From these remarks it can thus be concluded that the autolytic system of *L. helveticus* is composed of at least two autolysins, with apparent molecular masses in the ranges of 30 and 42.4 kDa, respectively. In previous works, the number of autolysins revealed by using renaturing SDS-PAGE as described in this work was highly variable, from 17 for *E. coli* to 7 for *B. subtilis* and 3 for *Enterococcus hirae* (4, 25).

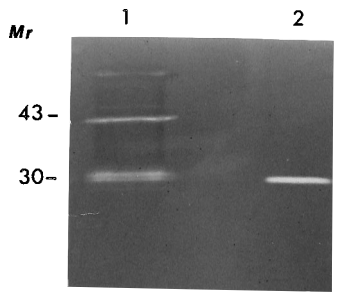

FIG. 8. Zymogram assay for comparison of the autolysins of *L. helveticus* ISLC5 contained in the S/P1M sample (lane 1) and the lytic activity contained in the concentrated crude lysate of bacteriophage 832-B1 (propagated in *L. helveticus* CNRZ 223).

The autolysin profile of strain ISLC5 was not modified when the preparation of samples was carried out without β-mercaptoethanol in the Laemmli buffer. This fact suggested that all these SDS-stable autolysins do not possess a disulfide bridge.

In addition, a peptidoglycan hydrolase with an apparent molecular mass of 37.5 kDa was detected in this system. In this work, two separate SDS-treated wall preparations with different muramic acid-to-dry weight ratios were incorporated into the acrylamide gels for the renaturing SDS-PAGE. By using SDS-treated walls with a ratio of 266 nmol/mg, autolysin A, one of the autolysins B, and the peptidoglycan hydrolase were detected. When SDS-treated walls with a higher peptidoglycan content (ratio of muramic acid to dry weight = 630 nmol/mg) were used, only the peptidoglycan hydrolase was detected. This may indicate that the autolysins of *L. helveticus* ISLC5 need non-covalently-bound cell envelope components to hydrolyze their substrates or at least to renature within the acrylamide gel.

Interestingly, the autolysin profiles of 30 other strains of *L. helveticus*, of various geographical origins and involved in different technological processes, were identical to the autolysin profile of strain ISLC5.

A concentrated crude lysate obtained after the propagation of the virulent bacteriophage 832-B1 in *L. helveticus* CNRZ 223 showed, in the zymogram assay, a lytic activity of 31.2 kDa. This activity may be the phage lysin; in this case its molecular mass would be very close to those of the autolysin(s) B. This preliminary observation has to be regarded in a more general view of lytic enzymes; indeed, García et al. (17) and Romero et al. (43) found homology between the phage lysin and one of the host autolysins of *Streptococcus pneumoniae* and therefore suggested an evolutionary relationship between these two groups of lytic enzymes. Nevertheless, the hypothesis that this 31.2-kDa lytic activity corresponded in fact to one of the autolysins B of *L. helveticus* CNRZ 223 released in growth medium during the propagation of the phage cannot be completely excluded without further experiments.

Regarding the temporal distribution of autolysins A and B during the growth of *L. helveticus* ISLC5, autolysin A appeared to be associated strictly with viable cells. This would suggest a possible involvement in elongation and/or division of the cells (19, 20, 48), but this hypothesis related to a key role of autolysins in cell life is controversial, in particular for other systems extensively studied at the gene level (2, 13, 14).

Previous work on the autolysis of *L. helveticus* ATCC 12046 in buffered solutions has shown that the largest extent of autolysis, attributed to a β-1,4-*N*-acetylmuramidase, occurred when whole cells harvested at the transition between the exponential and the stationary growth phases were used (27). In the present study, the best time to harvest cells of strain ISLC5 and to induce its autolysis was in the early exponential growth phase (OD_{650} = 0.2), but a second optimum, during the transition between the exponential growth phase and the stationary phase (OD_{650} = 1.7) like the strain ATCC 12046 optimum, was in fact noted. Interestingly this second optimum was concomitant with an increase in intensity of some autolysins B in zymogram assays.

Further work must be done before the determination of which one(s) among these lytic enzymes corresponds to the muramidase activity described as being mainly involved in the induced autolysis of *L. helveticus* ATCC 12046 is made. Autolysins B remained associated with the cell wall after the complete loss of viability of the culture. Since autolysins could have

no positive role for the cells at this stage, a recycling role of the peptidoglycan components as well as a progressive release of cytoplasmic contents, without a drastic autolysis, can be suggested.

Autolysins A and B of strain ISLC5 were extracted efficiently and with a relative selectivity from whole cells by using 1 M lithium chloride. LiCl was used previously to extract cell wall-associated proteins such as autolysins and S-layer-forming proteins (28, 29). From the results presented here, it can be noted that about 30% of the autolysins remained associated with the cell wall even when the LiCl molarity was increased to 5 M. However, the 1 M LiCl crude extract was the starting point of several purification steps, leading to a 20-fold increase in specific activity. Nevertheless, the autolysins A and B were not separated and attempts to determine their respective specificities could not be undertaken. For this purpose the fact that the autolysin A was strictly associated with viable cells could be a valuable observation; indeed, in order to obtain a crude extract of autolysins B and to determine their specificities, the LiCl extraction could be applied only to dead cells.

Some experiments undertaken in this study highlight the exceptional resistance of the autolysins, in particular to heating, to anionic detergent (SDS), and to high ionic strength (5 M LiCl) or to several of these factors at one time. The partially purified autolysin A and one of the autolysins B (seemingly B2 [29.6 kDa]) were still active after a 60-min treatment at 100°C. A similar observation was made with a partially purified glucosaminidase of *Staphylococcus aureus* which was resistant to boiling for 15 min at pH values below 6 (51). The heat resistance of the autolysins A and B was observed here in 1 M LiCl (pH 5.8), and it should be tested at various pH values.

Concerning more particularly the cell wall of *L. helveticus*, it has been previously described as a three-layered structure (28) with an oblique paracrystalline layer (S-layer) as the outermost layer (29). It was very interesting that the extracted autolysins A and B described in this work were able to hydrolyze whole cells of *L. helveticus* included in the polyacrylamide network, demonstrating that they can pass through the S-layer to reach the peptidoglycan. Taking into account the molecular masses of these autolysins (30 and 42.4 kDa), the molecular sieving function sometimes suggested for S-layers (30) appears unlikely. Nevertheless, electrophoresis was performed under denaturing conditions (with SDS in the gel and in the running buffer) which can induce a disorganization of the S-layer network of the enclosed cells, thus allowing the crossing of the autolysins.

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