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Murein Hydrolase Activity in the Surface Layer of *Lactobacillus acidophilus* ATCC 4356[▽]

Mariano Prado Acosta, María Mercedes Palomino, Mariana C. Allievi, Carmen Sanchez Rivas, and Sandra M. Ruzal*

Departamento de Química Biológica, Facultad Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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We describe a new enzymatic functionality for the surface layer (S-layer) of *Lactobacillus acidophilus* ATCC 4356, namely, an endopeptidase activity against the cell wall of *Salmonella enterica* serovar Newport, assayed via zymograms and identified by Western blotting. Based on amino acid sequence comparisons, the hydrolase activity was predicted to be located at the C terminus. Subsequent cloning and expression of the C-terminal domain in *Bacillus subtilis* resulted in the functional verification of the enzymatic activity.

Surface layers (S-layers) have been recognized ubiquitously in both *Eubacteria* and *Archaea*. Their respective structures and functionalities have been investigated intensively in the past (3, 4). Several species of the genus *Lactobacillus* possess an S-layer. The S-layer of *Lactobacillus acidophilus* ATCC 4356 is composed of a single S-protein (S_A protein) of around 45 kDa, which has been extensively characterized by Peter H. Pouwels and coworkers (5, 6, 7, 8, 9, 23, 24, 25). The function of the S-layers of these organisms is unknown, but they may be important for bacterial adhesion to intestinal epithelial cells and extracellular matrix components (11, 12, 15, 16). *Lactobacillus acidophilus* is one of the main species of the genus *Lactobacillus* found in human and animal intestines. Several lactobacilli have been proposed to have probiotic characteristics. Probiotics are live microorganisms, usually contained in food, traditionally regarded as safe for human and animal use. When ingested in sufficient numbers, probiotics are believed to play an important role in the control of the host intestinal microbiota and in the modulation of host immune responses (26). An antagonist action of the *Lactobacillus* S-layer bearing S traits has been suggested (11, 15). Different groups have found probiotic activity in *Lactobacillus acidophilus* strains (13, 14, 15, 18), particularly in strain ATCC 4356, used in this study (12, 18, 21).

In an attempt to evaluate whether there was any cell wall polymer hydrolysis activity, purified S-layers extracted from *L. acidophilus* cells (statically grown overnight in MRS medium at 37°C) by using 6 M LiCl as described previously (6, 9) were subjected to a zymogram assay. For that purpose, 2% purified peptidoglycan (PG) was prepared from several species according to the method of Bousfield et al. (10) and was incorporated into a 12% polyacrylamide gel. Zymography was performed as described by Kakikawa et al. (17) and was used to detect the lytic activity. Gels were cast with only 0.01% sodium dodecyl sulfate (SDS). After the run, hydrolase activity was detected by a clear zone (27). We observed an intense clear band when PG from *Salmonella enterica* serovar Newport was used (Fig. 1C). The lytic band corresponds to the molecular weight of S_A protein (Fig. 1A) and was confirmed by Western blotting (Fig. 1B) with an antibody against the S-layer. A polyclonal anti-S-layer antibody was produced by injecting the protein obtained from SDS-polyacrylamide gels into a mouse and was used at a 1:10,000 dilution. A biotin-conjugated anti-mouse antibody was detected, with a second biotinylated antibody conjugated to alkaline phosphatase, by chemiluminescence using CDP-Star (GE-Biosciences).

Also, PGs from *Escherichia coli* and *Micrococcus luteus* gave positive results, whereas no activity was observed against PGs from *Bacillus cereus*, *Lactobacillus casei*, and *L. acidophilus* (data not shown). Although *L. acidophilus* ATCC 4356 has been extensively characterized by Peter H. Pouwels and coworkers (5, 6, 7, 8, 9, 23, 24, 25), this novel activity had not been visualized previously. Here we describe a true uncharacterized murein hydrolase activity associated with this S-layer, with lytic activity toward the cell walls of several *Eubacteria*. A role of murein hydrolases for an S-layer protein had previously been described only for *Bacillus anthracis* (1). This activity of the S-layer protein as a murein hydrolase is also expected for proteins sharing extensive homology with the S_A protein in the carboxy-terminal portion, such as those described by Boot et al. (8).

To compare this in vitro effect, in vivo assays with whole viable cells were also performed. Viable cells and cell walls from *Salmonella* serovar Newport were isolated, washed once with phosphate-buffered saline, and mixed with S-layer protein at the concentrations indicated. Lysis was monitored by the decrease in absorbance (optical density at 600 nm [OD_{600}]) (Fig. 2A and B). With cell wall preparations, the best lytic performance was achieved at the lowest S-layer concentration (0.02 to 0.1 mg). Reassembling of S-proteins at high concentrations may mask the lytic activity, which would explain the decrease in the lytic activity observed (Fig. 2A). With viable cells at this low S-layer concentration, no significant decrease in the OD was observed (data not shown); this may be due to the presence of lipopolysaccharides in the *Salmonella* cell wall. However, at higher S-layer concentrations (0.5 to 1 mg), lysis was obtained but took longer.

* Corresponding author. Mailing address: Departamento de Química Biológica, Ciudad Universitaria Pabellón II, 4° piso, (1428) Buenos Aires, Argentina. Phone and fax: 54 11 4576 3342. E-mail: sandra@qb.fcen.uba.ar.
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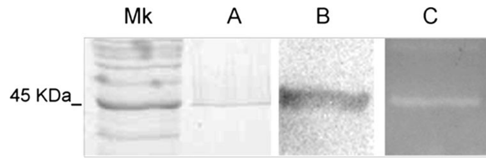

FIG. 1. SDS, Western blot, and zymography profiles of the S-layer obtained from *L. acidophilus*. (A) SDS-polyacrylamide gel electrophoresis after isolation with LiCl and staining with Coomassie blue; (B) Western blot detection with the anti-S-layer antibody; (C) zymogram of the S-layer showing lytic activity over the cell wall from *Salmonella* serovar Newport. Mk, molecular mass standard.

To determine the specificity of the lytic activity, we analyzed the newly exposed groups of the PG after hydrolysis with the S-layer protein. An increase in the number of sugar-reducing groups will be detected if the enzyme is a muramidase or a glucosaminidase, and an increase in the number of free amino groups will be detected if the enzyme is an amidase or an endopeptidase. Free amino groups released during hydrolysis of the cell walls were determined in the presence of 2,4-dinitrofluorobenzene (DNFB) and analyzed by thin-layer chromatography (TLC) as described previously (20). The lysis of the *Salmonella* serovar Newport cell walls was accompanied by an increase in the level of free amino groups; particularly, we were able to quantify the increase in the level of the dinitrophenyl-alanine (DNF-Ala) (Fig. 3), while there was no change in the number of reducing ends, determined, as described previously (20), with 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS; Molecular Probes) (data not shown). These results are consistent with the absence of activity toward the glycan strand of PG but allow us to postulate that this lytic activity works as an endopeptidase or even a PG amidase as defined by Vollmer et al. (28).

In silico sequence analysis with the BLASTp tool (2) showed that the C-terminal motif has homology with lytic enzymes of several *Lactobacillus* strains. ClustalW alignments (http://www.ebi.ac.uk/Tools/clustalw2/index.html) are shown in Fig. 4.

Primers were designed to amplify the carboxy-terminal domain of the protein, and a 968-bp amplicon was obtained, cloned into the pGEM-T Easy vector (Promega), and sequenced. Primers 5′-CAGAAAATGCAGGTAAGACTGTT

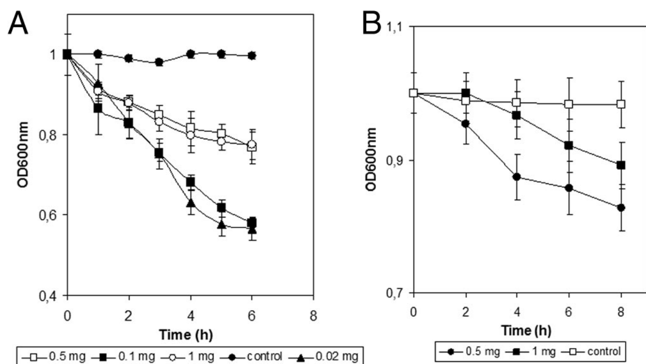

FIG. 2. Hydrolase activities against isolated cell walls and viable cells of *Salmonella* serovar Newport. Mixtures of cell walls (0.5 mg/ml) (A) or whole cells (OD_{660}, 1) (B) and the S-layer at the indicated mass were incubated, and the OD_{600} was measured.

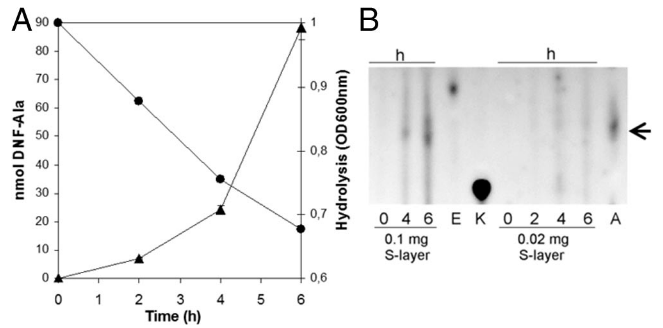

FIG. 3. Correlation between hydrolysis and increased levels of DNFB-treated free amino groups. (A) Hydrolysis with 0.1 mg of S-layer protein was followed by determination of the OD_{600} (●) and the amount of DNF-Ala (▲) by TLC and densitometry. (B) TLC analysis of hydrolysis with two S-layer concentrations and different times (in hours) as indicated. DNF-amino acids (100 nmol) are Glu (E), Lys (K), and Ala (A). The arrow indicates the position of DNF-Ala.

A-3′ (forward) and 5′-GCGGAATTCGAGCTCAGCGTTAGTGCTACGACT-3′ (reverse) were used. Complete homology with the previously reported sequence (EMBL accession number X89375) (4) was obtained. The 968-bp fragment (323 amino acids) in the pHCMC05 shuttle plasmid, kindly pro-

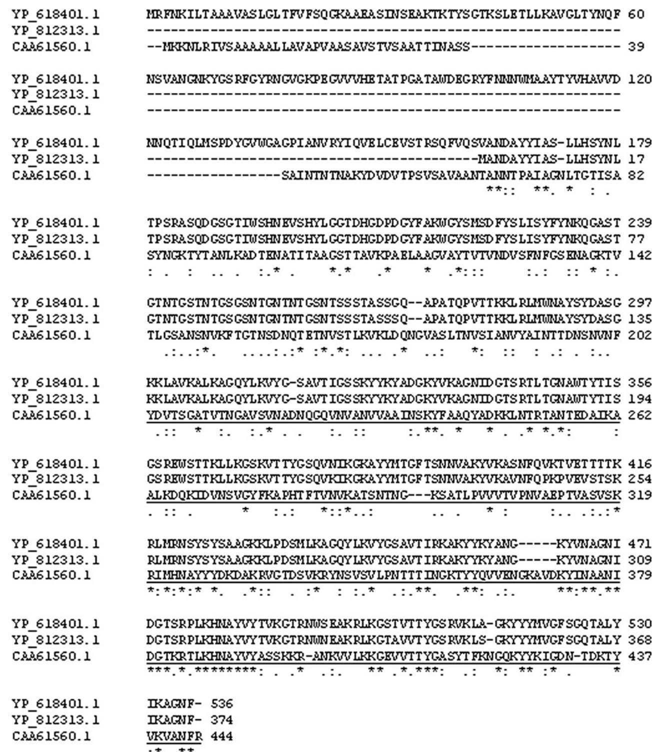

FIG. 4. Identities of the primary sequence of the S_A protein to those of other lytic enzymes analyzed by ClustalW. The cloned C-terminal portion of the S_A protein is underlined. Asterisks indicate residues identical in all sequences in the alignment; colons, conserved substitutions; periods, semiconserved substitutions. CAA61560, S_A protein (*Lactobacillus acidophilus* ATCC 4356); YP_618401, putative amidase (*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842); YP_812313, *N*-acetylmuramoyl-L-alanine amidase (*L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365).

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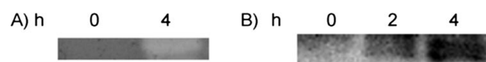

FIG. 5. Heterologous expression. (A) Zymogram of the C terminus showing lytic activity over the *Salmonella* serovar Newport cell wall. (B) Western blot detection with anti-S-layer. Times (measured in hours) after the addition of 0.5 mM IPTG are shown.

vided by the *Bacillus* Genetic Stock Center (19), was subcloned in order to express this fragment under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P*spac* promoter, allowing us to produce the pC1C2 plasmid (recombinant plasmid of pHCMC05 containing the 968-bp insert). When introduced into *E. coli* JM109 (grown in LB medium at 37°C with aeration), the plasmid showed high instability and decreased growth, particularly when IPTG was present (data not shown). The lack of success in expressing the cloned hydrolase motif in *E. coli* was certainly related to the PG structure of this host bacterium, since we found that it was a substrate for this lytic activity. This may explain the instability that other authors (9, 25) encountered when they attempted to clone the entire *slpA* gene of ATCC 4356 into *E. coli*.

Therefore, we introduced the plasmid into *Bacillus subtilis* 168 competent cells. For expression, cells were induced by the addition of 0.5 mM IPTG. *B. subtilis* was grown in LB medium at 37°C with aeration in the presence of 1 M NaCl to avoid the presence of proteases (22) that might degrade the heterologous product. To prepare for the analysis of expression by zymograms and Western blotting, cells were disrupted by sonication after harvest at the indicated times. Western blot and zymogram analyses showed the predicted 33-kDa band when the plasmid was expressed in *Bacillus subtilis* (Fig. 5A). When IPTG was added to the growth medium, a lytic band corresponding to the molecular mass of this fragment was seen; expression of this fragment was confirmed by Western blotting with an antibody against the S-layer (Fig. 5B).

Murein or PG hydrolases comprise a large family of enzymes with roles in daughter cell separation, PG turnover, autolysis, spore formation, and antibiotic-induced lysis within their own cells (27, 28, 29). However, these enzymes are also important for an adaptive response to environmental conditions that might result from an antagonistic relationship in the same ecological niche. The gastrointestinal tract will determine the coexistence of gram-negative and gram-positive species, dealing with high osmotic strength, pH gradients, anaerobic conditions, and nutrient variability, resulting in a competition for survival. The murein hydrolase activity provides S-layer-bearing strains of lactobacilli with an additional means to succeed and survive. Due to the lytic activity against whole cells of *Salmonella* serovar Newport that we observed, one might wonder if these S-layer characteristics may account for the probiotic properties of *Lactobacillus acidophilus*. In vivo assays would be required to address the question of whether the inhibition of adhesion of gram-negative bacteria and the competitive exclusion of pathogens that have been reported for S-layer-bearing strains (12, 13, 14) are associated with the antibacterial activity reported here. The precise characterization of murein hydrolase activity will be the aim of our future investigation.

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