Lytic activity of the recombinant staphylococcal bacteriophage ΦH5 endolysin active against *Staphylococcus aureus* in milk

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ABSTRACT

The endolysin gene (*lys*H5) from the genome of the *Staphylococcus aureus* bacteriophage ΦH5 was cloned in *Escherichia coli* and characterized. The *lys*H5 gene encoded a protein (LysH5) whose calculated molecular mass and pI were 53.7 kDa and 8.7, respectively. Comparative analysis revealed that LysH5 significantly resembled other murein hydrolases encoded by staphylococcal phages. The modular organization of LysH5 comprised three putative domains, namely, CHAP (cysteine, histidine-dependent amidohydrolase/peptidase), amidase (L-muramoyl-L-alanine amidase), and SH3b (cell wall recognition). In turbidity reduction assays, the purified protein lysed bovine and human *S. aureus*, and human *Staphylococcus epidermidis* strains. Other bacteria belonging to different genera were not affected. The lytic activity was optimal at pH 7.0, 37 °C, and sensitive to high temperatures. The purified protein was able to kill rapidly *S. aureus* growing in pasteurized milk and the pathogen was not detected after 4 h of incubation at 37 °C. As far as we know, this is the first report to assess the antimicrobial activity of a phage endolysin which might be useful for novel biocontrol strategies in dairying.

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1. Introduction

Staphylococcus aureus is capable of producing enterotoxins responsible for staphylococcal food poisoning, one of the most prevalent causes of gastroenteritis worldwide (Dinges et al., 2000). This pathogen is recognized as a frequent cause of subclinical intramammary infections in dairy cows (Gruet et al., 2001) and is commonly isolated from raw milk of dairy cattle suffering from mastitis. Its presence in raw milk is a major concern for the safety and quality of traditionally produced cheeses (Delbes et al., 2006; Cremonesi et al., 2007). In this context, it is relevant to develop alternative strategies to ensure the hygienic quality of dairy products.

Bacteriophage endolysins are mureolytic enzymes that directly target bonds in the peptidoglycan of the bacterial cell wall. They are encoded by the bacteriophage genome and are synthesized at the end of the phage lytic life cycle to lyse the host cell and release the newly produced virions. Besides this "lysis from within", endolysins from phages of Gram-positive hosts are also able to quickly lyse the bacteria when they are applied exogenously (Loessner, 2005). As potential antibacterials, endolysins possess several relevant features, namely, a distinct mode of action, highly specific, and active against bacteria regardless of their antibiotic susceptibility pattern (Borysowski et al., 2006). On the other hand, there is a low probability of developing resistance against the activity of bacteriophage endolysins linked to the fact that they target unique and highly conserved bonds in the peptidoglycan (Loeffler et al., 2001). Recombinant phage endolysins have been reported to inhibit a variety of pathogens, and have recently been claimed as alternative antimicrobials for treatment of bacterial infections caused by Gram-positive bacteria (Fischetti, 2003; Loessner, 2005). The effectiveness of phage lysins in clearing certain infections has been well documented in mouse models (Loeffler et al., 2001; Nelson et al., 2001; Schuch, Nelson and Fischetti, 2002; Cheng et al., 2005; Rashel et al., 2007) as well as transgenic murine and bovine mammary glands (Kerr et al., 2001; Wall et al., 2005). *S. aureus*, *Streptococcus uberis* and *Streptococcus agalactiae* bacteriophage endolysins have also been characterized to be applied in mastitis cow's treatment (Donovan et al., 2006a,b; Celia et al., 2008).

In spite of the high antimicrobial potential of phage endolysins, little has been done to assess their use for the biocontrol of pathogens in food. The heterologous production of a *Listeria monocytogenes* phage endolysin by starter lactic acid bacteria has been achieved. However, this approach was unsuccessful to effectively reduce *L. monocytogenes* growth (Gaeng et al., 2000; Turner et al., 2007). Transgenic plants carrying phage endolysins genes showed increased resistance to pathogen attack (de Vries et al., 1999).

So far, a few staphylococcal phage endolysins have been characterized such as those of phages phi11 (Wang et al., 1991; Sass and Bierbaum, 2007), Twort (Loessner et al., 1998), 187 (Loessner et al., 1999), P68 (Takac et al., 2005), phiWMY (Yokoi et al., 2005), and phage K (O'Flaherty et al., 2005) but none have been tested as a biopreservative in foodstuffs. These staphylococcal endolysins have a modular organization with

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enzymatic (D-alanylglycyl endopeptidase, L-muramoyl-L-alanine amidase, N-acetyl-glucosaminidase) and cell wall recognition domains (Navarre et al., 1999; Loessner et al., 1998).

We have isolated and characterized two staphylococcal bacteriophages, ΦH5 and ΦA72, from dairy samples which were able to inhibit *S. aureus* grown in milk and curd manufacturing processes (García et al., 2007). In this approach, we have cloned and heterologously overexpressed the endolysin gene of the bacteriophage ΦH5 in *Escherichia coli* for subsequent characterization of the lytic activity. The antimicrobial activity of the purified protein was assayed in pasteurized milk against *S. aureus*.

2. Materials and methods

2.1. Bacteria, phages and growth conditions

The bacterial strains used in this study are summarized in Table 1. Additionally, 21 *S. aureus* isolates from mastitic milks as well as 31 and 25 clinical human isolates of *S. aureus* and *S. epidermidis*, respectively, were also used in lytic assays. These clinical isolates were kindly supplied by Dr. Rodríguez and Dr. Delgado (Universidad Complutense, Madrid, Spain). *E. coli* transformants were selected with 100 μg/ml ampicillin and/or 25 μg/ml chloramphenicol, as appropriate.

Bacteriophage ΦH5 was routinely propagated on *S. aureus* Sa9 (García et al., 2007).

2.2. DNA manipulations

Plasmid DNA was obtained by the alkaline lysis method (Birnboim and Doly, 1979). Analytical and preparative gel electrophoresis of plasmid DNA and restriction fragments was carried out in 0.8% (w/vol) agarose–Tris-Acetate horizontal slab gels. Phage ΦH5 DNA was extracted and purified as described previously (García et al., 2003).

Table 1
Bacterial strains and growth conditions used in this study

Strain	Growth conditions	Observations	Source/reference
E. coli DH10B	2× YT, 37 °C, shaking	Cloning host	Invitrogen
E. coli BL21(DE3)/pLysS	2× YT, 37 °C, shaking	Expression host	Invitrogen
E. coli pUC18-LysH5	2× YT, 37 °C, shaking	*lysH5* cloning	This work
E. coli BL21(DE3)pRSET-LysH5	2× YT, 37 °C, shaking	*lysH5* expression	This work
S. aureus Sa9	2× YT, 37 °C, shaking	phiH5 host and LysH5 indicator	Laboratory collection
Bacillus cereus ATCC 9139	2× YT, 37 °C, shaking	LysH5 assay	ATCC
Streptococcus pneumoniae R6	2× YT, 37 °C, shaking	LysH5 assay	Laboratory Collection
Clostridium tyrobutiricum CECT 4012	BHI, 30 °C, anaerobic	LysH5 assay	CECT
Clostridium sporogenes LMG13570	BHI, 30 °C, anaerobic	LysH5 assay	LMG
Lactococcus lactis IPLA 947	M17, 30 °C, static	LysH5 assay	Laboratory Collection
L. lactis ssp. *lactis* biovar *diacetylactis* IPLA 838	M17, 30 °C, static	LysH5 assay	Laboratory Collection
Leuconostoc citreum IPLA 567	MRS, 30 °C, static	LysH5 assay	Laboratory Collection
Leuconostoc citreum IPLA 979	MRS, 30 °C, static	LysH5 assay	Laboratory Collection
Lactobacillus plantarum IPLA LL441	MRS, 30 °C, static	LysH5 assay	Laboratory Collection
Lactobacillus casei ATCC 393	MRS, 30 °C, static	LysH5 assay	ATCC
Listeria monocytogenes ScottA	2× YT, 37 °C, shaking	LysH5 assay	Laboratory collection
Listeria inocua CECT 910	2× YT, 37 °C, shaking	LysH5 assay	CECT
Enterococcus faecalis CNRZ 1352	2× YT, 37 °C, shaking	LysH5 assay	CNRZ

CECT: Spanish Type Culture Collection; LMG: culture collection of University of Gent, Belgium. ATCC: American Type Culture Collection; CNRZ: National Centre for Zootechnical Research.
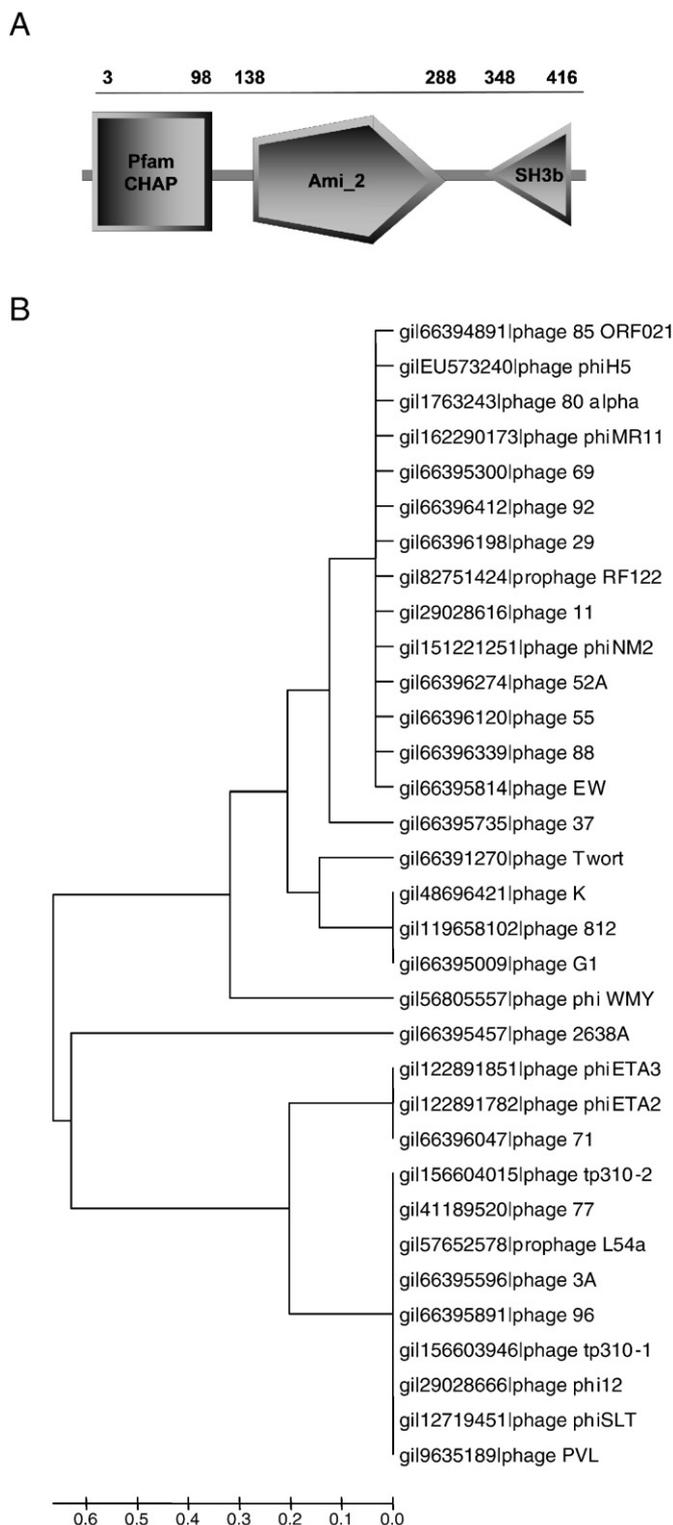

Fig. 1. Sequence analysis and phylogenetic position of the phage H5 endolysin. A) Domain organization of LysH5 as displayed by SMART (http://smart.embl-heidelberg.de) containing CHAP (cysteine, histidine-dependent amidohydrolases/peptidases), Ami_2 (*N*-acetylmuramyl-L-alanine amidase) and SH3b (bacterial cell recognition). Numbers indicate the amino acid positions in LysH5. B) Phylogenetic position of LysH5 compared to several phage endolysins. The tree was constructed using the Neighbor-Joining method. The phylogenetic tree was linearized and drawn to scale. The evolutionary distances were computed using the Poisson correction method and are expressed in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).
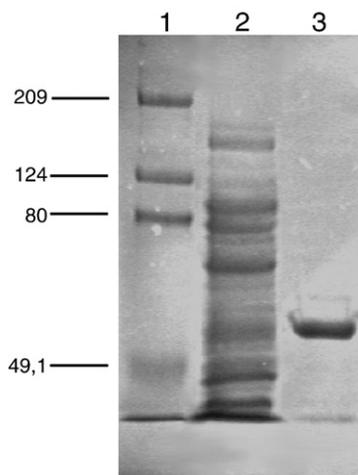
Fig. 2. Purification of the recombinant LysH5 endolysin from *E. coli* BL21(DE3)/pLys pRSET*lys*H5. Lane 1: Standard molecular weight marker in kDa (Broad Range Prestained SDS-PAGE Standards, BioRad); lane 2, supernatant of the lysate induced culture; lane 3, fraction eluted from cation exchange chromatography containing purified LysH5.
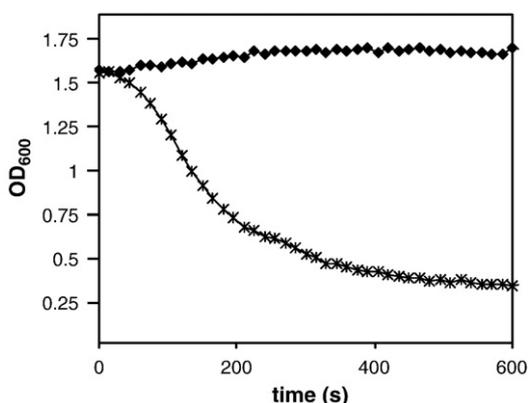

Fig. 3. Lysis of *S. aureus* Sa9 cells by exogenously added recombinant LysH5. The decrease in optical density (OD) (*y* axis) over time (*x* axis) following addition of the enzyme (5 U/ml) to a standardized cell suspensions is shown. Symbols: * *S. aureus* plus LysH5; ♦ negative control (no enzyme added).

The DNA was digested with EcoRI (Takara, Otsu, Shiga, Japan) and random fragments were cloned in pUC18 in *E. coli* DH10B. Plasmid DNA from ninety-six white colonies were extracted and analyzed. Sequences obtained were BLAST searched against the NCBI protein database.

Table 2
Temperature stability of the endolysin LysH5

Temperature (°C)	Time	% Activity
100	1 min	0
72	15 s	0
63	30 min	0
37	30 min	100±0.6
20	30 min	96.6±0.3
4	48 h	95.5±0.1
−20	30 min	67.1±0.1

Activity was determined after incubation of the protein (5 U/ml) at the temperatures and times indicated.

2.3. Cloning and overexpression of the recombinant LysH5 endolysin

A 1490-bp DNA segment containing the *lys*H5 gene was obtained by PCR amplification with the primers Ami1: 5′-ATTATGGAGGATCC-GACAATGCAAG-3′ and Ami2: 5′-GACTCACTGCAGTTTTATATTAACGT-3′ and digested with the restriction enzymes PstI and BamHI (Takara, Otsu, Shiga, Japan). The amplification product was cloned in pUC18 for sequencing and in the expression vector pRSETB (Invitrogen, Carlsbad, CA). The plasmids, pUC18-*lys*H5 and pRSETB-*lys*H5 were electroporated in *E. coli* DH10B and in *E. coli* BL21(DE3)/pLys, respectively. pRSETB-*lys*H5 construction was used to overexpress *lys*H5. Exponentially growing cultures ($OD_{600\ nm}$ of 0.6–0.8) were induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside), followed by 18 h shaking at 19 °C. Cells were pelleted, washed with lysis buffer (20 mM NaH_2PO4, 500 mM NaCl, 20 mM Imidazole, pH 7.4) and frozen at −20 °C. For protein purification, 500 ml culture cell pellets were resuspended in 10 ml lysis buffer, sonicated (15×5 s pulses with 15 s recovery on ice) and centrifuged at 10.000 ×*g*. The supernatant was added to 5 ml Ni-NTA (nickel matrix) slurry and eluted according to the manufacturer's instructions (Qiagen, Valencia, CA). Fractions containing LysH5 were dialyzed against 20 mM NaH_2PO4 buffer, pH 6.0. This sample was loaded onto a CM column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer and the protein eluted with a NaCl gradient (0 to 1 M). Protein fractions were analyzed in 15% (w/v) SDS-PAGE gels. Electrophoresis was conducted in Tris–Glycine buffer at 20 mA for 1 h in the BioRad Mini-Protean gel apparatus. The fractions containing pure LysH5 (as judged by SDS-PAGE) were pooled, diluted in glycerol (50% final concentration), and stored at −20 °C. Protein was quantified by the Quick Start Bradford Protein Assay (BioRad, Hercules, CA).

2.4. Quantification of LysH5 activity

S. aureus Sa9 strain was grown to an $OD_{600\ nm}$ of 0.5, centrifuged, and suspended in 50 mM phosphate buffer, pH 7.0, to a final $OD_{600\ nm}$
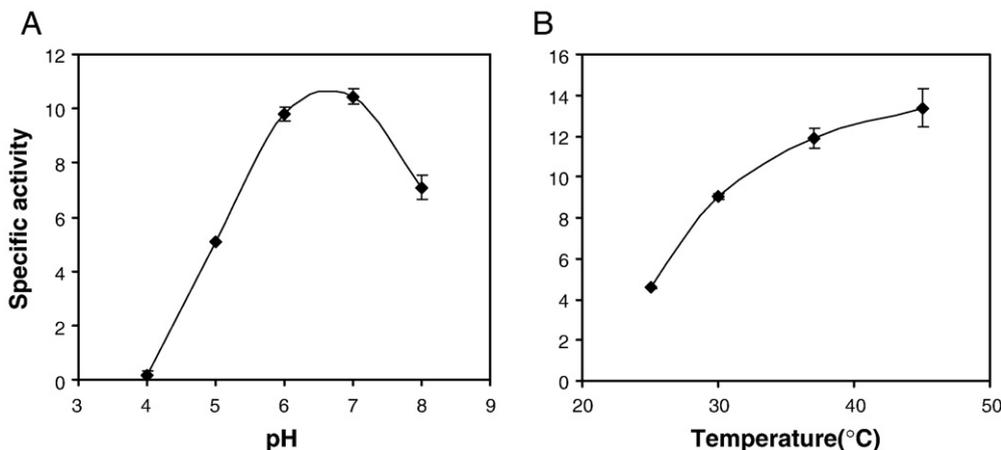

Fig. 4. Influence of pH (A) and temperature (B) on the specific activity of the ΦH5 endolysin. The endolysin (5 U/ml) was tested at various pHs in 50 mM sodium acetate (pH4–pH6) and in 20 mM sodium phosphate buffer (pH7–pH8) under standard assay conditions. Values are the mean of three independent experiments. Error bars are also shown.

of 1.5. Bacterial suspensions (0.1 ml) were added to serial dilutions of purified LysH5 (0.1 ml) in sterile, uncoated polystyrene 96-well plates, and the decrease in $OD_{600\ nm}$ was monitored every 15 s for 15 min, at 37 °C, in a Microplate Spectrophotometer Benchmark Plus (BioRad, Hercules, CA). The activity of LysH5, expressed in units per millilitre (U/ml), was defined as the reciprocal of the highest dilution that decreased the OD by 50% in 15 min. Specific activity was calculated as the change in $OD_{600\ nm}$ per mg protein per min. The lytic spectrum of LysH5 was determined in a similar fashion using 15 U/ml. The enzyme activity was determined over a pH range 4.0 to 6.0 in 50 mM Na-acetate buffer, and pH 7.0 to pH 8.0 in 20 mM Na-phosphate buffer and at temperatures ranging from 25 °C to 45 °C. Temperature stability was determined by incubation of the protein at different temperatures prior to the standard activity assay. All these experiments were performed in triplicate.

2.5. Antimicrobial activity in milk

The lytic activity of LysH5 on *S. aureus* was tested in commercial whole-fat pasteurized milk. Milk was inoculated with exponentially
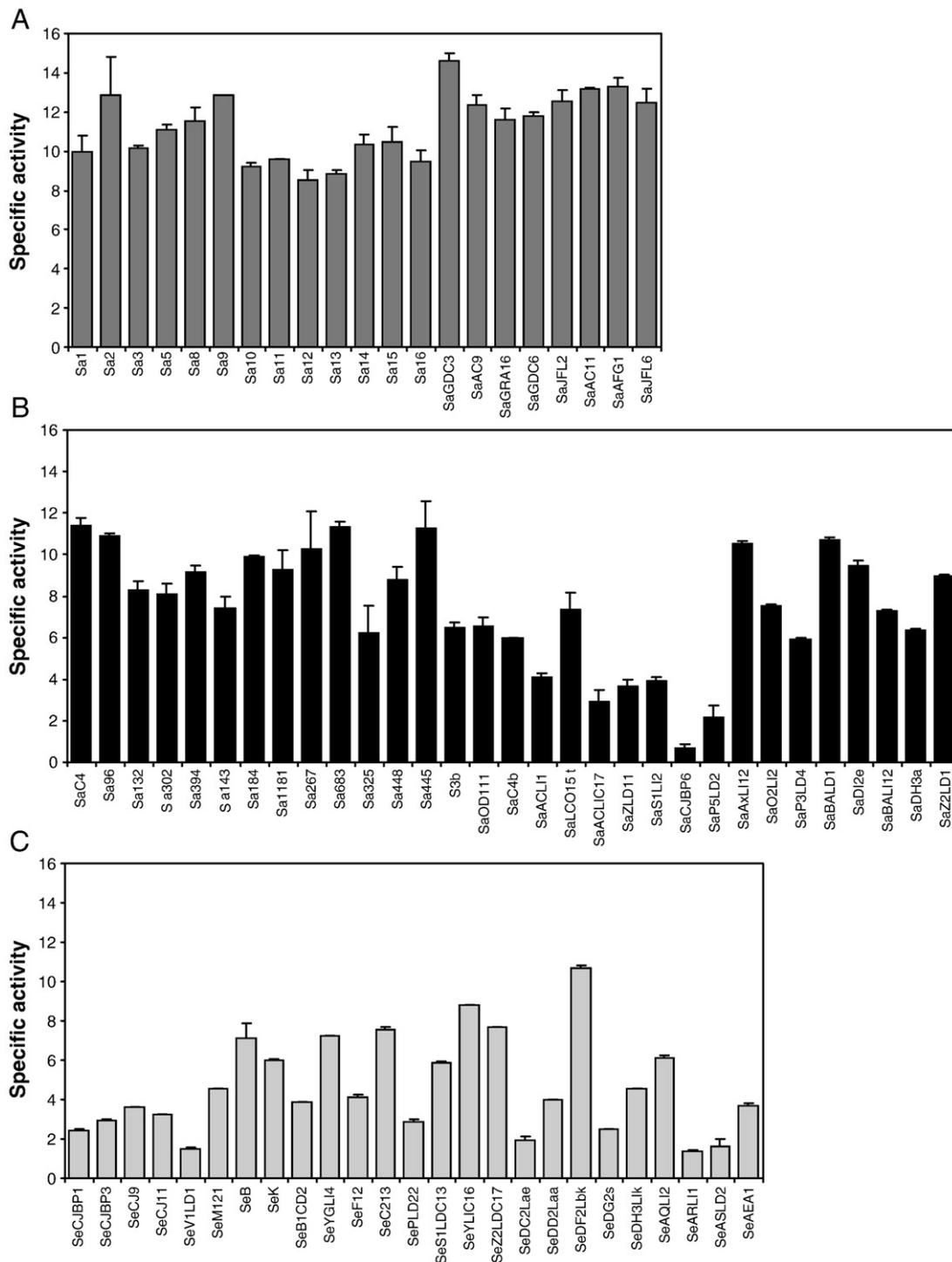

Fig. 5. Lytic spectrum of the endolysin LysH5. A) *S. aureus* bovine strains. B) *S. aureus* clinical strains. C) *S. epidermidis* clinical strains. Values are the mean of three independent experiments. Error bars are also shown.

growing cultures of *S. aureus* Sa9 (10^6 and 10^3 CFU/ml) and purified LysH5 was added at 160, 80 and 45 U/ml. The cultures were incubated at 37 °C without shaking. Samples were taken at different time intervals and scored for *S. aureus* viables on Baird Parker Agar plates supplemented with egg yolk tellurite (Scharlau Chemie, S.A. Barcelona, Spain). The absence of *S. aureus* in non-inoculated milk was verified by direct plating.

2.6. Statistical analysis

The results were compared using the one-way ANOVA analysis (SPSS 11.0 software for windows; SPSS, Chicago, IL, USA).

3. Results

3.1. Identification and sequence analysis of the bacteriophage ΦH5 endolysin

Twenty recombinant plasmids were randomly chosen from a shotgun library of ΦH5 DNA and sequenced. One of the plasmids carried a partial insert (1.5 kbp) highly homologous to a phage-related amidase encoded by the *S. aureus* RF122 prophage genome (accession number AJ938182.1). Based on the known sequence of RF122, oligonucleotides were designed to amplify the whole putative ΦH5 endolysin gene (*lys*H5) by PCR, cloned into pUC18 and sequenced (GeneBank Accession number EU573240). *lys*H5 (1446 bp) was identical to the putative endolysin gene of the *S. aureus* RF122 prophage. Analysis of the amino acid sequence (481 aa) revealed that LysH5 is a modular enzyme with three distinct domains, namely, an N-terminal CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain with hydrolytic function, a central amidase domain (*N*-acetylmuramyl-L-alanine amidase), and a C-terminal SH3b domain which might be involved in cell wall recognition (Fig. 1A). Comparative sequence analysis with other phage endolysins found in the databases indicated that LysH5 clustered together (≥97% identity) with others encoded well-characterized staphylococcal phages such as phiNM2, phi11, phi29 and phage 80 alpha (Fig. 1B). Other *S. aureus* endolysins from phage Twort, phage K, *S. warneri* phage phiWMY, phi 12, phiPVL, phiSLT were less related to LysH5.

3.2. Overexpression and characterization of the bacteriophage ΦH5 endolysin

The recombinant phage endolysin was synthesized as an N-terminally 6×-His-tagged fusion which allowed the purification by immobilized metal chelate affinity chromatography. An extra cation exchange chromatographic purification step was necessary to remove contaminants. The active fractions were pooled and analyzed by SDS-PAGE (Fig. 2). A major protein band of an estimated molecular mass of 55 kDa was observed which correlated well with the calculated molecular mass for LysH5 (53.7 kDa). Yields of 3.6 U per ml of induced *E. coli* cultures were routinely achieved with a specific activity of 1.8 U/μg. The recombinant LysH5 was able to lyse resting *S. aureus* cells. The initial $OD_{600\ nm}$ dropped to baseline within 6 min, indicating a rapid rate of cell lysis (Fig. 3).

Purified preparations were assayed at different pHs and temperature conditions. As shown in Fig. 4A, the highest specific activity was obtained at relatively neutral pH. The enzyme was slightly inactivated at pH 6.0 and significantly reduced at lower pHs. Levels of 48% and 1% activity were detected at pHs 5.0 and 4.0, respectively. The lytic activity was also temperature-dependent. The protein efficiently lysed the cells in a temperature range from 30 °C to, at least, 45 °C but decreased at lower temperatures (Fig. 4B). Stability of LysH5 was also tested under different heat treatments (Table 2). The endolysin was very sensitive to high temperatures and standard pasteurization processes, i.e. 30 min at 63 °C and 1 min at 72 °C fully inactivated the protein. After −20 °C storage without any cryoprotectans, a 32% decrease of specific activity was observed.

3.3. Lytic spectrum of the bacteriophage ΦH5 endolysin

In addition to killing the host bacterial strain, LysH5 was able to lyse all the other *S. aureus* strains irrespectively of their bovine or human origin, including those not infected by ΦH5 (Fig. 5). However, LysH5 had a significant ($p < 0.001$) different killing effect on *S. aureus* depending on the strain origin. Higher susceptibility to the endolysin was observed on *S. aureus* bovine strains with an average specific activity of 11.3 ± 1.7 while on clinical strains this value was 7.5 ± 2.9. A larger variability was also observed within the clinical strains. *S. epidermidis* isolated from humans were also sensitive although the lytic activity of LysH5 was significantly lower (4.6 ± 2.4). No lytic activity against several lactic acid bacteria and strains belonging to *Bacillus*, *Streptococcus*, *Clostridium*, *Listeria*, and *Enterococcus* was detected (data not shown).

3.4. Antimicrobial activity of LysH5 on S. aureus in milk

The effect of purified LysH5 was tested against an exponentially growing *S. aureus* Sa9 strain in milk at two contamination levels (Fig. 6). At higher contamination levels (10^6 CFU/ml), the addition of 160 U/ml (88 μg/ml) of LysH5 to pasteurized milk reduced the viable counts to undetectable levels in 4 h. The inhibitory effect of the
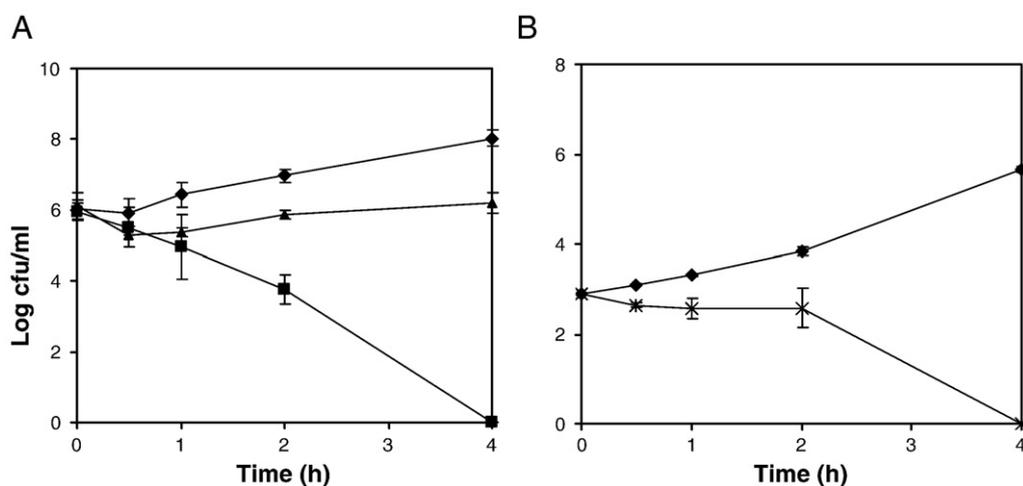

Fig. 6. Killing of *S. aureus* Sa9 with purified LysH5 in pasteurized whole milk. A) ♦, cell numbers of *S. aureus* Sa9; ■, cell numbers of *S. aureus* Sa9 plus LysH5 (160 U/ml); ▲, cell numbers of *S. aureus* Sa9 plus LysH5 (80 U/ml). B) ♦, cell numbers of *S. aureus* Sa9; X, cell numbers of *S. aureus* Sa9 plus LysH5 (45 U/ml). Values are the means of two independent experiments with standard deviation indicated by vertical bars.

endolysin was already significant ($p<0.05$) after 60 min and the counts were more than 1 log unit below the control culture. When less LysH5 was used, the inhibitory effect was only observed in the first 60 min (Fig. 6A). At lower contamination levels (10^3 CFU/ml), the addition of 45 U/ml eliminated *S. aureus* in 4 h (Fig. 6B). These results showed that LysH5 was capable of killing staphylococci which are actively multiplying in milk in these conditions.

4. Discussion

In this work, phage endolysin LysH5 was cloned in *E. coli* and the lytic activity of the purified protein was characterized. Preliminary experiments showed also that LysH5 was able to inhibit *S. aureus* growth in pasteurized milk. While a number of staphylococcal endolysin have been characterized, to our knowledge, none has been assessed as an antimicrobial additive for preventing the growth of *S. aureus* in dairy products.

LysH5 displayed a modular organization similar to other staphylococcal endolysins previously described (Navarre et al., 1999; Yokoi et al., 2005; O'Flaherty et al., 2005). According to the domains found, LysH5 should display a cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) endopeptidase activity that cleaves at D-alanyl-glycyl moieties and an amidase domain that cleaves at *N*-acetylmuramyl-L-alanyl bonds but this has not been experimentally proved yet. The SH3b domain, thought to be involved in cell wall recognition, was also detected. Several phage endolysins of Gram-positive bacteria carry a SH3b domain in their C-terminal (Sugahara et al., 2007; Donovan et al., 2006b; Porter et al., 2007). In *L. monocytogenes* phage endolysins Ply118 and Ply500 the C-terminal cell wall binding domains confer the specificity necessary to direct the murein hydrolases to the bacterial cell wall (Loessner et al., 2002; Kretzer et al., 2007).

The ΦH5 endolysin protein showed substantial similarity to those of *S. aureus* phages and even to the *S. warneri* phage phiWMY. However, in contrast to the endolysin LysWMY that exhibits lytic activity against other Gram-positive genera (Yokoi et al., 2005) LysH5 is only active against *Staphylococcus* indicating distinct cell wall recognition signals. The full-length and 182-amino-acid C-terminally truncated *S. agalactiae* bacteriophage B30 endolysins also displayed lytic activity against *Streptococcus thermophilus* and *Leuconostoc cremoris* strains (Donovan et al., 2006b). The narrow spectrum of LysH5 is of practical relevance for its deliberate use in biopreservation of dairy products. Several dairy products are fermented by lactic acid bacteria that are mostly responsible for their organoleptic properties. In this scenario, it is crucial to specifically target the undesirable bacteria, leaving the natural microbial communities undisturbed.

Although LysH5 lysed human *S. aureus* and *S. epidermidis*, its activity is remarkably lower compared to *S. aureus* of bovine origin which were highly susceptible. It appears that endolysins derived from phages isolated from the dairy environment have co-evolved with their hosts. Therefore, they would specifically target the *S. aureus* clones most commonly found in the dairy environment and would be more suitable as antimicrobials to be used in biopreservation of milk and dairy products. Nevertheless, the specific action of LysH5 on *S. aureus* and *S. epidermidis* of human origin should not preclude other potential applications as disinfectants and even in human therapy against multiple-drug-resistant *Staphylococcus.* Bacterial biofilm formation is another pathogenic factor often shown by both bacteria. Recently, it has been shown that the phi11 endolysin lyses the complex structure of staphylococcal biofilms (Sass and Bierbaum, 2007).

Our results have shown that staphylococcal phage endolysins encoded by phages of dairy origin might be useful as an additional hurdle to prevent *S. aureus* in milk and presumably in dairy products. The *in vitro* activity assays performed with purified LysH5 under several conditions of pH indicated that LysH5 could be active during the milk coagulation process but not below pH 5 as the activity was seriously compromised. The endolysin was sensitive to high tempera-

ture. Therefore, it should be added after heat treatment of milk. On the other hand, the protein remained active at 4 °C. In case of temperature abuse, the presence of the endolysin could presumably hamper *S. aureus* growth and prevent enterotoxin production. Further biochemical characterization of LysH5 is in progress to optimize and define the scope of application in dairying.

Acknowledgments

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