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

Running Title: Bacteriophage ΦH5 endolysin activity 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.

Keywords: Endolysin, natural antimicrobials, biopreservation, *Staphylococcus aureus*.

1. Introduction

Staphylococcus aureus is capable of producing enterotoxins responsible for staphylococcal food poisoning, one of the most prevalent causes of gastroenteritis worldwide (Dinges, Orwin & Schlievert, 2000). This pathogen is recognized as a frequent cause of subclinical intramammary infections in dairy cows (Gruet, Maicent, Berthelot & Kaltsatos, 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, Alomar, Chougui, Martin & Montel, 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 synthesised 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, Weber-Dabrowska & Gorski, 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, Nelson & Fischetti, 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, Loomis & Fischetti, 2001; Schuch, Nelson & Fischetti, 2002; Cheng, Nelson, Zhu & Fischetti, 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 *Steptococcus agalactiae* bacteriophage endolysins have also been characterized to be applied in mastitis cow's treatment (Donovan, Lardeo & Foster-Frey, 2006a; Donovan, Foster-Frey, Dong, Rousseau, Moineau & Pritchard, 2006b; Celia, Nelson & Kerr, 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, Scherer, Neve & Loessner, 2000; Turner, Waldherr, Loessner & Giffard, 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, Wilkinson & Jayaswal, 1991; Sass & Bierbaum, 2007), Twort (Loessner, Gaeng, Wendlinger, Maier & Scherer, 1998), 187 (Loessner, Gaeng & Scherer, 1999), P68 (Takac, Witte & Blasi, 2005), phiWMY (Yokoi et al. 2005), and phage K (O'Flaherty, Coffey, Meaney, Fitzgerald & Ross, 2005) but none have been tested as a biopreservative in foodstuffs. These staphylococcal endolysins have a modular

84 organization with enzymatic (d-alanylglycyl endopeptidase, l-muramoyl-l-alanine
85 amidase, N-acetyl-glucosaminidase) and cell wall recognition domains (Navarre, Ton-
86 That, Faull & Schneewind, 1999; Loessner et al. 1998).

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

94

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 & 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, Ladero & Suárez, 2003). The DNA was digested with *EcoR*I (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.

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'-ATTATGGAGGATCCGACAATGCAAG-3' and Ami2: 5'-GACTCACTGCAGTTTTATATTAACGT-3' and digested with the restriction enzymes *Pst*I and *BamH*I (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_{600nm} of 0.6-0.8) were induced with 1 mM IPTG (isopropyl- beta-D-thiogalactopyranoside), followed by 18 h shaking at 19 ºC. Cells were pelleted, washed with lysis buffer (20 mM NaH_2PO4, 500 mM NaCl, 20 mM Imidazole, pH 7.4) and frozen at -20 ºC. For protein purification, 500 ml culture cell pellets were resuspended in 10 ml lysis buffer, sonicated (15x 5 s pulses with 15 s recovery on ice) and centrifuged at 10.000 x 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 (Bio-Rad, Hercules, CA).

141

142 *2.4. Quantification of LysH5 activity.*

143 *S. aureus* Sa9 strain was grown to an OD_{600nm} of 0.5, centrifuged, and suspended
144 in 50 mM phosphate buffer, pH 7.0, to a final OD_{600nm} of 1.5. Bacterial suspensions (0.1
145 ml) were added to serial dilutions of purified LysH5 (0.1 ml) in sterile, uncoated
146 polystyrene 96-well plates, and the decrease in OD_{600nm} was monitored every 15 s for 15
147 min, at 37°C, in a Microplate Spectrophotometer Benchmark Plus (BioRad, Hercules,
148 CA). The activity of LysH5, expressed in units per millilitre (U/ml), was defined as the
149 reciprocal of the highest dilution that decreased the OD by 50% in 15 min. Specific
150 activity was calculated as the change in OD_{600nm} per mg protein per min. The lytic
151 spectrum of LysH5 was determined in a similar fashion using 15 U/ml. The enzyme
152 activity was determined over a pH range 4.0 to 6.0 in 50 mM Na-acetate buffer, and pH
153 7.0 to pH 8.0 in 20 mM Na-phosphate buffer and at temperatures ranging from 25 °C to
154 45 °C. Temperature stability was determined by incubation of the protein at different
155 temperatures prior to the standard activity assay. All these experiments were performed in
156 triplicate.

157

158 *2.5. Antimicrobial activity in milk.*

159 The lytic activity of LysH5 on *S. aureus* was tested in commercial whole-fat
160 pasteurized milk. Milk was inoculated with exponentially growing cultures of *S. aureus*
161 Sa9 (10^6 and 10^3 CFU/ml) and purified LysH5 was added at 160, 80 and 45 U/ml. The
162 cultures were incubated at 37 ºC without shaking. Samples were taken at different time
163 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 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

187 LysH5 clustered together (≥ 97% identity) with others encoded well-characterized
188 staphylococcal phages such as phiNM2, phi11, phi29 and phage 80 alpha (Fig 1B). Other
189 *S. aureus* endolysins from phage Twort, phage K, *S. warneri* phage phiWMY, phi 12,
190 phiPVL, phiSLT were less related to LysH5.
191
192 *3.2. Overexpression and characterization of the bacteriophage ΦH5 endolysin.*
193 The recombinant phage endolysin was synthesized as an N-terminally 6x-His-
194 tagged fusion which allowed the purification by immobilized metal chelate affinity
195 chromatography. An extra cation exchange chromatographic purification step was
196 necessary to remove contaminants. The active fractions were pooled and analyzed by
197 SDS-PAGE (Fig. 2). A major protein band of an estimated molecular mass of 55 kDa was
198 observed which correlated well with the calculated molecular mass for LysH5 (53.7
199 kDa). Yields of 3.6 U per ml of induced *E. coli* cultures were routinely achieved with a
200 specific activity of 1.8 U/μg. The recombinant LysH5 was able to lyse resting *S. aureus*
201 cells. The initial OD_{600nm} dropped to baseline within 6 min, indicating a rapid rate of cell
202 lysis (Fig. 3).
203 Purified preparations were assayed at different pHs and temperature conditions.
204 As shown in Fig. 4A, the highest specific activity was obtained at relatively neutral pH.
205 The enzyme was slightly inactivated at pH 6.0 and significantly reduced at lower pHs.
206 Levels of 48% and 1% activity were detected at pHs 5.0 and 4.0, respectively. The lytic
207 activity was also temperature-dependent. The protein efficiently lysed the cells in a
208 temperature range from 30 ºC to, at least, 45 ºC but decreased at lower temperatures (Fig.
209 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 ºC and 1 min at 72 ºC 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 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

233 only observed in the first 60 min (Fig. 6A). At lower contamination levels (10^3 CFU/ml),
234 the addition of 45 U/ml eliminated *S. aureus* in 4 h (Fig. 6B). These results showed that
235 LysH5 was capable of killing staphylococci which are actively multiplying in milk in
236 these conditions.
237
238
239 **4. Discussion**
240
241 In this work, phage endolysin LysH5 was cloned in *E. coli* and the lytic activity of
242 the purified protein was characterized. Preliminary experiments showed also that LysH5
243 was able to inhibit *S. aureus* growth in pasteurized milk. While a number of
244 staphylococcal endolysin have been characterized, to our knowledge, none has been
245 assessed as an antimicrobial additive for preventing the growth of *S. aureus* in dairy
246 products.
247 LysH5 displayed a modular organization similar to other staphylococcal
248 endolysins previously described (Navarre et al. 1999; Yokoi et al. 2005; O'Flaherty et al.
249 2005). According to the domains found, LysH5 should display a cysteine, histidine
250 dependent amidohydrolases/peptidase (CHAP) endopeptidase activity that cleaves at D-
251 alanyl-glycyl moieties and an amidase domain that cleaves at N-acetylmuramyl-L-alanyl
252 bonds but this has not been experimentally proved yet. The SH3b domain, thought to be
253 involved in cell wall recognition, was also detected. Several phage endolysins of Gram-
254 positive bacteria carry a SH3b domain in their C-terminal (Sugahara et al. 2007; Donovan
255 et al. 2006b; Porter et al. 2007). In *L. monocytogenes* phage endolysins Ply118 and

256 Ply500 the C-terminal cell wall binding domains confer the specificity necessary to direct
257 the murein hydrolases to the bacterial cell wall (Loessner, Kramer, Ebel & Scherer, 2002;
258 Kretzer et al. 2007).

259 The ΦH5 endolysin protein showed substantial similarity to those of *S. aureus*
260 phages and even to the *S. warneri* phage phiWMY. However, in contrast to the endolysin
261 LysWMY that exhibits lytic activity against other Gram positive genera (Yokoi et al.
262 2005) LysH5 is only active against *Staphylococcus* indicating distinct cell-wall-
263 recognition signals. The full-length and 182-amino-acid C-terminally truncated *S.*
264 *agalactiae* bacteriophage B30 endolysins also displayed lytic activity against
265 *Streptococcus thermophilus* and *Leuconostoc cremoris* strains (Donovan et al. 2006b).
266 The narrow spectrum of LysH5 is of practical relevance for its deliberate use in
267 biopreservation of dairy products. Several dairy products are fermented by lactic acid
268 bacteria that are mostly responsible for their organoleptic properties. In this scenario, it is
269 crucial to specifically target the undesirable bacteria, leaving the natural microbial
270 communities undisturbed.

271 Although LysH5 lysed human *S. aureus* and *S. epidermidis*, its activity is
272 remarkable lower compared to *S. aureus* of bovine origin which were highly susceptible.
273 It appears that endolysins derived from phages isolated from the dairy environment have
274 co-evolved with their hosts. Therefore, they would specifically target the *S. aureus* clones
275 most commonly found in the dairy environment and would be more suitable as
276 antimicrobials to be used in biopreservation of milk and dairy products. Nevertheless, the
277 specific action of LysH5 on *S. aureus* and *S. epidermidis* of human origin should not
278 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 & 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 temperature. Therefore, it should be added after heat treatment of milk. On the other hand, the protein remained active at 4 ºC. 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.

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7. Figure legends

Figure 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, Dudley, Nei & Kumar, 2007).

Figure 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.

Figure 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

438 enzyme (5 U/ml) to a standardized cell suspensions is shown. Symbols: * *S. aureus* plus
439 LysH5; ♦ negative control (no enzyme added).
440
441 Figure 4.- Influence of pH (A) and temperature (B) on the specific activity of the ΦH5
442 endolysin. The endolysin (5 U/ml) was tested at various pHs in 50 mM sodium acetate
443 (pH4-pH6) and in 20 mM sodium phosphate buffer (pH7-pH8) under standard assay
444 conditions. Values are the mean of three independent experiments. Error bars are also
445 shown.
446
447 Figure 5.- Lytic spectrum of the endolysin LysH5. A) *S. aureus* bovine strains. B) *S.*
448 *aureus* clinical strains. C) *S. epidermidis* clinical strains. Values are the mean of three
449 independent experiments. Error bars are also shown.
450
451 Figure 6.- Killing of *S. aureus* Sa9 with purified LysH5 in pasteurized whole milk. A) ♦,
452 cell numbers of *S. aureus* Sa9; ■, cell numbers of *S. aureus* Sa9 plus LysH5 (160 U/ml);
453 ▲, cell numbers of *S. aureus* Sa9 plus LysH5 (80 U/ml). B) ♦, cell numbers of *S. aureus*
454 Sa9; X, cell numbers of *S. aureus* Sa9 plus LysH5 (45 U/ml). Values are the means of
455 two independent experiments with standard deviation indicated by vertical bars.
456
457 Figure 1
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Figure 1. Obeso et al.

A

B

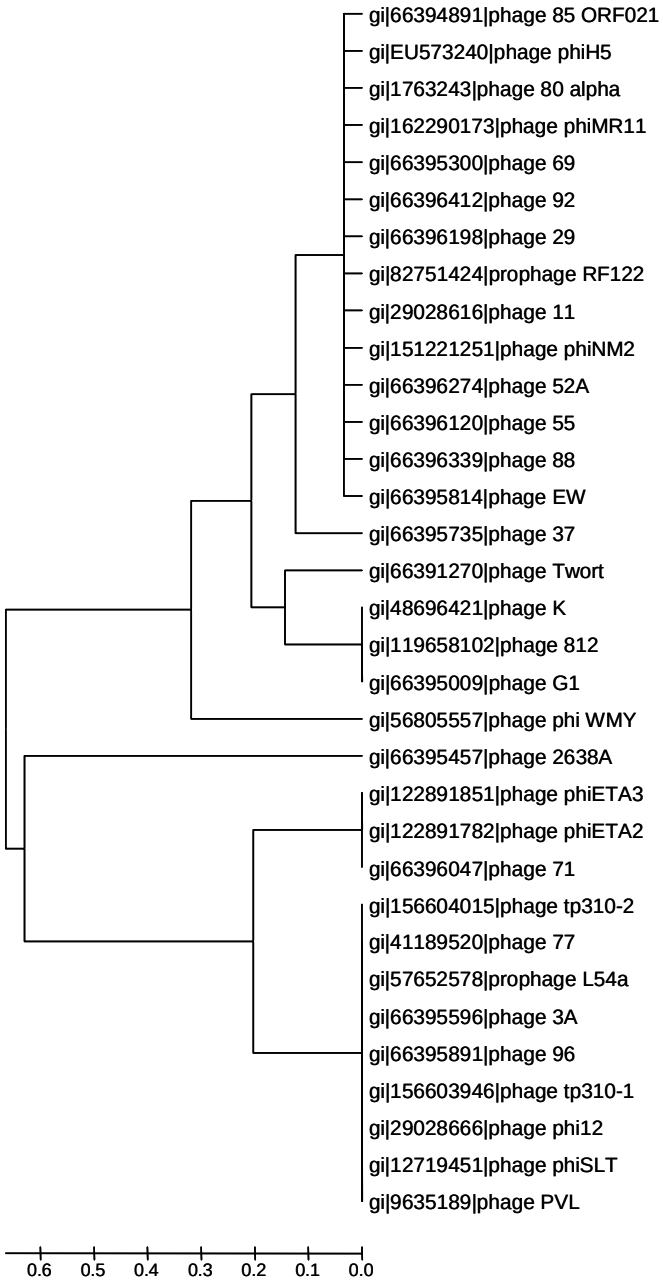
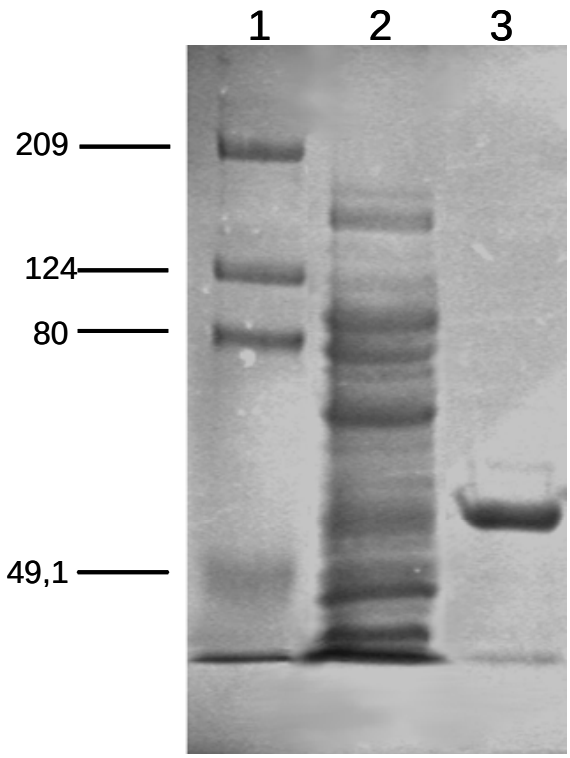


Figure 2. Obeso et al.



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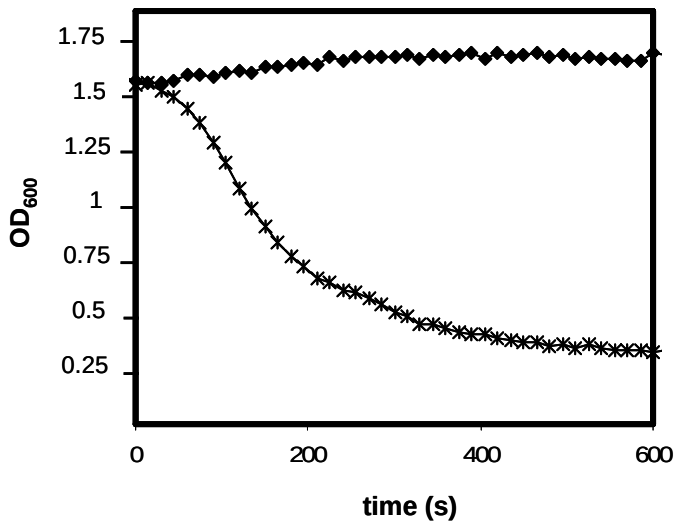

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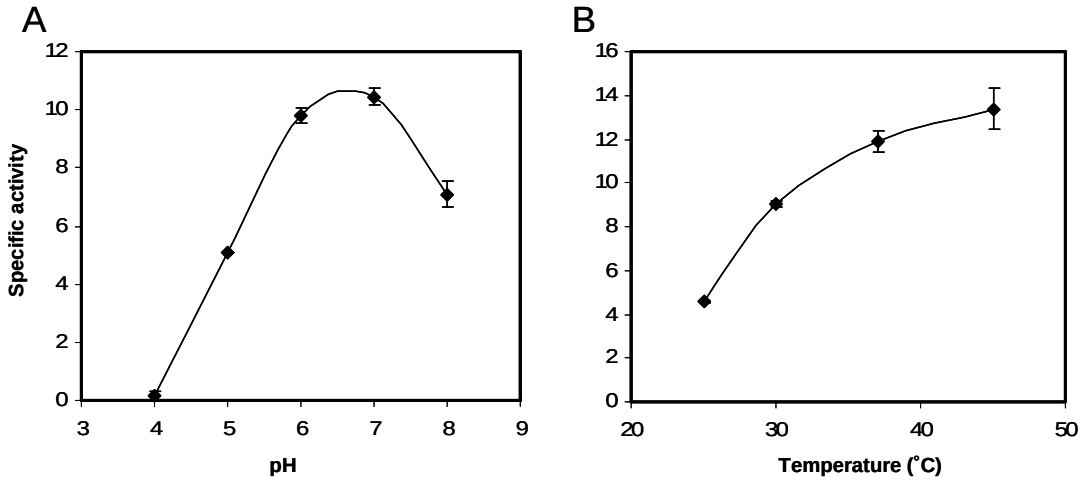

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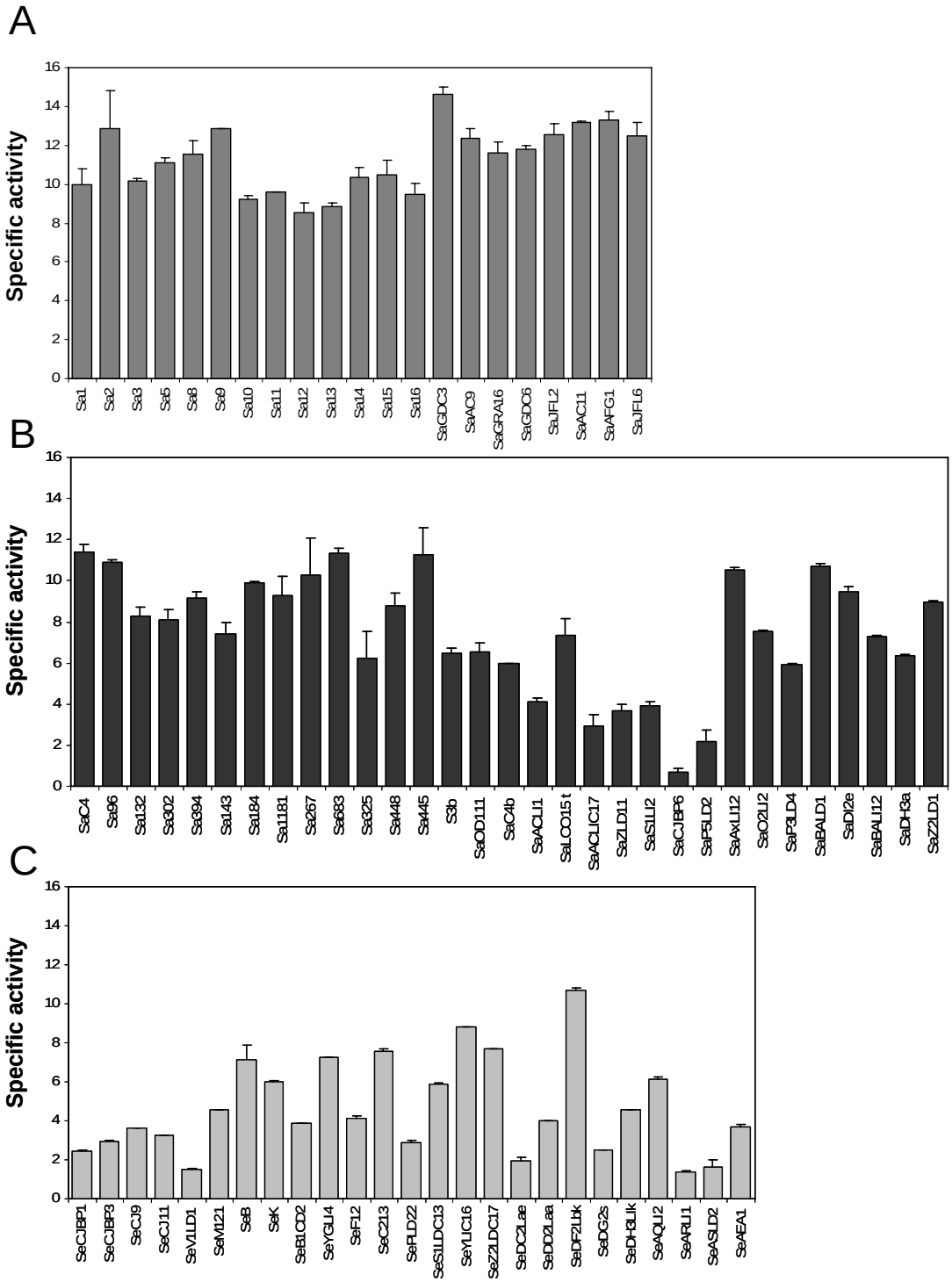

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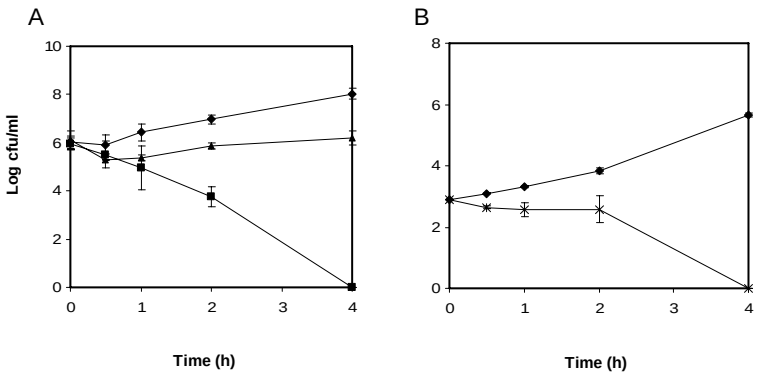
Figure 6. Obeso et al.,



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