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

4

5 **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*.

38 **1. Introduction**

39

40 *Staphylococcus aureus* is capable of producing enterotoxins responsible for
41 staphylococcal food poisoning, one of the most prevalent causes of gastroenteritis
42 worldwide (Dinges, Orwin & Schlievert, 2000). This pathogen is recognized as a frequent
43 cause of subclinical intramammary infections in dairy cows (Gruet, Maicent, Berthelot &
44 Kaltsatos, 2001) and is commonly isolated from raw milk of dairy cattle suffering from
45 mastitis. Its presence in raw milk is a major concern for the safety and quality of
46 traditionally produced cheeses (Delbes, Alomar, Chougui, Martin & Montel, 2006;
47 Cremonesi et al. 2007). In this context, it is relevant to develop alternative strategies to
48 ensure the hygienic quality of dairy products.

49 Bacteriophage endolysins are mureolytic enzymes that directly target bonds in the
50 peptidoglycan of the bacterial cell wall. They are encoded by the bacteriophage genome
51 and are synthesised at the end of the phage lytic life cycle to lyse the host cell and release
52 the newly produced virions. Besides this "lysis from within", endolysins from phages of
53 gram-positive hosts are also able to quickly lyse the bacteria when they are applied
54 exogenously (Loessner, 2005). As potential antibacterials, endolysins possess several
55 relevant features, namely, a distinct mode of action, highly specific, and active against
56 bacteria regardless of their antibiotic susceptibility pattern (Borysowski, Weber-
57 Dabrowska & Gorski, 2006). On the other hand, there is a low probability of developing
58 resistance against the activity of bacteriophage endolysins linked to the fact that they
59 target unique and highly conserved bonds in the peptidoglycan (Loeffler, Nelson &
60 Fischetti, 2001). Recombinant phage endolysins have been reported to inhibit a variety of

61 pathogens, and have recently been claimed as alternative antimicrobials for treatment of
62 bacterial infections caused by gram-positive bacteria (Fischetti, 2003; Loessner, 2005).
63 The effectiveness of phage lysins in clearing certain infections has been well documented
64 in mouse models (Loeffler et al. 2001; Nelson, Loomis & Fischetti, 2001; Schuch, Nelson
65 & Fischetti, 2002; Cheng, Nelson, Zhu & Fischetti, 2005; Rashel et al. 2007) as well as
66 transgenic murine and bovine mammary glands (Kerr et al. 2001; Wall et al. 2005). *S.*
67 *aureus, Streptococcus uberis* and *Steptococcus agalactiae* bacteriophage endolysins have
68 also been characterized to be applied in mastitis cow's treatment (Donovan, Lardeo &
69 Foster-Frey, 2006a; Donovan, Foster-Frey, Dong, Rousseau, Moineau & Pritchard,
70 2006b; Celia, Nelson & Kerr, 2008).

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

78 So far, a few staphylococcal phage endolysins have been characterized such as
79 those of phages phi11 (Wang, Wilkinson & Jayaswal, 1991; Sass & Bierbaum, 2007),
80 Twort (Loessner, Gaeng, Wendlinger, Maier & Scherer, 1998), 187 (Loessner, Gaeng &
81 Scherer, 1999), P68 (Takac, Witte & Blasi, 2005), phiWMY (Yokoi et al. 2005), and
82 phage K (O'Flaherty, Coffey, Meaney, Fitzgerald & Ross, 2005) but none have been
83 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

95 **2. Materials and Methods**

96

97 *2.1. Bacteria, phages and growth conditions.*

98 The bacterial strains used in this study are summarized in Table 1. Additionally,
99 21 *S. aureus* isolates from mastitic milks as well as 31 and 25 clinical human isolates of
100 *S. aureus* and *S. epidermidis*, respectively, were also used in lytic assays. These clinical
101 isolates were kindly supplied by Dr. Rodríguez and Dr. Delgado (Universidad
102 Complutense, Madrid, Spain). *E. coli* transformants were selected with 100 μg/ml
103 ampicillin and/or 25 μg/ml chloramphenicol, as appropriate.

104 Bacteriophage ΦH5 was routinely propagated on *S. aureus* Sa9 (García et al.
105 2007).

106

107 *2.2. DNA manipulations.*

108 Plasmid DNA was obtained by the alkaline lysis method (Birnboim & Doly,
109 1979). Analytical and preparative gel electrophoresis of plasmid DNA and restriction
110 fragments was carried out in 0.8% (w/vol) agarose–Tris-Acetate horizontal slab gels.
111 Phage ΦH5 DNA was extracted and purified as described previously (García, Ladero &
112 Suárez, 2003). The DNA was digested with *EcoR*I (Takara, Otsu, Shiga, Japan) and
113 random fragments were cloned in pUC18 in *E. coli* DH10B. Plasmid DNA from ninety-
114 six white colonies were extracted and analyzed. Sequences obtained were BLAST
115 searched against the NCBI protein database.

116

117 *2.3. Cloning and overexpression of the recombinant LysH5 endolysin.*

118 A 1490-bp DNA segment containing the *lys*H5 gene was obtained by PCR
119 amplification with the primers Ami1: 5'-ATTATGGAGGATCCGACAATGCAAG-3'
120 and Ami2: 5'-GACTCACTGCAGTTTTATATTAACGT-3' and digested with the
121 restriction enzymes *Pst*I and *BamH*I (Takara, Otsu, Shiga, Japan). The amplification
122 product was cloned in pUC18 for sequencing and in the expression vector pRSETB
123 (Invitrogen, Carlsbad, CA). The plasmids, pUC18- *lys*H5 and pRSETB-*lys*H5 were
124 electroporated in *E. coli* DH10B and in *E. coli* BL21(DE3)/pLys, respectively. pRSETB-
125 *lys*H5 construction was used to overexpress *lys*H5. Exponentially growing cultures
126 (OD_{600nm} of 0.6-0.8) were induced with 1 mM IPTG (isopropyl- beta-D-
127 thiogalactopyranoside), followed by 18 h shaking at 19 ºC. Cells were pelleted, washed
128 with lysis buffer (20 mM NaH_2PO4, 500 mM NaCl, 20 mM Imidazole, pH 7.4) and
129 frozen at -20 ºC. For protein purification, 500 ml culture cell pellets were resuspended in
130 10 ml lysis buffer, sonicated (15x 5 s pulses with 15 s recovery on ice) and centrifuged at
131 10.000 x g. The supernatant was added to 5 ml Ni-NTA (nickel matrix) slurry and eluted
132 according to the manufacturer's instructions (Qiagen, Valencia, CA). Fractions
133 containing LysH5 were dialyzed against 20 mM NaH_2PO4 buffer, pH 6.0. This sample
134 was loaded onto a CM column (Pharmacia, Uppsala, Sweden) equilibrated with the same
135 buffer and the protein eluted with a NaCl gradient (0 to 1 M). Protein fractions were
136 analyzed in 15% (w/v) SDS-PAGE gels. Electrophoresis was conducted in Tris-Glycine
137 buffer at 20 mA for 1 h in the BioRad Mini-Protean gel apparatus. The fractions
138 containing pure LysH5 (as judged by SDS-PAGE) were pooled, diluted in glycerol (50%
139 final concentration), and stored at -20 °C. Protein was quantified by the Quick Start
140 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

164 egg yolk tellurite (Scharlau Chemie, S.A. Barcelona, Spain). The absence of *S. aureus* in
165 non-inoculated milk was verified by direct plating.
166
167 *2.6. Statistical analysis.*
168 The results were compared using one-way ANOVA analysis (SPSS 11.0 software for
169 windows; SPSS, Chicago,IL, USA).
170
171 **3. Results**
172
173 *3.1. Identification and sequence analysis of the bacteriophage ΦH5 endolysin.*
174 Twenty recombinant plasmids were randomly chosen from a shotgun library of
175 ΦH5 DNA and sequenced. One of the plasmids carried a partial insert (1.5 kbp) highly
176 homologous to a phage-related amidase encoded by the *S. aureus* RF122 prophage
177 genome (accession number AJ938182.1). Based on the known sequence of RF122,
178 oligonucleotides were designed to amplify the whole putative ΦH5 endolysin gene
179 (*lys*H5) by PCR, cloned into pUC18 and sequenced (GeneBank Accession number
180 EU573240). *lys*H5 (1446 bp) was identical to the putative endolysin gene of the *S. aureus*
181 RF122 prophage. Analysis of the amino acid sequence (481 aa) revealed that LysH5 is a
182 modular enzyme with three distinct domains, namely, an N-terminal CHAP (cysteine,
183 histidine-dependent amidohydrolase/peptidase) domain with hydrolytic function, a
184 central amidase domain (N-acetylmuramyl-L-alanine amidase), and a C-terminal SH3b
185 domain which might be involved in cell wall recognition (Fig. 1A). Comparative
186 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

210 endolysin was very sensitive to high temperatures and standard pasteurization processes,
211 i.e. 30 min at 63 ºC and 1 min at 72 ºC fully inactivated the protein. After -20 ˚C storage
212 without any cryoprotectans, a 32% decrease of specific activity was observed.
213
214 *3.3. Lytic spectrum of the bacteriophage ΦH5 endolysin.*
215 In addition to killing the host bacterial strain, LysH5 was able to lyse all the other
216 *S. aureus* strains irrespectively of their bovine or human origin, including those not
217 infected by ΦH5 (Fig. 5). However, LysH5 had a significant ($p<0.001$) different killing
218 effect on *S. aureus* depending on the strain origin. Higher susceptibility to the endolysin
219 was observed on *S. aureus* bovine strains with an average specific activity of 11.3±1.7
220 while on clinical strains this value was 7.5±2.9. A larger variability was also observed
221 within the clinical strains. *S. epidermidis* isolated from humans were also sensitive
222 although the lytic activity of LysH5 was significantly lower (4.6±2.4). No lytic activity
223 against several lactic acid bacteria and strains belonging to *Bacillus*, *Streptococcus*,
224 *Clostridium*, *Listeria*, and *Enterococcus* was detected (data not shown).
225
226 *3.4. Antimicrobial activity of LysH5 on S. aureus in milk.*
227 The effect of purified LysH5 was tested against an exponentially growing *S.*
228 *aureus* Sa9 strain in milk at two contamination levels (Fig. 6). At higher contamination
229 levels (10^6 CFU/ml), the addition of 160 U/ml **(**88 µg/ml) of LysH5 to pasteurized milk
230 reduced the viable counts to undetectable levels in 4 h. The inhibitory effect of the
231 endolysin was already significant ($p<0.05$) after 60 min and the counts were more than 1
232 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

279 multiple-drug-resistant *Staphylococcus.* Bacterial biofilm formation is another pathogenic
280 factor often shown by both bacteria. Recently, it has been shown that the phi11 endolysin
281 lyses the complex structure of staphylococal biofilms (Sass & Bierbaum, 2007).
282 Our results have shown that staphylococcal phage endolysins encoded by phages
283 of dairy origin might be useful as an additional hurdle to prevent *S. aureus* in milk and
284 presumably in dairy products. The *in vitro* activity assays performed with purified LysH5
285 under several conditions of pH indicated that LysH5 could be active during the milk
286 coagulation process but not below pH 5 as the activity was seriously compromised. The
287 endolysin was sensitive to high temperature. Therefore, it should be added after heat
288 treatment of milk. On the other hand, the protein remained active at 4 ºC. In case of
289 temperature abuse, the presence of the endolysin could presumably hamper *S. aureus*
290 growth and prevent enterotoxin production. Further biochemical characterization of
291 LysH5 is in progress to optimize and define the scope of application in dairying.
292
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294
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301

302 **6. References**

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416 **7. Figure legends**

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418 Figure 1. Sequence analysis and phylogenetic position of the phage H5 endolysin. A)
419 Domain organization of LysH5 as displayed by SMART (http://smart.embl-
420 heidelberg.de) containing CHAP (cysteine, histidine-dependent amidohydrolases/
421 peptidases), Ami_2 (N-acetylmuramyl-L-alanine amidase) and SH3b (bacterial cell
422 recognition). Numbers indicate the amino acid positions in LysH5. B) Phylogenetic
423 position of LysH5 compared to several phage endolysins. The tree was constructed using
424 the Neighbor-Joining method. The phylogenetic tree was linearized and drawn to scale.
425 The evolutionary distances were computed using the Poisson correction method and are
426 expressed in the units of the number of amino acid substitutions per site. All positions
427 containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses
428 were conducted in MEGA4 (Tamura, Dudley, Nei & Kumar, 2007).

429

430 Figure 2.- Purification of the recombinant LysH5 endolysin from *E. coli*
431 BL21(DE3)/pLys pRSET*lys*H5. Lane 1: Standard molecular weight marker in kDa
432 (Broad Range Prestained SDS-PAGE Standards, BioRad); lane 2, supernatant of the
433 lysate induced culture; lane 3, fraction eluted from cation exchange chromatography
434 containing purified LysH5.

435

436 Figure 3.- Lysis of *S. aureus* Sa9 cells by exogenously added recombinant LysH5. The
437 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.
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457 Figure 1
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Figure 1. Obeso et al.

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463 A

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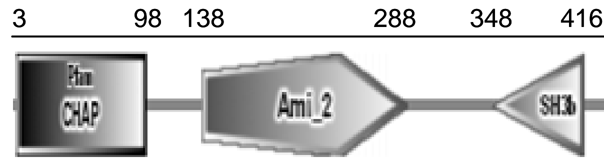

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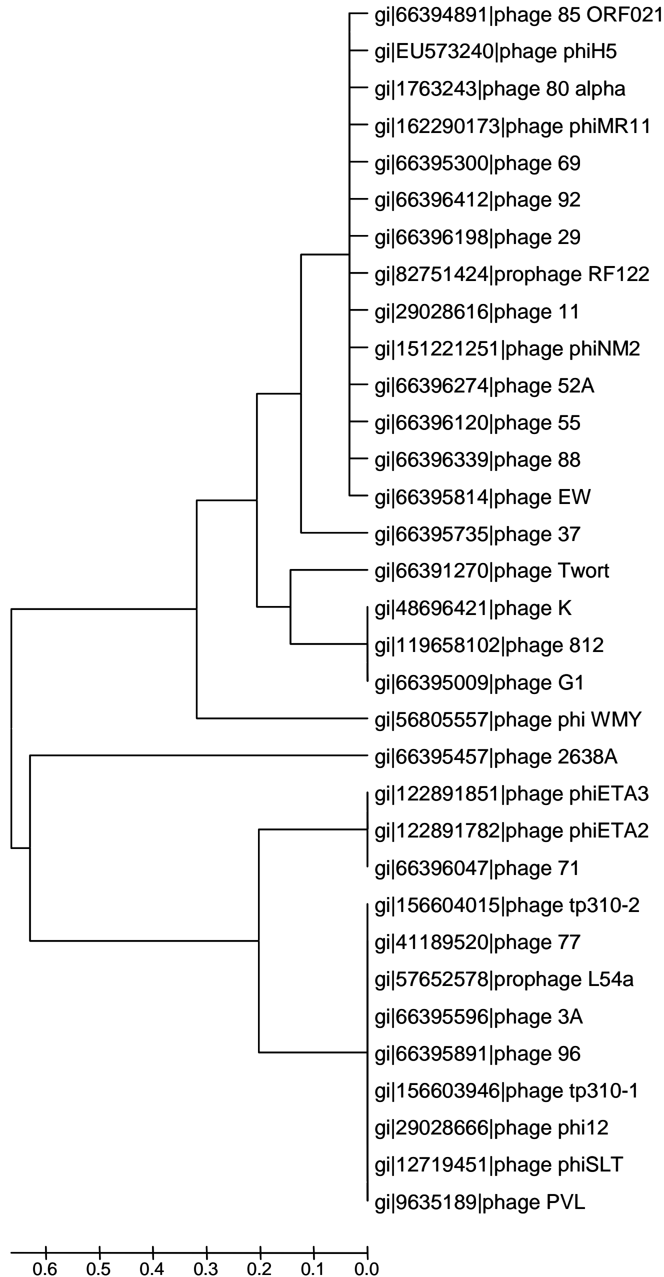

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485 Figure 2. Obeso et al.

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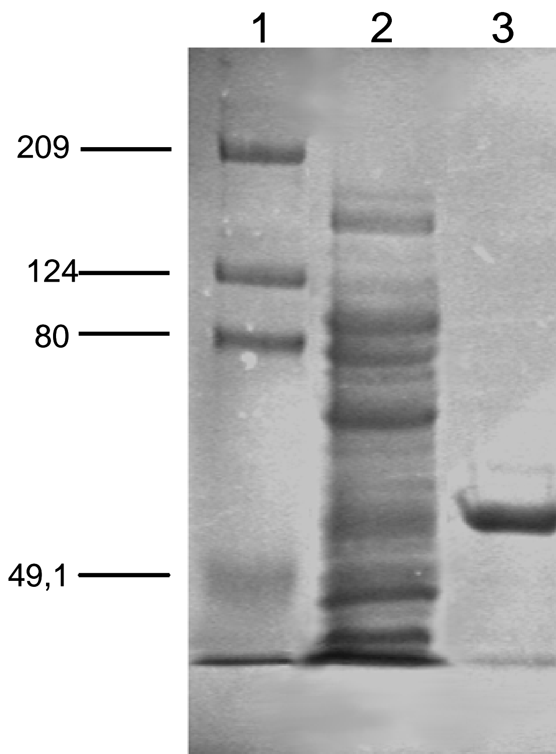

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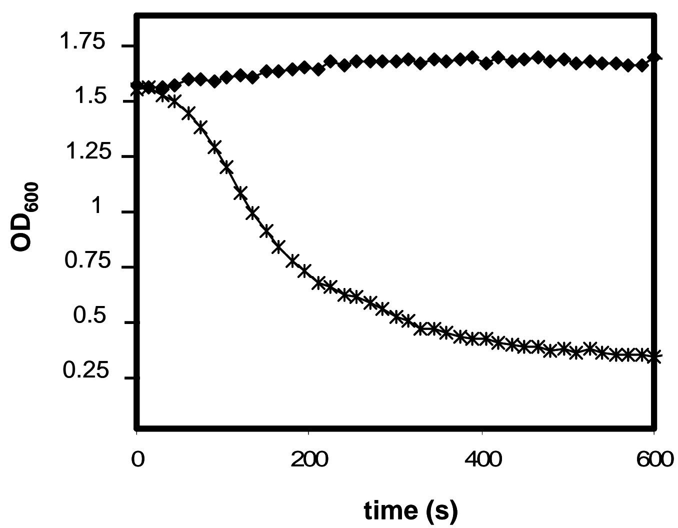

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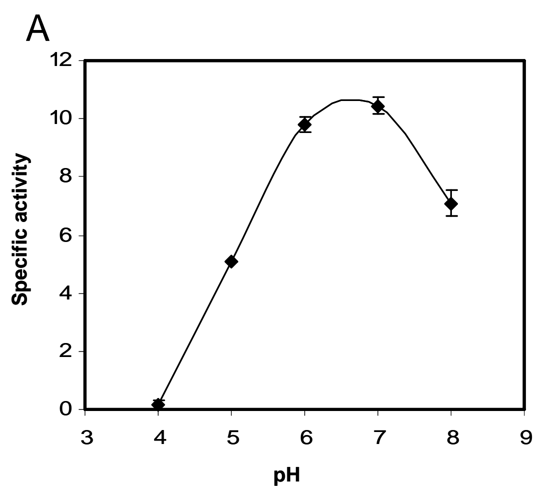

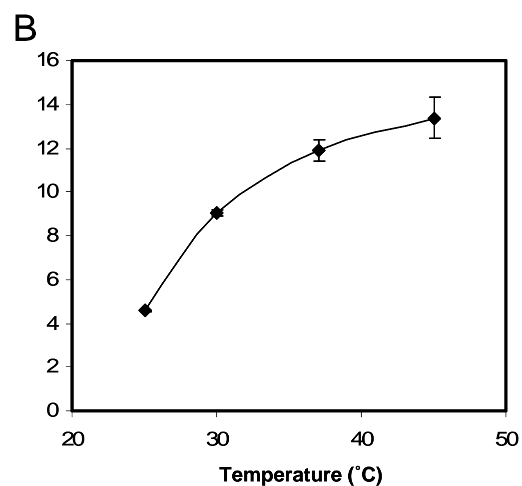

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532 Figure 5. Obeso et al.,

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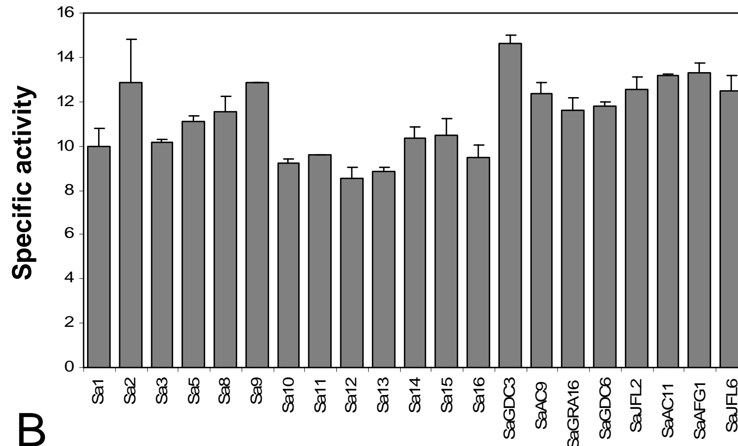

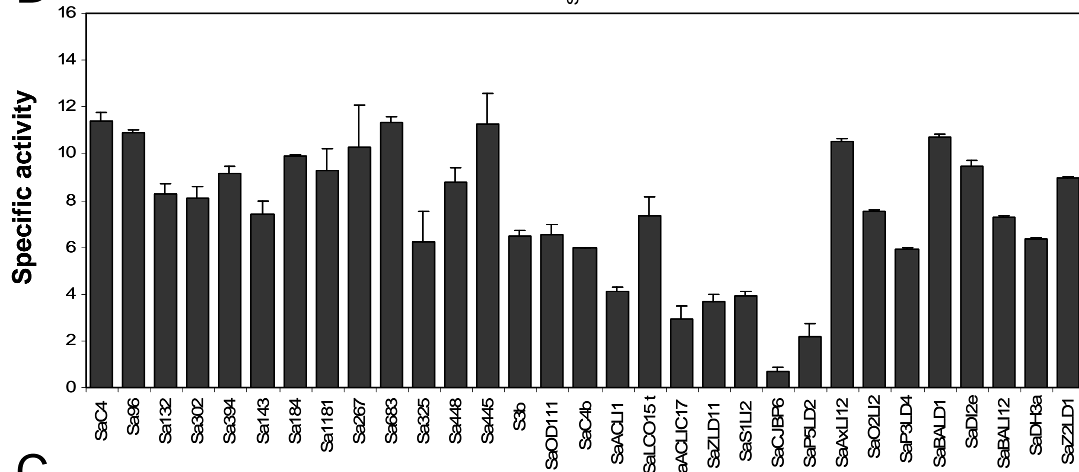

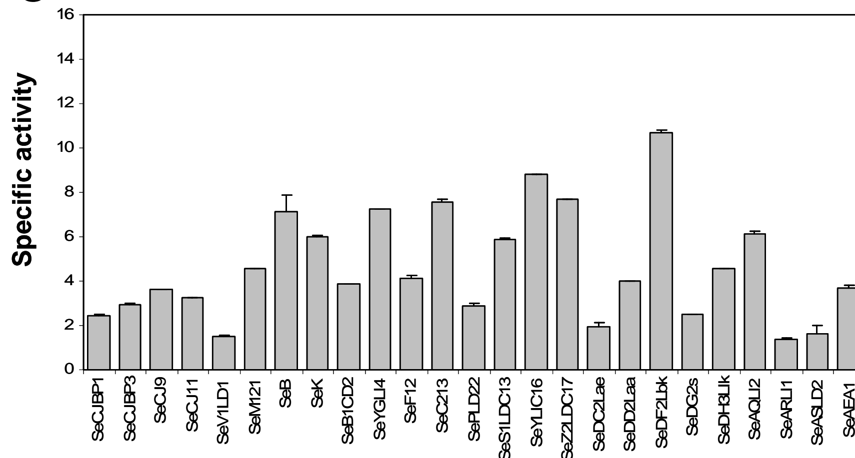

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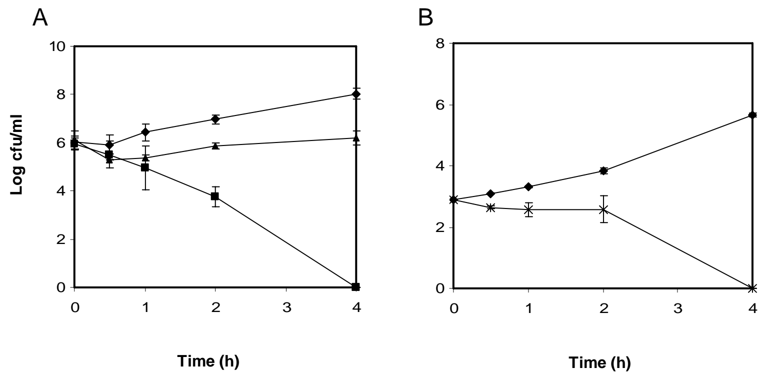
Figure 6. Obeso et al.,



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