

LambdaSa1 and LambdaSa2 Prophage Lysins of *Streptococcus agalactiae*

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1 Putative N-acetylmuramoyl-L-alanine amidase genes from LambdaSa1 and LambdaSa2
2 prophages of *Streptococcus agalactiae* were cloned and expressed in *Escherichia coli*. The
3 purified enzymes lysed the cell walls of *Streptococcus agalactiae*, *Streptococcus pneumoniae*,
4 and *Staphylococcus aureus*. The peptidoglycan digestion products in the cell wall lysates were
5 not consistent with amidase activity. Instead, the structure of the muropeptide digestion
6 fragments indicated that LambdaSa1 and LambdaSa2 lysins both exhibited γ -D-glutaminyll-
7 lysine endopeptidase activity. The endopeptidase cleavage specificity of the lysins was
8 confirmed using a synthetic peptide substrate corresponding to a portion of the stem peptide and
9 cross-bridge of *Streptococcus agalactiae* peptidoglycan. The LambdaSa2 lysin also displayed β -
10 D-N-acetylglucosaminidase activity.

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Bacteriophages often lyse their bacterial hosts at the end of an infective cycle and release their progeny into the surrounding medium. Phages of gram-positive bacteria usually accomplish this by employing a two-component lytic system consisting of a holin and a lysin (11). The holin makes the bacterial cell membrane porous, thereby exposing the peptidoglycan layer of the cell wall to the degradative action of the lysin. Phage lysins and the related endogenous bacterial cell wall hydrolases have been found that cleave many different bonds in peptidoglycan (11, 12). They include β -D-N-acetylmuramidases, β -D-N-acetylglucosaminidases, lytic transglycosylases, N-acetylmuramyl-L-alanine amidases, and various endopeptidases that cleave either the stem peptide or cross-bridge peptide of peptidoglycan (11, 12).

The observation that many phage lysins specifically lyse susceptible gram-positive bacteria, which have been exposed to the enzyme externally, has led to recent intense interest in using them as antimicrobial agents. For example, Nelson and coworkers showed that nasopharyngeal colonization of mice with *Streptococcus pyogenes* could be cleared using the C1 phage lysin from a group C streptococcus (14). Schuch *et al.* reported that the phage enzyme, PlyG, killed vegetative cells of *B. anthracis* and protected mice from an otherwise fatal intraperitoneal infection with *Bacillus cereus* (16). Similarly, Jado and coworkers showed that a single intraperitoneal injection of either of the pneumococcal phage lysins Pal, an amidase, or Dpl-1, a lysozyme, was sufficient to protect mice in a murine model of pneumococcal sepsis (7). These workers also demonstrated a synergistic effect on pneumococcal killing when the two enzymes were administered together. Similar synergism among different peptidoglycan cleavage activities may be why many phage lysins evolved with more than one type of lytic activity. The different peptidoglycan cleavage activities are usually located on distinct

34 enzymatic domains, often along with one or more cell wall binding domains (11, 12). We
35 recently described the cloning and elucidation of cleavage specificities of the B30 lysin from a
36 bacteriophage of *Streptococcus agalactiae* (1, 15). It contains an endopeptidase domain, a
37 glycosidase domain, and an SH3b putative cell wall binding domain (1, 15). The endopeptidase
38 activity was shown to cleave between the D-alanine of the stem peptide and L-alanine in the
39 cross-bridge, and the glycosidase had the specificity of an N-acetylmuramidase (1). We initially
40 cloned the genes for the two lysins described in this report expecting them to exhibit N-
41 acetylmuramyl-L-alanine amidase activity, however, as described below, characterization of
42 their cleavage specificities showed that they were not amidases.

44 MATERIALS AND METHODS

45 **Bacterial strains and culture conditions.** *Streptococcus agalactiae* and *Staphylococcus*
46 *aureus* strains from our collections were routinely plated on Trypticase soy agar (BBL)
47 containing 5% sheep blood or cultured in Todd-Hewitt broth (Difco) at 37° C without shaking.
48 *S. pneumoniae* were cultured in Todd-Hewitt broth containing 0.5% (w/v) yeast extract.
49 Serotype V strain 2603 V/R *Streptococcus agalactiae* was obtained from the ATCC (No. BAA-
50 611). *Escherichia coli* strains INV α F' and BL21/DE3 were routinely grown at 37°C with
51 shaking in Luria-Bertani (LB) broth. However, for protein expression, cultures were grown at
52 room temperature.

53
54 **Cloning the genes for LambdaSa1 and LambdaSa2 lysins.** Chromosomal DNA was
55 prepared from GBS strain 2603 V/R by a procedure described previously (9). Briefly, this
56 involved digesting washed cells with mutanolysin at 60° C for 30 min, adding sodium dodecyl

57 sulfate (2% final concentration) to lyse the cells, cooling to 37° C and treating with ribonuclease,
58 purifying the DNA by phenol-chloroform extraction, and finally precipitating the DNA with
59 isopropanol. The two lysin genes were PCR amplified with chromosomal DNA as template.
60 The LambdaSa1 gene was amplified using the primers SAG0604F, 5' GTA CAG TCC ATA
61 TGG TAA TTA ATA TTG AGC AAG CTA TC 3' and SAG0604R, 5' CGT ACA TGC TCG
62 AGC ATA TCT GTT GCA TCA ATT AAG TAA G 3'. The primers for the LambdaSa2 gene
63 were SAG1837F, 5' GTA CAG TCC ATA TGG AAA TCA ACA CTG AAA TAG CCA TTG 3'
64 and SAG1837R, 5'CGT ACA TGC TCG AGA ACT GGC TTT TTA GTC AGT TCA T 3'. The
65 PCR products were cloned into the pGEM T-vector (Promega, Madison, WI) and transformed
66 into *E. coli* strain INV α F'. Purified plasmid DNA was cleaved with the restriction
67 endonucleases NdeI and XhoI and the inserts cloned into similarly digested expression vector
68 pET21a (Novagen). This construct was then transformed into *E. coli* strain BL21/DE3 for
69 enzyme expression. The sequence of the resulting plasmid was confirmed by DNA sequencing.

70
71 **Expression and purification of cloned lysins.** Starter cultures of *E. coli* BL21/DE3 containing
72 the recombinant plasmids were grown overnight in LB medium containing 50 μ g/ml ampicillin
73 at 37°C with shaking at 250 rpm. This culture was diluted with twenty volumes of fresh medium
74 (3 liters) and grown at 37°C until it reached mid log phase (OD_{550 nm} of 0.5). Lysin expression
75 was induced by adding isopropyl- β -D-thiogalactopyranoside to give a final concentration of 1
76 mM. After growing for an additional 4.5 h at room temperature, the cells were harvested by
77 centrifugation and washed three times with extraction buffer (50 mM NaH₂PO₄, 300 mM NaCl,
78 10 mM imidazole, pH 8.0). The pellet was resuspended in 100 ml of extraction buffer and
79 sonicated in an ice bath for a total of 5 min at 60% of maximum power using a model 300 Fisher

80 Sonic Dismembrator. Cell debris was removed by centrifugation and the resulting supernate
81 passed over a 10 ml Ni-NTA affinity column (Qiagen), which was then washed and eluted
82 according to the manufacturer's instructions. Imidazole was removed from the sample by
83 passing it through a 2.5 x 14 cm column of Bio-Gel P4 run in 50 mM ammonium acetate, pH
84 6.8. Fractions with enzyme activity were pooled and concentrated by ultrafiltration to give final
85 approximate concentrations of 10 mg/ml for LambdaSa1 lysin and 1 mg/ml for LambdaSa2
86 lysin.

87 Turbidity reduction assays were carried out on washed cells or bacterial cell walls
88 essentially as we described previously (15). However, the buffer used was 50 mM ammonium
89 acetate, pH 6.8. Bacterial cell walls were also prepared as described previously (15). Plate
90 assays for lytic activity were performed in 1% agarose in the above buffer containing a
91 suspension of cells or cell walls sufficient to make the layers turbid. Holes (5 mm) were
92 punched in the layers and 10 μ l samples were added. After incubation at 37° C for several hours,
93 or overnight, distinctive clear zones form around sample wells containing lysin activity.

94 Lysin endopeptidase activity was estimated by measuring the free amino groups released
95 during digestions. Cell walls were enzymatically digested as described above, but with 50 mM
96 sodium acetate substituting for ammonium acetate. After centrifugation of the digests, aliquots
97 of the supernatants were assayed for free amino groups by a spectrophotometric method
98 employing picryl sulphonic acid (13). In order to determine the site of endopeptidase cleavage, a
99 synthetic peptide corresponding to a portion of the stem peptide and cross-bridge of GBS
100 peptidoglycan (Fig.1) was lysin digested. The hexapeptide Ala-D- γ -Glu-Lys-D-Ala-Ala-Ala was
101 custom synthesized by Genscript Corp. (Piscataway, NJ) and the two carboxyl groups of the

102 peptide were amidated (8, 17) to yield Ala-D- γ -Gln-Lys-D-Ala-Ala-Ala-amide. Digestion
103 products were characterized by mass spectrometry.

104
105 **Sample preparation for mass spectrometry.** Washed cell walls of different bacterial
106 species were treated with purified lysins overnight. In some cases double digests using both
107 mutanolysin and a phage lysin were carried out. In all cases the digestion mixtures were
108 centrifuged and the supernates were passed through a 5 K cutoff centrifugal ultrafilter (Ultrafree-
109 MC Centrifugal filter, Millipore Corp., Billerica, MA) prior to analysis in order to remove
110 proteins and capsular polysaccharides. Alkaline de-O-acetylation of glycopeptides was
111 accomplished by treating samples with 5% triethylamine at room temperature for 30 min
112 followed by evaporation to dryness in a vacuum centrifuge. Acetate released from glycopeptides
113 was measured enzymatically using a kit supplied by R-Biopharm Inc. (Marshall, MI 49068).

114
115 **Electrospray ionization mass spectrometry.** This was performed on a Micromass Q-
116 TOF2 mass spectrometer (Micromass Ltd., Manchester, U.K.). Samples were dissolved in either
117 water or dilute ammonium acetate buffer. Sodium salts were avoided to minimize the formation
118 of sodium adducts. Online injections were made into a stream of 50/50 acetonitrile/water
119 containing 0.1% formic acid pumped at a rate of 1 μ L/min. Negative ion mass spectra were
120 obtained using a needle voltage of 2.8 kV. The mass spectral data was processed using the Max-
121 Ent3 module of MassLynx 3.5.

122
123 **Determining the mode of glycosidic cleavage.** This was accomplished by identifying the
124 amino sugar at the reducing terminus of glycopeptides obtained upon digestion with a lysin.

125 Procedures for the borohydride reduction, propanolysis, re-N-acetylation, and trimethylsilylation
126 of samples have been described previously (1). Derivatized samples were analyzed on a
127 GLC/MS (Varian 4000, Varian Inc. Palo Alto, CA) fitted with a 30-m (0.25 mm in diameter)
128 VF-5 capillary column. Column temperature was maintained at 100°C for 5 min, then increased
129 to 275°C at 20°C/min, and finally held at 275°C for 5 min. In an improved version of the
130 original procedure used in this work, the column effluent was analyzed by chemical ionization
131 mass spectrometry using acetonitrile as the reagent gas. This relatively gentle means of sample
132 ionization results in little fragmentation and often yields ions that are highly characteristic of a
133 particular sugar or alditol.

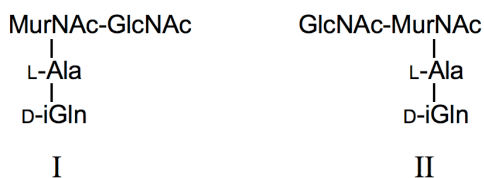
135 RESULTS AND DISCUSSION

136 The increasing incidence of antibiotic resistance in pathogenic bacteria has contributed to
137 the recent interest in bacteriophage lysins as potential antimicrobial agents. Encouraging results
138 from several animal experiments are likely to prompt further work directed towards using phage
139 lysins to prevent or treat human bacterial diseases (1, 3, 6, 7, 10, 14, 16).

140 The sequencing of bacterial genomes often reveals the presence of prophage lysin genes.
141 Such genes are usually identified by their sequence similarities to known lysin genes and on this
142 basis they are generally annotated as coding for amidases, lysozymes, endopeptidases, or other
143 types of lysins. Occasionally, lysin genes are annotated incorrectly. The sequenced genome of
144 strain 2603V/R *S. agalactiae* contains several lysin genes, including two putative amidases from
145 LambdaSa1 and LambdaSa2 prophages (19). These two lysin genes were cloned and expressed
146 as described above. Crude cell lysates obtained by sonic disruption of the *E. coli* cells were
147 found to rapidly lyse *S. agalactiae* cells and cell walls in a turbidity reduction assay and also

148 gave distinct zones of clearing in the lysin plate assay (data not shown). Interestingly, although
 149 both LambdaSa1 and LambdaSa2 lysins gave slightly smaller clear zones in the agar plate than
 150 did the B30 lysin we described previously, the zones showed complete clearing with no residual
 151 turbidity, unlike the B30 lysin. Presumably this is because some of the products of B30
 152 digestion are large enough to remain insoluble. Since digestion of *S. agalactiae* cell walls with
 153 affinity-purified LambdaSa1 or LambdaSa2 lysins led to a significant increase in free amino
 154 groups, it was evident that the lysins possessed either N-acetylmuramyl-L-alanine amidase or
 155 endopeptidase activities. Further characterization of the lysin digestion products was necessary
 156 to identify the type of enzymatic activity present.

157 Lysin digestion products of *S. agalactiae* cell walls that had been passed through a 5 K-
 158 cutoff ultrafilter were examined by electrospray mass spectrometry in the negative ion mode. No
 159 significant peaks above background were found in LambdaSa1 lysin digests. So although
 160 LambdaSa1 lysin rapidly decreases the turbidity of a suspension of *S. agalactiae* cell walls, it
 161 does not yield fragments small enough to pass through a 5 K-cutoff ultrafilter. However,
 162 LambdaSa2 lysin digests contained low-molecular weight products that gave remarkably simple
 163 mass spectra (Fig 2A). Two prominent peaks, 694 m/z and 736 m/z were observed. The first
 164 peak corresponds to the M-1 of a disaccharide containing N-acetylglucosamine and N-
 165 acetylmuramic acid linked to Ala-Gln and will have the structure of I or II illustrated below.



166
 167 The production of this glycopeptide indicates that both endopeptidase and glycosidase
 168 activity are present. The second peak at 736 m/z is 42 mass units higher than the first peak and

169 appeared to be the same glycopeptide with an additional acetyl group. Brief alkaline treatment
170 of the sample with aqueous triethylamine resulted in the loss of the 736 m/z peak (Fig 2B),
171 consistent with the presence of a labile O-linked acetyl group. That acetate was released was
172 confirmed by enzymatic measurement of released acetate (data not shown).

173 The presence of alanine and glutamine in the muropeptides is evidence that the lysin
174 possesses an endopeptidase activity capable of cleaving between D- γ -glutamine and lysine in the
175 stem peptide of the peptidoglycan. In order to support this finding, the peptide Ala-D- γ -Gln-Lys-
176 D-Ala-Ala-Ala-amide was synthesized from Ala-D- γ -Glu-Lys-D-Ala-Ala-Ala as described in the
177 Materials and Methods section. Digesting this peptide with LambdaSa2 lysin resulted in the near
178 complete loss of substrate peptide ($M+1 = 558$) and the appearance of a peak at 339 m/z
179 corresponding to the $M+1$ ion of Lys-D-Ala-Ala-Ala-amide, confirming that it is the γ -linkage
180 between D-Gln and L-Lys that is cleaved by this lysin.

181 The amino acid sequence of LambdaSa2 lysin contains two domains, annotated as
182 amidase-5 and amidase-4 domains (see Fig. 1). In contrast, the smaller LambdaSa1 lysin lacks
183 the amidase-4 domain and its amidase-5 domain has 71% amino acid identity to that of the
184 LambdaSa2 lysin. Since digestion of cell walls with either lysin results in the exposure of free
185 amino groups it was predicted that the amidase-5 domain is responsible for endopeptidase
186 activity in both cases. The observed glycosidase activity of the LambdaSa2 lysin would then be
187 expected to be associated with the amidase-4 domain. Since this domain is absent in the
188 LambdaSa1 lysin we predicted that double digestion of *S. agalactiae* cell walls with both
189 LambdaSa1 lysin and the muramidase mutanolysin would yield glycopeptide II. As shown in
190 Fig. 2C the double digest gave the same two muropeptide fragments observed in LambdaSa2

191 digests. Therefore, the amidase-5 domain of the LambdaSa1 lysin is also responsible for
192 peptidoglycan cleavage at its D- γ -Gln-Lys linkage.

193 LambdaSa1 and LambdaSa2 lysin also degrade the cell walls of *Staphylococcus aureus*
194 and *S. pneumoniae*, but the relative proportions of the glycopeptides in LambdaSa2 digests
195 varies. LambdaSa2 digests of *S. agalactiae* cell walls (Fig. 2A) contain approximately equal
196 amounts of a glycopeptide composed of alanine, glutamine, N-acetylglucosamine and N-
197 acetylmuramic acid (m/z 694) and a similar glycopeptide containing an additional acetyl group
198 (m/z 736). However, as shown in Fig. 2D, LambdaSa2 digests of *Staphylococcus aureus* cell
199 walls have a much higher proportion of O-acetylated muropeptide (736 m/z). This O-acetylated
200 glycopeptide was a minor component in digests of *S. pneumoniae* cell walls (Fig 2E). These
201 digests contained about equal amounts of the core disaccharide-dipeptide and an N-deacetylated
202 form of the compound that gives a peak at 652 m/z. Acetylation of *S. aureus* cell walls occurs at
203 the C6-OH of the N-acetylmuramic acid residues and has been reported to render the cell walls
204 resistant to lysozyme (2). Similarly, N-deacetylation of N-acetylglucosamine residues in *S.*
205 *pneumoniae* peptidoglycan has also been shown to make the cell walls resistant to lysozyme
206 (20). Clearly, the presence of O-acetylated and N-deacetylated glycopeptides in LambdaSa2
207 lysin digests show that LambdaSa2 lysin, unlike lysozyme, is capable of cleaving peptidoglycan
208 with these two types of modifications.

209 In order to determine whether the glycosidase activity of LambdaSa2 lysin cleaves after
210 an N-acetylmuramic acid or an N-acetylglucosamine residue, it was necessary to establish their
211 sequence in the glycopeptide digestion products, i.e., whether the dual action of LambdaSa2's
212 glycosidase and endopeptidase activities release glycopeptides I or II, or both. We were unable
213 to confidently determine this by electrospray mass spectrometry alone. We therefore reduced the

214 glycopeptides with sodium borohydride and identified the resulting amino sugar alditol by a
215 GC/MS procedure. As described previously, depolymerization of samples using propanolysis
216 greatly improves the gas chromatographic separation of the amino sugars and alditols (1). In
217 addition, the use of chemical ionization results in very limited fragmentation and yields high-
218 molecular mass ions that can be selectively monitored to identify the amino sugar or alditol.
219 Unexpectedly, the mass spectra of the derivatized four sugars and alditols all possessed
220 molecular ions indicating that not only the hydroxyl groups, but also the nitrogen of the
221 acetamido groups, had been trimethylsilylated (data not shown). Panel 3A shows the total ion
222 chromatogram obtained for a mixture of GlcNAc, GlcNAc-ol (reduced GlcNAc), MurNAc, and
223 MurNAc-ol (reduced MurNAc). Selected ion monitoring of the indicated fragment ions clearly
224 distinguishes each sugar or alditol (Figs. 3B-3E). Fig. 3F shows the results obtained for
225 LambdaSa2 lysin digestion products when the indicated four ions were monitored. Two main
226 peaks are present corresponding to N-acetylglucosaminitol and N-acetylmuramic acid, showing
227 that glycopeptide I was present and was the product of N-acetylglucosaminidase action. That is
228 to say, the LambdaSa2 lysin possesses N-acetylglucosaminidase and not N-acetylmuramidase
229 activity. A much smaller peak due to MurNAc-ol was also present in the chromatogram showing
230 that some MurNAc residues were at the reducing termini of digestion fragments. However, as
231 we pointed out previously, this is most likely because peptidoglycan chains are biosynthesized
232 from lipid-linked glycopeptide precursor units (lipid II) that have MurNAc residues at their
233 reducing termini, and the polysaccharide chains of GBS peptidoglycan are short (1). Analysis of
234 the double digest with LambdaSa1 lysin and mutanolysin gave peaks due to N-
235 acetylglucosamine and N-acetylmuramitol indicating that glycopeptide II was present in this
236 digest. This is consistent with the known N-acetylmuramidase activity of mutanolysin. Thus, it

237 is concluded that the GBS prophage LambdaSa2 lysin possesses both N-acetylglucosaminidase
238 and γ -D-glutamyl-L-lysine endopeptidase activities. The smaller LambdaSa1 lysin possesses
239 only the endopeptidase activity.

240 We previously described the cleavage specificities of the B30 phage lysin of *S.*
241 *agalactiae* (1). We found that it possessed two enzymatic domains, an endopeptidase domain
242 and an N-acetylmuramidase domain. Using synthetic peptide substrates mimicking the stem
243 peptide and cross-bridge peptide of GBS peptidoglycan we showed that the endopeptidase
244 specifically cleaves between the D-Ala of the stem peptide and an L-Ala of the cross bridge.
245 However, the endopeptidase activities of the two lysins described in this paper cleave the stem
246 peptide between D- γ -glutamine and L-lysine. It is of interest, and possible significance, that the
247 B30 lysin and both lysins described in the current study cleave after D-amino acid residues, i.e. at
248 D-Ala-L-Ala or at D- γ -Gln-L-Lys. Cleavage of linkages involving a D-amino acid may help
249 ensure that the endopeptidases target cell wall peptidoglycan and not protein components of the
250 cell wall.

251 Lopez and Garcia pointed out that lysins typically possess both substrate binding and
252 bond cleavage specificities (12). Cell wall binding domains of lysins are responsible for
253 conferring substrate specificity whereas the enzymatic domains determine which bonds of the
254 peptidoglycan are cleaved. The putative cell wall binding domain of the LambdaSa1 lysin is an
255 SH3b domain, while the two putative cell wall binding domains of the LambdaSa2 lysin are of
256 the Cpl-7 type (Fig. 1A). We focused on the enzymatic domains, however, in the current study.

257 Chimeric lysins composed of enzymatic and cell wall binding domains from different
258 sources have been constructed (4-6, 18). Some of these combinations have unique, potentially
259 useful properties. For example, Donovan *et al.* recently fused the gene for lysostaphin to either

260 the entire B30 lysin gene or just the endopeptidase domain of the B30 lysin. Expression of these
261 constructs yielded chimeric enzymes that displayed lytic activity against all three major mastitis-
262 causing pathogens of dairy cattle, *Streptococcus agalactiae* (GBS), *Streptococcus uberis*, and
263 *Staphylococcus aureus* (6). Knowledge of the precise cleavage specificities of phage lysins
264 therefore is important for the rational design of such engineered chimeric enzymes.

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272 Research.

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REFERENCES

276 1. **Baker, J. R., C. Liu, S. Dong, and D. G. Pritchard.** 2006. The endopeptidase and
277 glycosidase activities of the bacteriophage B30 lysin. *Appl. Environ. Microbiol.* **72**:6825-
278 6828.

279 2. **Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz.** 2005. Why are pathogenic
280 staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the
281 major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.*
282 **55**:778-87.

283 3. **Cheng, Q., D. Nelson, S. Zhu, and V. A. Fischetti.** 2005. Removal of group B
284 streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic
285 enzyme. *Antimicrob. Agents Chemother.* **49**:111-7.

286 4. **Croux, C., C. Ronda, R. Lopez, and J. L. Garcia.** 1993. Interchange of functional
287 domains switches enzyme specificity: construction of a chimeric pneumococcal-
288 clostridial cell wall lytic enzyme. *Mol. Microbiol.* **9**:1019-25.

289 5. **Diaz, E., R. Lopez, and J. L. Garcia.** 1991. Chimeric pneumococcal cell wall lytic
290 enzymes reveal important physiological and evolutionary traits. *J. Biol. Chem.* **266**:5464-
291 71.

292 6. **Donovan, D. M., S. Dong, W. Garrett, G. M. Rousseau, S. Moineau, and D. G.**
293 **Pritchard.** 2006. Peptidoglycan hydrolase fusions maintain their parental specificities.
294 *Appl. Environ. Microbiol.* **72** 2988-2996.

- 295 7. **Jado, I., R. Lopez, E. Garcia, A. Fenoll, J. Casal, and P. Garcia.** 2003. Phage lytic
296 enzymes as therapy for antibiotic-resistant *Streptococcus pneumoniae* infection in a
297 murine sepsis model. J. Antimicrob. Chemother. **52**:967-73.
- 298 8. **Lewis, S. D., and J. A. Shafer.** 1973. Conversion of exposed aspartyl and glutamyl
299 residues in proteins to asparaginyl and glutaminyl residues. Biochim. Biophys. Acta
300 **303**:284-291.
- 301 9. **Lin, B., S. K. Hollingshead, J. E. Coligan, M. L. Egan, J. R. Baker, and D. G.**
302 **Pritchard.** 1994. Cloning and expression of the gene for group B streptococcal
303 hyaluronate lyase. J. Biol. Chem. **269**:30113-6.
- 304 10. **Loeffler, J. M., D. Nelson, and V. A. Fischetti.** 2001. Rapid killing of *Streptococcus*
305 *pneumoniae* with a bacteriophage cell wall hydrolase. Science **294**:2170-2.
- 306 11. **Loessner, M. J.** 2005. Bacteriophage endolysins - current state of research and
307 applications. Curr. Opin. Microbiol. **8**:480-7.
- 308 12. **Lopez, R., and E. Garcia.** 2004. Recent trends on the molecular biology of
309 pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol. Rev.
310 **28**:553-80.
- 311 13. **Mokrasch, L. C.** 1967. Use of 2,4,6-trinitrobenzenesulfonic acid for the coestimation of
312 amines, amino acids and proteins in mixtures. Anal. Biochem. **18**:64-71.
- 313 14. **Nelson, D., L. Loomis, and V. A. Fischetti.** 2001. Prevention and elimination of upper
314 respiratory colonization of mice by group A streptococci by using a bacteriophage lytic
315 enzyme. Proc. Natl. Acad. Sci. U S A **98**:4107-12.

- 316 15. **Pritchard, D. G., S. Dong, J. R. Baker, and J. A. Engler.** 2004. The bifunctional
317 peptidoglycan lysin of *Streptococcus agalactiae* bacteriophage B30. *Microbiology*
318 **150**:2079-87.
- 319 16. **Schuch, R., D. Nelson, and V. A. Fischetti.** 2002. A bacteriolytic agent that detects and
320 kills *Bacillus anthracis*. *Nature* **418**:884-9.
- 321 17. **Sehgal, D., and I. K. Vijay.** 1994. A method for the high efficiency of water-soluble
322 carbodiimide-mediated amidation. *Anal. Biochem.* **218**:87-91.
- 323 18. **Sheehan, M. M., J. L. Garcia, R. Lopez, and P. Garcia.** 1996. Analysis of the catalytic
324 domain of the lysin of the lactococcal bacteriophage Tuc2009 by chimeric gene
325 assembling. *FEMS Microbiol. Lett.* **140**:23-8.
- 326 19. **Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels,**
327 **I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J.**
328 **Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay,**
329 **R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S.**
330 **Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E.**
331 **T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaudo, R.**
332 **Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser.** 2002. Complete
333 genome sequence and comparative genomic analysis of an emerging human pathogen,
334 serotype V *Streptococcus agalactiae*. *Proc. Natl. Acad. Sci. U S A* **99**:12391-6.
- 335 20. **Vollmer, W., and A. Tomasz.** 2002. Peptidoglycan N-acetylglucosamine deacetylase, a
336 putative virulence factor in *Streptococcus pneumoniae*. *Infect. Immunol.* **70**:7176-8.
- 337
- 338

339

FIGURE LEGENDS

340

341 **FIG. 1.** Domain structure of the cloned LambdaSa1 and LambdaSa2 lysins (A) and structure of
342 GBS peptidoglycan showing the glycosidic bond cleaved by LambdaSa2 lysin and the
343 endopeptidase cleavage site for both lysins (B).

344

345 **FIG. 2.** Negative ion mass spectra of the low-molecular weight products of lysin-digested
346 bacterial cell walls. (A) LambdaSa2-digested GBS cell walls, (B) LambdaSa2-digested GBS cell
347 walls treated with 5% (v/v) aqueous triethylamine, (C) LambdaSa1 plus mutanolysin-digested
348 GBS cell walls, (D) LambdaSa2-digested *Staphylococcus aureus* cell walls, and (E) LambdaSa2-
349 digested *Streptococcus pneumoniae* cell walls. The 694.4 m/z fragment is due to a disaccharide
350 dipeptide composed of N-acetylglucosamine, N-acetylmuramic acid, alanine and glutamine. The
351 652.4 m/z fragment has one less acetyl group and the 736.4 m/z fragment has one more acetyl
352 group than the core glycopeptide (694.4).

353

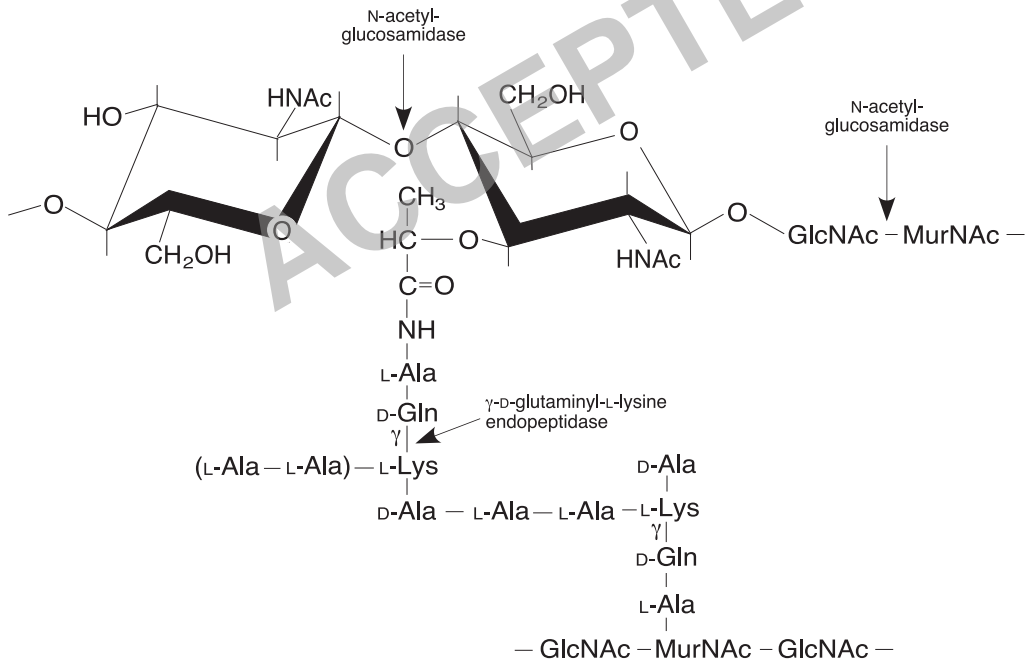
354 **FIG. 3.** Identification of the site of glycosidic bond cleavage by GC/MS. (A) Total ion
355 chromatogram (TIC) of a mixture of derivatized N-acetylglucosamine, N-acetylglucosaminitol,
356 N-acetylmuramic acid, and N-acetylmuramitol. (B) Selected ion chromatograms of the same
357 sample for m/z 584 (GlcNAC-ol), (C) m/z 552 (GlcNAc), (D) m/z 626 (MurNAc-ol), and (E)
358 m/z 462 (MurNAc). (F) Selected ion chromatogram for the four ions of m/z 462, 552, 584, and
359 626 for a LambdaSa2 digest of GBS cell walls. (G) Selected ion chromatogram for the same
360 four masses for a double digest of GBS cell walls by LambdaSa1 and mutanolysin.

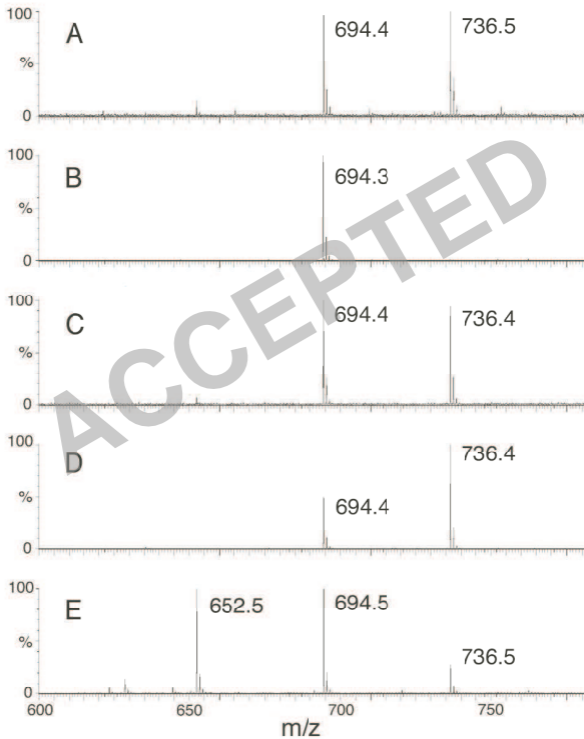
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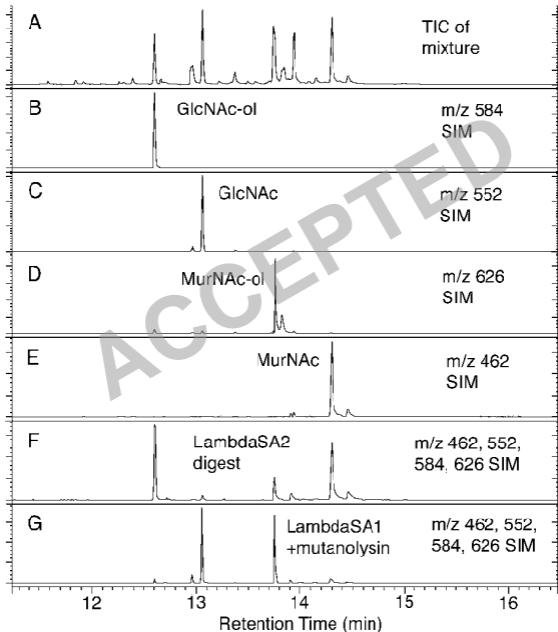
LambdaSa1
lysin



LambdaSa2
lysin

**B**





MurNAc-GlcNAc

|

L-Ala

|

D-iGln

I

GlcNAc-MurNAc

|

L-Ala

|

D-iGln

II

ACCEPTED