The ability of many phage endolysins to specifically lyse various gram-positive bacteria exposed to the enzymes externally has led to recent intense interest in using endolysins as antimicrobial agents (3–6, 9, 12–14, 18). Endolysins have been found that are capable of cleaving most bonds in peptidoglycan (10). These include glycosidases, amidases, and endopeptidases. Glycosidases cleave the polysaccharide backbone of peptidoglycan and include N-acetyl-β-D-glucosaminidases, N-acetyl-β-D-muramidases, and lytic transglycosylases. N-Acetylmuramyl-L-alanine amidases cleave the amide bond between the lactic acid side chain of N-acetylmuramic acid (MurNAc) and the L-alanine of the stem peptide. Endopeptidases capable of cleaving either the stem peptide or cross bridges of peptidoglycan have also been described (8, 11, 17). Unfortunately, assignments of cleavage specificities of lysins are often based upon sequence homologies and, in the absence of experimental evidence, may not be correct. The widespread misnaming of lysins with known cleavage specificities compounds this problem. For example, T7 phage amidase is often referred to as T7 lysozyme.

We previously described the cloning of the gene for a group B streptococcal (GBS) bacteriophage lysin and partially characterized the expressed enzyme (14). The enzyme, termed GBS bacteriophage B30 lysin, lysed several beta-hemolytic streptococci, including group A, B, C, E, and G streptococci. It possesses both glycosidase and endopeptidase activities, since reducing end groups as well as N-terminal alanine residues are generated during cell wall lysis. Site-directed mutagenesis established that the endopeptidase activity was located in the N-terminal cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) domain (1, 15) and that the glycosidase activity associated with the central acetylmuramidase (Acm) domain (Fig. 1A). The phage B30 lysin also contains a C-terminal SH3b putative cell wall-binding domain (19). Donovan and coworkers recently reported that enzymatic activity associated with the CHAP domain is primarily responsible for target cell lysis from without (5).

**Enzyme purification and substrates.** Recombinant bacteriophage B30 lysin was expressed and purified, GBS strain 3331 cell walls were prepared, and an aliquot of cell walls was acetylated as described previously (14). Peptides corresponding to the portions of the stem peptide and cross bridges of GBS peptidoglycan shown in Table 1 (16) were custom synthesized by Genscript Corp. (Piscataway, NJ). Ala-Ala and D-Ala–Ala–Ala–Ala were obtained from Sigma Chemical Co. (St. Louis, MO). For some experiments, peptide substrates were N-acetylated by dissolving each peptide (<20 μg) in 40 μl water, followed by the addition of 140 μl ethanol, 20 μl triethylamine, and 20 μl acetic anhydride. After 30 min at room temperature, the mixture was rapidly dried by vacuum centrifugation and resuspended in 95 μl water plus 5 μl triethylamine. After being dried again, the acetylated peptide was dissolved in water at approximately 2 μg of peptide/μl.

**Digestion of peptides by phage B30 lysin.** Peptides (10 μg) were incubated with lysin (5 μg) in 0.05 M sodium acetate, 10 mM CaCl₂, pH 6.0 (total volume of 25 μl), at 30°C for 18 h. Digests were diluted with water (200 μl), and peptide digestion products were separated using an HP capillary electrophoresis (CE) system (Hewlett-Packard Co., Palo Alto, CA). Samples were injected hydrostatically (20 s at 5 kPa) onto an uncoupled silica capillary (50 μm by 72 cm). Electrophoresis was done at 20 kV in a phosphate-borate-sodium dodecyl sulfate buffer, pH 9.3, at 40°C for 15 min (2).

**CE analysis of a digest of Ala–D-γ-Glu–Lys–D-Ala–Ala–Ala,** corresponding to the stem peptide and cross bridge of GBS peptidoglycan (Fig. 1), gave peaks at 8.7 and 11.4 min, which corresponded to Ala–D-γ-Glu–Lys–D-Ala and Ala-Ala, respectively (Fig. 2A). Coelectrophoresis of the digest with the subunit showed that digestion was complete (Fig. 2B). Electrospray mass spectrometry of the digest, carried out on a Micromass Q-TOF 2 mass spectrometer, confirmed the presence of peptides with the expected masses of 417 Da for Ala–D-γ-Glu–Lys–D-Ala and 160 Da for Ala-Ala.

The peptide D-γ-Glu–Lys–D-Ala–Ala–Ala, which lacks the N-terminal Ala, also was cleaved by the enzyme. However, a truncated peptide lacking the C-terminal Ala, Ala–D-γ-Glu–Lys–D-Ala–Ala, was not cleaved. Substituting an L-Glu for D-Glu gave γ-Glu–Lys–D-Ala–Ala–Ala, which was also cleaved by the enzyme. However, replacing the γ-linked Glu with α-linked Glu gave Ala–D-Glu–Lys–D-Ala–Ala–Ala, which was not cleaved. Replacing the D-Ala with L-Ala gave D-γ-Glu–Lys–D-Ala–Ala–Ala, which was not cleaved. Lys–D-Ala–Ala–Ala and D-Ala–Ala–Ala were also not cleaved (Table 1).

The above experiments showed that the minimum peptide sequence necessary in a substrate for the phage B30 lysin is...
DL-γ-Glu–Lys–D-Ala–Ala–Ala. There is also an absolute requirement for D-Ala, but either D-Glu or L-Glu may be present, provided that they are γ-linked, not α-linked, to the Lys of the stem peptide. In addition, both alanines of the cross bridge must be present for cleavage to occur.

Identification of reducing sugar termini exposed by digestion of GBS cell walls with phage B30 lysin. Cell wall suspensions (1.0 ml) in 20 mM ammonium acetate, 5 mM CaCl₂, pH 6.8, with an initial optical density at 550 nm (OD₅₅₀) of 1.0 were incubated with 0.76 mg of lysin (40 μl of 19-mg/ml lysin) for 2 h or 18 h at 37°C, and the reduction in turbidity (i.e., OD₅₅₀) was measured (Table 2). Reducing end groups in cell wall peptidoglycan were reduced by incubation at 4°C for 16 h with an equal volume of ice-cold 20-mg/ml NaBH₄ in 0.2 M NaHCO₃. Borohydride and bicarbonate were decomposed by carefully adding an excess of methanol-acetic acid (9:1 [vol/vol]) to the samples, which were then dried by vacuum centrifugation in a SpeedVac (Savant Instruments, Hicksville, NY). Borate was removed as methyl borate by adding acidified methanol and drying the samples four more times. The samples were resuspended in 1.0 ml of water, and 200-μl aliquots were dried under vacuum in 2-ml glass vials, resuspended in 0.4 ml propanolic HCl, and placed in a heating block at 80°C for

TABLE 1. Susceptibilities of synthetic peptides to cleavage by phage B30 endopeptidase

<table>
<thead>
<tr>
<th>Substrate Products</th>
<th>Cleaved substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d–γ-Glu–Lys–d–Ala–Ala–Ala–Ala–Ala–Ala–Ala</td>
</tr>
<tr>
<td></td>
<td>d–γ-Glu–Lys–d–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala</td>
</tr>
<tr>
<td></td>
<td>Ala–d–γ-Glu–Lys–d–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala–Ala</td>
</tr>
</tbody>
</table>

A

B

FIG. 1. Domain structure of cloned bacteriophage B30 lysin (A) and structure of GBS peptidoglycan with sites of B30 lysin cleavage indicated (B). SH3b, putative bacterial cell wall-binding domain; His₆, hexahistidine affinity tag. The highlighted area depicts the minimum peptide structure required for endopeptidase cleavage.

TABLE 2. Gas chromatographic analysis of amino sugars and alditols in cell walls after B30 lysin digestion

<table>
<thead>
<tr>
<th>GBS cell wall state</th>
<th>Presence of phage B30 lysin</th>
<th>Incubation time (h) at 37°C</th>
<th>% MurNAc-ol&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>+</td>
<td>2</td>
<td>74.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>94.3</td>
</tr>
<tr>
<td>Not reduced</td>
<td>−</td>
<td>ND</td>
<td>19.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> After B30 lysin digestion for 2 h and 18 h, the turbidities of the digests (OD<sub>550</sub>) had decreased 38% and 49%, respectively.

18 h. Propanolic HCl was freshly prepared by carefully adding 0.5 ml acetyl chloride to 4.5 ml 1-propanol and allowing the mixture to react for 30 min prior to use. After propanolysis, insoluble NaCl in the sample was removed by centrifugation, and an aliquot of the supernatant (200 μl) was dried by vacuum centrifugation. Re-N-acetylation of amino sugars in the samples was accomplished by dissolving them in 200 μl methanol and adding 20 μl acetic anhydride followed by 20 μl pyridine. After 30 min at room temperature, the samples were dried under vacuum, redissolved in 100 μl of methanol, and transferred to glass conical inserts in sample vials. After evaporation to dryness, samples were trimethylsilylated by dissolving them in 50 μl of Tri-Sil (Pierce Chemical Co., Rockford, IL) under argon. Derivatized sugars and alditols were separated and quantitated on an HP 5890 gas chromatograph (Hewlett Packard) equipped with a 30-m HP-1 wide-bored fused-silica column coated with a 0.88-μm layer of cross-linked methylsilicone gum. Aliquots (1 μl) were applied to the column with an automatic sample injector, and peaks were monitored using a flame ionization detector. After injection, the oven temperature was maintained at 100°C for 5 min and then increased to 275°C at a rate of 20°C per min. Retention times for derivatized N-acetylglucosaminol, N-acetylglucosamine, N-acetylmuramitol, and N-acetylmuramic acid were 12,888, 13,187, 14,009, and 14,383 min, respectively.

Approximately one-fifth of N-acetylmuramic acid residues in undigested cell walls, but no N-acetylglucosamine residues, were reduced (Table 2). Presumably, this is because peptidoglycan chains are biosynthesized via a lipid-linked disaccharide-peptide precursor (lipid II) that has an N-acetylmuramic acid residue at its reducing terminus (7) and because the carbohydrate chains are relatively short. Digestion of GBS cell walls with phage B30 lysin for 2 h increased the percentage of MurNAc that was reduced from 19.7% to 74.7%, and overnight digestion resulted in 94.3% reduction (Table 2). Figure 3 shows the gas chromatogram obtained for the 2-h digest. This data clearly show that phage B30 lysin cleaves N-acetylmuramic acid–N-acetylmuramic acid bonds in GBS peptidoglycan and is therefore an N-acetyl-β-D-muramidase.

Donovan and coworkers recently described the construction and properties of chimeric lysins containing both lysostaphin and either the entire phage B30 lysin or only its endopeptidase domain (4). These chimeric lysins displayed lytic activity against all three major mastitis-causing pathogens of dairy cattle, i.e., *Streptococcus agalactiae* (GBS), *Streptococcus uberis*, and *Staphylococcus aureus*. Knowledge of the precise cleavage specificities of phage lysins will be important for the rational design of such engineered and chimeric enzymes.

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**REFERENCES**


