The bifunctional peptidoglycan lysin of \textit{Streptococcus agalactiae} bacteriophage B30

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A group B streptococcal (GBS) bacteriophage lysin gene was cloned and expressed in \textit{Escherichia coli}. The purified recombinant enzyme, calculated to have a molecular mass of 49,677 Da, lysed GBS cells. The susceptibility of GBS cells to lysis by the enzyme depended upon the growth stage at which they were harvested, with early exponential phase cells most sensitive. Calcium ions enhanced the activity of the enzyme. The enzyme also lysed other \(\beta\)-haemolytic streptococci, including groups A, C, E and G streptococci, but not common oral streptococci, including \textit{Streptococcus mutans}. The generation of both reducing activity and N-terminal alanine residues during lysis indicated that the lysin is a bifunctional enzyme, possessing both glycosidase and endopeptidase activities. This is consistent with the presence of two conserved sequence domains, an Acm (acetylmuramidase) domain associated with lysozyme activity, and a CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain associated with endopeptidase activity. Site-directed mutagenesis of conserved cysteine and histidine residues in the CHAP domain and conserved aspartate and glutamate residues in the Acm domain confirmed their importance for lysozyme and endopeptidase activity respectively.

INTRODUCTION

Group B streptococci (GBS) remain a major cause of serious neonatal bacterial infections (Centers for Disease Control and Prevention, 1996). Babies with early-onset GBS infections acquire the bacteria from the birth canal of their mothers (Yow \textit{et al.}, 1980). Since about one-fifth of pregnant women are vaginally colonized with GBS at the time of delivery, a high percentage of babies are born colonized with the organism and many develop potentially fatal infections (Regan \textit{et al.}, 1996).

GBS, like most other bacterial pathogens, often harbour bacteriophages. Bacteriophages of GBS were first isolated from bovine strains of the bacteria in 1969 (Russell \textit{et al.}, 1969). Phages were subsequently reported to be ubiquitous in human GBS strains and they formed the basis for various phage-typing systems for GBS (Haug \textit{et al.}, 1981; Stringer, 1980). Double-stranded DNA bacteriophages usually utilize a two-component lytic system that includes both a holin and a lysin (Grundling \textit{et al.}, 2001). A phage holin released intracellularly first disrupts the bacterial cell membrane, exposing the peptidoglycan to the degradative action of the lysin, which leads to cell lysis and the release of progeny phage. The lysin alone, however, is often sufficient to lyse the bacteria when they are exposed to preparations of the enzyme externally (Loeffler \textit{et al.}, 2001).

The use of bacteriophage lysins to selectively destroy their bacterial targets has been the subject of three recent reports. In one case, nasopharyngeal colonization of mice by group A streptococci was eliminated by treatment with a purified lysin isolated from a group C streptococcal phage lysate (Nelson \textit{et al.}, 2001). This C1 phage lysin specifically killed groups A, C and E streptococci but not 14 other commensal streptococci. It also did not affect GBS or streptococci belonging to groups D, F, G, L and N. In another report, nasopharyngeal colonization of mice by pneumococci was eliminated by treatment with a recombinant pneumococcal phage lysin (Loeffler \textit{et al.}, 2001). This enzyme was highly specific for \textit{Streptococcus pneumoniae}. In a third report, the PlyG lysin isolated from the \(\gamma\) phage of \textit{Bacillus anthracis} was shown to specifically kill the bacteria both \textit{in vitro} and \textit{in vivo} (Schuch \textit{et al.}, 2002). There are also many examples of micro-organisms secreting lysins to prevent the growth of other bacteria. For example, lysostaphin, a bacteriocin produced by \textit{Staphylococcus simulans}, has long been known to specifically cleave the pentaglycine cross-bridges in the peptidoglycan of other susceptible staphylococci (Schindler & Schuhardt, 1964). The enzyme was reported to dramatically reduce aortic valve vegetation bacterial counts in a rabbit model of

Abbreviations: Acm domain, 1,4-\(\beta\)-N-acetylmuramidase domain; CHAP domain, cysteine histidine-dependent amidohydrolases/peptidases domain; GBS, group B streptococci (\textit{Streptococcus agalactiae}); TNBS, 2,4,6-trinitrobenzenesulfonic acid.

The GenBank accession number for the nucleotide sequence of the GBS phage lysin and flanking regions reported in this paper is AY149214.
Staphylococcus aureus endocarditis (Patron et al., 1999). However, there have been no reports describing the lysins of GBS phages. In this paper we describe the bifunctional lysin of a GBS bacteriophage with a view to its use in the prevention of GBS infections.

**METHODS**

**Bacterial strains and culture conditions.** GBS strains were stored at −70°C in sheep blood and routinely plated on trypticase soy agar containing 5% (v/v) sheep blood or cultured in Todd–Hewitt broth (Difco) at 37°C without shaking. Bacteriophages were detected and propagated on a solid medium consisting of 30 g Todd–Hewitt broth base, 2 g yeast extract, 12 mg CaCl₂ and 10 mg L-tryptophan l⁻¹ (Stringer, 1980). Groups A, C, E and G streptococci, Streptococcus faecalis, Streptococcus mutans serotypes c (strain AHT), e (strain LM-7) and f (strain DMZ 175), Streptococcus cricetus (strain AHT), Streptococcus sobrinus (strain SL-1), Streptococcus mitis and Staph. aureus were also grown in Todd–Hewitt broth. The autolysis-deficient mutant of *Strep. pneumoniae*, L82016 (LyTA⁻), kindly provided by Dr Susan Hollingshead, Dept of Microbiology, University of Alabama at Birmingham, was grown in Todd–Hewitt broth containing 5 g l⁻¹ yeast extract. Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus brevis and Lactobacillus delbruecki, kindly provided by Dr Jane Schwebke, Dept of Medicine, University of Birmingham, were grown in MRS broth (Difco).

*Escherichia coli* strains INV-A² and BL21(DE3) were routinely grown with shaking in Luria–Bertani (LB) broth at 37°C or at 30°C in Terrific broth (TB) (per litre: 4.8 g Bacto tryptone, 9.6 g Bacto yeast extract, 2.35 g glycerol, adjusted after autoclaving to pH 7 with KH₂PO₄) for protein expression. Recombinant clones were grown in media supplemented with 50 μg ampicillin ml⁻¹.

**Induction and isolation of GBS bacteriophages.** GBS strains from our collections were screened for lysogenic bacteriophages by exposing cultures of the bacteria to mitomycin C. The complete lysin gene was isolated from the phage genomic DNA by PCR amplification, using a forward primer containing an Ndel site (5’ GCA CTA CAT ATG GCA ACT TATCAA GAA TAA AG 3’) and a reverse primer containing an Xhol site (5’ GTG GTG CTC GAG CAT ATC TGTT GTC GTT AAC TAA GTA GC 3’). The PCR product was cloned into the pGEM T-vector (Promega) and transformed into INV-A². Purified plasmid DNA was then cleaved with restriction endonucleases Ndel and Xhol and the insert cloned into the expression vector pET21a (Novagen) and transformed into INV-A². Additional amino acids were added to the carboxyl-terminal end of the protein by sequences included in the pET21a plasmid, including a 6x histidine tag to aid with affinity purification. The sequence of the resulting plasmid was confirmed by DNA sequencing. The plasmid was then transformed into E. coli strain BL21(DE3) for enzyme expression.

**Expression and purification of the cloned GBS phage lysin.** A starter culture of *E. coli* BL21(DE3) containing the recombinant plasmid was grown overnight in TB medium containing 50 μg ampicillin ml⁻¹ at 37°C with shaking at 250 r.p.m. This culture was diluted with 20 vols fresh medium (3 l) and grown at 37°C until it reached mid-exponential phase (OD₅₅₀ 0.5). Lysin expression was induced by adding IPTG to give a final concentration of 1 mM. After growing for an additional 4.5 h at 30°C, the cells were harvested by centrifugation and washed three times with extraction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The pellet was resuspended in 100 ml extraction buffer and sonicated in an ice bath for a total of 5 min at 60% of maximum power using a model 300 Fisher Sonic Dismembrator. Sonication was carried out for 30 s periods, allowing sufficient time between each period to prevent overheating the sample. Cell debris was removed by centrifugation and the resulting supernatant passed over a 10 ml Ni–NTA affinity column (Qiagen), according to the manufacturer’s instructions. The column was washed with 50 ml 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0, and the His-tagged lysin was eluted with 50 ml 50 mM NaH₂PO₄, 300 mM NaCl, 200 mM imidazole, pH 8.0. The eluate was concentrated by ultrafiltration using a Centriprep-30 ultrafiltration device (Amicon) and the buffer exchanged three times with 50 mM ammonium acetate, 1 mM DTT, pH 6.2, to remove phosphate ions, and then once with lysin buffer A (50 mM ammonium acetate, 10 mM CaCl₂, 1 mM DTT, pH 6.2). The final product gave a single band with a molecular mass of 51 kDa upon SDS-PAGE.

**Turbidity reduction assay for GBS phage lysin.** GBS lysin activity was assessed by following the reduction in turbidity of a suspension of strain 3331 GBS cells after enzyme addition. Buffered GBS cell suspensions do not exhibit detectable autolysis and may be stored in the refrigerator for many weeks. In order to optimize assay conditions, the effect of calcium ion concentration on lysin activity was determined at 30°C in 50 mM ammonium acetate, 1 mM DTT, pH 6.2, containing different concentrations of CaCl₂.
The pH optimum of the phage lysin was then determined in the presence of 10 mM CaCl₂, 1 mM DTT. A unit of activity was defined as the amount of enzyme that reduces the optical density of 1 ml of a suspension of strain 3331 GBS cells (adjusted to an initial OD₅₅₀ of 0.8) by 0.001 min⁻¹ at 30°C. The susceptibility of other species of bacteria to lysis by GBS phage lysin was also assessed by monitoring turbidity reduction. In all cases the cells were grown to an OD₅₅₀ of 0.4, harvested by centrifugation, washed three times in lysin buffer A, and resuspended in the same buffer to give an OD₅₅₀ of 0.8. Lysin (10 μl) was added to 0.4 ml of the cell suspension at 30°C and the rate of decrease in turbidity during the first 2 min was determined.

**Preparation of cell walls.** A crude cell wall preparation made from GBS strain 3331 cells was also a suitable substrate for the phage lysin. It was made by passing a suspension of GBS cells, grown to an OD₅₅₀ of 0.4, through a French press operated at approximately 15 000 p.s.i. (1·0·10⁸ Pa). Unbroken cells were removed by low-speed centrifugation (5000 g) for 5 min, and the cell walls were recovered from the supernatant by centrifugation at 20 000 g for 45 min. The cell walls were washed three times in lysin buffer A. For studies of the glycosidase and endopeptidase activities of the GBS phage lysin, the cell walls were washed an additional three times with water. The final stock cell wall preparation, adjusted to an OD₅₅₀ of 1.0, was stored at 4°C.

Cell walls were acetylated by a modified standard procedure (Riordan & Vallee, 1967). Stock GBS cell walls in water (2·0 ml) were diluted with an equal volume of saturated aqueous sodium acetate at 4°C and acetylated by adding 5 x 40 μl aliquots of acetic anhydride over a period of 1 h with constant stirring. The acetylated cell walls were washed three times with water, then diluted with water to give a suspension with an OD₅₅₀ of 1.0 at 550 nm and stored at 4°C. A test for free amino groups using TNBS (Mokrasch, 1967) gave a negative reaction for acetylated cell walls (i.e. the cell walls remained white), whereas the nonacetylated cell walls were strongly positive (i.e. they became yellow–brown in colour).

**Measurement of endopeptidase activity.** Incubation mixtures (50 μl) in lysin buffer B (50 mM sodium acetate, 10 mM calcium chloride 1 mM DTT, pH 5.6) contained lysin (3-5 μg) and cell walls to give an OD₅₅₀ of 0.90. Incubations were at 30°C for 18 h and were stopped by heating at 100°C for 5 min. Undigested cell walls were pelleted by centrifugation at 5000 g for 15 min and the supernatant passed through a 5000 MW cutoff filter (Ultrafree, Millipore). Aliquots (20 μl) of the filtrate were assayed for free amino groups using the TNBS method (Mokrasch, 1967) and serine as the standard.

The soluble products released from GBS cell walls by digestion with phage lysin were also analysed by N-terminal amino acid sequencing. In this case, incubation mixtures were clarified by centrifugation, the supernatants passed through Ultrafree-MC 5000 MW cutoff filters, and 20 μl aliquots applied to protein support disks for N-terminal sequencing. Insoluble cell wall residues from the same incubation mixtures were recovered by centrifugation, washed once with lysin buffer B, twice with water, and an equivalent aliquot applied to a protein support disk. Cell wall controls, not digested with the lysin, were treated similarly. All samples were subjected to three or four cycles of Edman degradation and the released PTH-amino acid(s) were identified following separation by RP-HPLC. Analyses were performed using a Beckman peptide microsequencer (model PI 2090E).

**Measurement of glycosidase activity of GBS phage lysin.** Lysin digests of cell walls were assayed for cleavage of the peptido-glycan polysaccharide backbone using a modified Park–Johnson method (Spiro, 1966). Briefly, digests were clarified by centrifugation, when necessary, and aliquots (0·2 ml) were heated with 0·05% (w/v) aqueous potassium ferricyanide (0·2 ml) and 0·53% (w/v) sodium carbonate/0·065% (w/v) potassium cyanide in water (0·2 ml) at 100°C for 15 min. Under these alkaline conditions, reducing groups liberated by the cleavage of glycosidic bonds in the peptidoglycan glycan chain will stoichiometrically reduce ferricyanide to ferrocyanide. Upon cooling, 1·0 ml of a ferric iron reagent [0·15% (w/v) ferric ammonium sulfate/0·1% (w/v) SDS in 0·025 M sulfuric acid] was added, which forms a stable prussian blue colour. After 15 min at room temperature, the Δ₅₉₀ was measured. Data were converted to glucose equivalents using a standard curve.

**Site-directed mutagenesis.** Plasmid DNA was prepared using a Qagen Midiprep Kit. Specific mutations in the lysin gene were made using a QuickChange Site-Directed Mutagenesis Kit (Strategene Cloning Systems). The following mutagenesis forward primers were used: C26S, 5’ GGC TAC GCA GAT TAC AGG TAT CTA GGA ATG C 3’; C34S, 5’ GGC TAC GCA GAT TAC AGT AAG TAT CTA GGA CGT C3’; C44S, 5’ GCC ATA CGC AAA CAG TAC AAA TAC AGG ATA CGC 3’; C171S, 5’ GCA GAT TTA ACA ACG ACT AGT CAG CAC CGG GCC 3’; H91A, 5’ GAC GGT GTA ACG CCT TAC AGT GCT GTA GCA ATT TTT GAC AGC G 3’; D158A, 5’ AAT AAA GGT GAT TAC TTT ATC GCT GTA TCG GCT TAT CAA CAA GC 3’; E185A, 5’ CGA TTA TCA AGG TAT CCG GGT CAA TTG CCT GGC TGT CTG ACA G 3’. The reverse primers were the exact complements. The PCR reaction mixtures contained 80 ng plasmid DNA template, 12 pmol each of the forward and reverse primers containing the desired mutation, nucleoside triphosphates, reaction buffer and Pfu Turbo DNA polymerase (Strategene Cloning Systems). After overlying with mineral oil, each sample was placed in a thermal cycler and denatured for 1 min at 95°C and then subjected to 25 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 14 min. The reaction mixtures were then cooled and digested with 1 μl DpnI restriction enzyme to digest the parental DNA template. A 10 μl aliquot of the DpnI-treated DNA was used to transform E. coli INV-45’ competent cells (Invitrogen). Transformed cells were plated on LB agar containing 50 μg ampicillin ml⁻¹, and after overnight growth, colonies were picked and minipreps were prepared from the clones. The plasmid inserts were sequenced to confirm that the desired nucleotide sequence change had been introduced.

**RESULTS**

**Cloning and sequencing the GBS bacteriophage B30 lysin gene.**

GBS strains often contain prophages that can be induced by various treatments (Haug et al., 1981). We exposed serotype III GBS strain 3330 to mitomycin C and recovered bacteriophages that gave small (~0·5 mm) clear plaques on a lawn of serotype III GBS strain 3331. Both strains were clinical isolates that did not produce hyaluronan lyase. We selected one phage clone (B30) for further study. Phage particles were purified by precipitation with PEG 8000 followed by CsCl density-gradient ultracentrifugation, and phage chromosomal DNA was isolated using standard procedures (Ausubel et al., 1998). A plasmid library of HindIII fragments of phage DNA was constructed in pUC19. The HindIII ends of inserts in randomly selected clones in the plasmid library were sequenced using vector-specific primers and a portion of putative phage lysin gene was found in one clone (plasmid 35). A 744 bp region of the insert exhibited 89% identity with the GBS phage lysin gene.
nucleotide sequence identity to a putative phage lysin in the chromosome of *Streptococcus pyogenes* M1 strain SF370 and 88% sequence identity to a *Strep. equi* phage lysin. Primers based on the sequenced region were then used to sequence the entire lysin gene in the intact phage chromosomal DNA.

The sequence of the gene and flanking regions revealed both an upstream (nucleotides 66–99) and a downstream (nucleotides 1512–1539) stem-and-loop structure that could serve as transcription terminators. A putative ribosome-binding site is present starting 13 nucleotides prior to the predicted ATG start codon for the lysin gene. There is also an in-frame TAA stop codon immediately prior to the ATG start, suggesting that the predicted lysin gene is part of some larger multi-domain protein. The calculated molecular mass of the 443 amino acid enzyme is 49,677 Da.

**Lytic specificity of bacteriophage B30**

We knew from previous studies that both the carrier strain of bacteriophage B30 (GBS 3330) and our propagation strain (GBS 3331) did not secrete hyaluronan lyase. Inability of GBS strains to secrete hyaluronan lyase arises because the *hylB* gene is interrupted by the insertion sequence IS1548 and is characteristic of a minor subtype of serotype III GBS strains, restriction–digestion pattern type III-2 (Takahashi et al., 2002). We therefore screened a collection of 33 GBS strains for their susceptibility to lysis by bacteriophage B30. Neither the serotype Ia (6), serotype Ib (4) and serotype II (3) GBS strains nor the eight hyaluronan-lyase-positive serotype III strains were lysed by the phage. Twelve hyaluronan lyase-negative serotype III strains were tested: nine were lysed and three were not. The three strains that were not lysed all contained a lysin gene that could be amplified by PCR, suggesting that they already contained a prophage. Attempted PCR amplification of the lysin gene in the other 30 GBS strains did not yield an amplicon, except for a single hyaluronan-lyase-negative serotype III strain.

**Expression of the GBS bacteriophage B30 lysin gene**

Based on the sequence data, the gene was amplified by PCR from phage chromosomal DNA, digested with restriction endonucleases, and cloned into the expression vector pET21a, as described in Methods. The entire sequence of the cloned gene was confirmed in both strands by DNA sequencing. The cloned phage lysin gene was then expressed in *E. coli* and the product was purified on a Ni-NTA nickel affinity column. The purified enzyme, which gave a single band upon SDS-PAGE with a molecular mass of 51 kDa, rapidly reduced the turbidity of a suspension of GBS strain 3331 cells.

**Properties of the phage lysin**

The effect of calcium ion concentration on lysin activity was determined while optimizing the assay conditions.

Initial experiments suggested that the susceptibility of GBS cells to lysis depended upon the growth stage at which they were harvested. Stationary phase cells appeared to be a poorer substrate than early exponential phase cells. This observation was confirmed by removing aliquots of a freshly inoculated culture of strain 3331 GBS cells at defined intervals during growth, washing the cells, and resuspending them to give an OD550 of approximately 0.8. The rate of lysis of the suspensions was then determined after addition of a fixed amount of the GBS phage lysin. As shown in Fig. 2, GBS cells harvested in early exponential phase (2–3 h after inoculation) were much more rapidly lysed than cells in late exponential phase (3.5 h) or stationary phase (4.5 h). Resistance to lysis did not increase further after overnight growth (data not shown). In all subsequent experiments, GBS cells to be used as a substrate for the phage lysin were grown to an OD550 of 0.4. Suspensions of washed GBS cells, either kept at 4°C or stored frozen at -70°C, were satisfactory substrates for the phage lysin, but GBS cells heat-killed by placing in a boiling water bath for 5 min were lysed much more slowly. Similarly, the enzyme rapidly lysed crude cell wall preparations, but only slowly lysed heat-treated cell walls (data not shown).

**Specificity of the phage lysin**

The ability of the GBS phage enzyme to lyse cells of other streptococcal species and other Gram-positive bacteria was determined while optimizing the assay conditions.
was also studied. Although phage B30 was highly specific and infected, and lysed only a small subgroup of type III GBS strains, the lysozyme itself was capable of lysing all \( \beta \)-haemolytic streptococci tested, including groups A, B, C, E and G streptococci (Fig. 3). In all cases, the test bacteria were harvested by centrifugation after they had reached an OD\(_{550}\) of 0.4, and resuspended in lysin buffer A to an OD\(_{550}\) of 0.8. As shown in Fig. 3, group A streptococci were most sensitive to lysis by the GBS phage lysozyme followed by groups C and G streptococci. GBS strain 3331 was lysed only about one-quarter as rapidly as group A streptococci. Strep. faecalis, group E streptococci and an autolysis-negative mutant of Strep. pneumoniae (LytA\(^{-}\)) were lysed very slowly. The GBS phage lysozyme, however, had no detectable effect on the following bacteria: Strep. cricetus AHT (serotype a), Strep. rattus FA-1 (serotype b), Strep. rattus BHT (serotype b), Strep. mutans MT8148 (serotype c), Strep. mutans GS-5 (serotype c), Strep. mutans GLM-7 (serotype e), Strep. mutans DMZ 175 (serotype f), Strep. mitis, Staph. aureus, L. acidophilus, L. brevis, L. casei and L. delbrueckii.

**Identification of enzymic activities**

Analysis of conserved domains within the predicted open reading frame for the lysozyme gene using the CDD search system at NCBI (Marchler-Bauer et al., 2002, 2003) revealed two conserved domains. A CHAP domain (pfam05257.1; amino acids 6–107) is present at the amino terminus and an Acm domain (COG3757.1; amino acids 145–344) is located in the central part of the protein. Since the Acm domain present in the GBS phage lysozyme is associated with a lysozyme-type activity in other proteins, we determined whether the enzyme possessed glycosidase activity consistent with lysozyme activity. GBS cell walls were digested with GBS phage lysozyme for 4 h at 30°C, while monitoring the extent of digestion by turbidimetry (Table 1). An aliquot of the digest was then assayed for reducing groups using a modified Park–Johnson method (Spiro, 1966). Digestion was clearly accompanied by an increase in reducing groups (Table 1), compared to a negligible level in the cell wall control (Table 1). This liberation of reducing groups is consistent with the lysozyme cleaving glycosidic bonds in the repeating disaccharide backbone of the peptidoglycan.

The second conserved domain in the GBS phage lysozyme, a CHAP domain, is also present in a lysozyme of staphylococcal phage \( \phi 11 \), where it was shown to be responsible for \( \delta \)-alanyl-glycine endopeptidase activity (Navarre et al., 1999). To determine whether the GBS phage lysozyme also exhibits endopeptidase activity, the soluble digestion products from a GBS cell wall digest were analysed by N-terminal amino acid sequencing. Alanine was identified as the predominant residue through three cycles of sequencing. Alanine was also seen in the cell wall control through cycles 1–3, but at much lower levels. Sequencing the insoluble cell wall residues remaining after digestion again showed alanine as the only major N-terminal residue, but the residue from the lysozyme digest contained significantly less N-terminal alanine than the cell wall control digest (results not shown). One possible explanation for this result is that.

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**Fig. 2.** Effect of growth stage of GBS cells on their sensitivity to GBS phage lysozyme. (a) The relative rates of lysis of GBS cells harvested at different times in the growth cycle were determined by a turbidity reduction assay after addition of a fixed amount of GBS phage lysozyme to each cell suspension, as described in Methods. (b) The growth curve of the culture of strain 3331 GBS cells from which aliquots were removed at various times.

**Fig. 3.** Relative activity of GBS phage lysozyme on cells of various species. GBS phage lysozyme (25 U) was added to suspensions of Strep. faecalis, Strep. pneumoniae (LytA\(^{-}\) mutant), and groups A, B, C, E and G streptococci harvested at early exponential phase, as described in Methods. The rate of lysis is expressed as the percentage decrease in turbidity in 2 min.
the glycosidase activity present yielded soluble muropeptide fragments possessing N-terminal alanine residues. In order to determine whether N-terminal alanine appearing in the soluble fraction was the direct result of endopeptidase activity, cell walls were acetylated to block pre-existing free amino groups and subsequently incubated with the lysin. The acetylated cell walls remained a satisfactory substrate for the lysin, as measured by a drop in turbidity during incubation (Table 1). N-terminal peptide sequencing of an ultratrace of the digest again showed alanine as the predominant residue through cycles 1–3 (Table 1), and the acetylated cell wall control gave only negligible levels of amino acids through three cycles of sequencing. These results clearly show that the GBS phage lysin possesses endopeptidase activity in addition to its glycosidase activity.

**Identification of amino acid residues important for enzymic activity**

The CHAP domain is present in a large number of enzymes that cleave peptidoglycan (Bateman & Rawlings, 2003). In the GBS phage lysin, the conserved cysteine of the CHAP domain corresponds to C26 and the conserved histidine is H91. Site-directed mutagenesis was used to convert C26 and the three other cysteine residues in the protein (C34, C44 and C171) to serine residues. Histidine 91 was replaced with an alanine. The effects of these substitutions on the two different enzymic activities of the lysin are shown in Table 2. It can be seen that replacement of either of the two acidic residues resulted in a major decrease in glycosidase (lysozyme) activity while causing no decrease in endopeptidase activity.

**Table 1. Glycosidase and endopeptidase activities of GBS phage lysin**

<table>
<thead>
<tr>
<th>Incubation mixture*</th>
<th>Turbidity reduction† (mean±SD)</th>
<th>Reducing activity‡ (µg glucose; mean±SD)</th>
<th>Edman degradation of muropeptide§ (cycle no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell walls + lysin</td>
<td>0.162±0.008</td>
<td>24.5±1.2</td>
<td>ND</td>
</tr>
<tr>
<td>Cell walls alone</td>
<td>0.014±0.002</td>
<td>1.32±0.07</td>
<td>ND</td>
</tr>
<tr>
<td>Acetylated cell walls + lysin</td>
<td>0.112±0.006</td>
<td>12.8±0.6</td>
<td>A (514)</td>
</tr>
<tr>
<td>Acetylated cell walls</td>
<td>0.008±0.002</td>
<td>0.39±0.04</td>
<td>A (4)</td>
</tr>
</tbody>
</table>

*Incubation mixtures (300 µl) contained: stock GBS cell walls (200 µg glucose equivalents released in the incubation mixture. Incubations were at 30 °C for 4 h.
†Reduction in OD_{550} of incubation mixtures.
‡Reducing activity is expressed as µg glucose equivalents released in the incubation mixture.
§See Methods for details. In each sample and cycle recorded, PTH-alanine is the predominant peak. The amount released, in pmol, is shown in parentheses. ND, Not determined.

The Acm domain of the GBS phage lysin possesses two acidic amino acid residues, D158 and E185, which are highly conserved in other proteins possessing the Acm domain. Site-directed mutagenesis was used to individually replace each of these acidic amino acid residues with an alanine residue. The effects of the substitutions on enzyme activity are shown in Table 2. It can be seen that replacement of either of the two acidic residues resulted in a major decrease in glycosidase (lysozyme) activity while causing no decrease in endopeptidase activity.

**Table 2. Glycosidase and endopeptidase activities of GBS phage lysin mutants**

Analysis of both glycosidase and endopeptidase activity was repeated with similar results.

<table>
<thead>
<tr>
<th>GBS phage lysin</th>
<th>Reducing groups released [µg glucose (50 µl)^−1]*</th>
<th>Amino groups released [nmol (50 µl)^−1]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1.92</td>
<td>15.8</td>
</tr>
<tr>
<td>C26S</td>
<td>1.95</td>
<td>0</td>
</tr>
<tr>
<td>C34S</td>
<td>1.88</td>
<td>2.6</td>
</tr>
<tr>
<td>C44S</td>
<td>1.25</td>
<td>0.38</td>
</tr>
<tr>
<td>H91A</td>
<td>1.21</td>
<td>0</td>
</tr>
<tr>
<td>D158A</td>
<td>0.23</td>
<td>19.1</td>
</tr>
<tr>
<td>E185A</td>
<td>0.24</td>
<td>14.3</td>
</tr>
</tbody>
</table>

*Incubation mixtures (50 µl) in lysin buffer B contained lysin (6.5 µg) and cell walls to give an OD_{550} of 0.75. Incubations were at 30 °C for 1 h and were stopped by heating at 100 °C for 5 min. Undigested cell walls were pelleted by centrifugation at 5000 g for 15 min. Aliquots (20 µl) of the clear supernatant were assayed for reducing activity (Spiro, 1966). Enzyme level limited the extent of glycosidase activity of the native enzyme under the conditions employed.
†Endopeptidase assays were carried out as described in the text.
DISCUSSION

This work was undertaken with the eventual goal of using the GBS phage lysin to prevent, or clear, existing vaginal colonization by GBS in pregnant women. This approach could lead to a significant reduction in the incidence of GBS neonatal infections. The pH optimum of GBS phage lysin, however, is between 5.5 and 6.0, somewhat higher than the frequently cited 'normal' vaginal pH of 4.5. This raises the concern that GBS phage lysin secreted by an engineered Lactobacillus sp. may not exhibit optimal bacteriocidal activity against GBS present in a woman's vagina. Vaginal pH, however, is often considerably higher than 4.5 (Hauth et al., 2003) and is likely to be so in women who are heavily colonized with GBS, since GBS grow very poorly at pH 4.5. GBS phage lysin, therefore, may be most effective in clearing GBS vaginal colonization in those women at greatest risk of delivering babies who develop GBS infections, since it has been known for many years that babies born to women heavily vaginally colonized with GBS are much more likely to develop early-onset GBS infections than those born to women lightly vaginally colonized with the organism (Anthony et al., 1978).

GBS bacteriophages have been used in a variety of phage typing systems since they are highly specific for different strains of the species (Haug et al., 1981; Stringer, 1980). In this work, we found that bacteriophage B30 lysed only a minor subgroup of serotype III GBS strains, those that do not secrete hyaluronate lyase. Such strains are classified as restriction-digestion pattern type III-2 strains (Takahashi et al., 2002).

The rate at which GBS cells are lysed by the GBS phage enzyme was found to be dependent upon the growth stage of the cells when they are harvested, with early-exponential phase cells lysed most rapidly. The reason for this dependence is not clear, but it may be due to modifications of the cell wall that take place in stationary phase culture that make the bacteria more resistant to lysis. Modifications that may be important include increased cross-linking of the peptidoglycan layer, deacetylation of the amino sugars, and an increase in the amount of cell wall-associated proteins and polysaccharides. Heating GBS cells or cell wall preparations in boiling water made them poorer substrates for the lysin. Again, the explanation is not clear, but denatured cell wall-associated proteins may be impeding access of the lysin to the peptidoglycan.

GBS peptidoglycan consists of a glycan backbone of alternating β-1,4-linked N-acetylglucosamine and β-1,4-linked N-acetylmuramic acid residues linked to a stem peptide composed of L-Ala-D-Gln-L-Lys-D-Ala-D-Ala (Schleifer & Kandler, 1972). As shown in Fig. 4, the stem peptides are cross-linked by an (L-Ala)_2 or L-Ala-L-Ser interpeptide bridge between the ε-amino group of the L-Lys of one stem peptide cross-bridge and the carboxylate of a D-Ala of another stem peptide (Karakawa & Krause, 1966; Reinscheid et al., 2002). This linkage is characteristic of an A3α type of peptidoglycan in the classification system described by Schleifer & Kandler (1972).

Phage lysins can potentially degrade GBS peptidoglycan by cleaving any of several different linkages (Young et al., 2000). Some lysins are glycosidases (e.g. N-acetylmuramidases or N-acetylglucosaminidases). Other lysins possess specific endopeptidase activity. Another very common type of lysin is an N-acetylmuramyl-L-alanyl amidase that cleaves the linkage between the stem peptide and N-acetylmuramic acid of the glycan chains (see Fig. 4). The GBS bacteriophage B30 lysin protein contains two conserved sequence domains, an N-terminal CHAP domain (formerly called an AXE domain) located between amino acids 6 and 107 and a large Acm domain located between amino acids 65 and 344, partially overlapping the CHAP domain. The location of a substrate-binding domain is not known but may be present at the C-terminal region of the protein as it is in many other lysins. Since the Acm domain present in the GBS phage lysin is associated with a lysozyme-type activity in other proteins, we determined whether the enzyme possessed glycosidase activity consistent with lysozyme activity. As shown in Table 1, digestion was accompanied by a large increase in reducing activity. Site-directed mutagenesis experiments further confirmed the presence of an active Acm (lysozyme) domain. Mutagenesis of D158 and

![Fig. 4. Portion of GBS peptidoglycan indicating possible lysin cleavage sites. The cleavage sites of N-acetylmuramyl-L-alanyl amidase (amidase), N-acetylglucosaminidase (glucosaminidase) and N-acetylmuramidase (muramidase) are indicated. Several autolytic and phage lysins are known to possess these activities. The indicated endopeptidase cleavage site is based on the results of this study and has not been described previously.](http://mic.sgmjournals.org)
E185, residues that are highly conserved in proteins possessing the Acm domain, resulted in a large decrease in glycosidase activity while causing no decrease in endopeptidase activity (Table 2).

In the GBS phage lysin, C26 and H91 are the highly conserved residues of the CHAP domain (Bateman & Rawlings, 2003). Site-directed mutagenesis of these residues confirmed that they were essential for the endopeptidase activity of the lysin (Table 2). The CHAP domain is also present in a lysin of staphylococcal phage φ11, where it was shown to be responsible for D-alanyl-glycine endopeptidase activity (Navarre et al., 1999). A separate central domain (amidase-2) conferring N-acetylmuramyl-L-alanyl amidase activity was also present in this bifunctional staphylococcal phage lysin. The ability of the staphylococcal phage φ11 lysin to release N-terminal alanine residues, in addition to the glycine residues of the pentapeptide bridge, was due to N-acetylmuramyl-L-alanyl amidase activity in the enzyme (Navarre et al., 1999). Using N-terminal amino acid sequencing, we also found that lysin was accompanied by an increase in N-terminal alanine residues. However, unlike the staphylococcal phage lysin, the GBS phage lysin lacks an N-acetylmuramyl-L-alanyl amidase domain. The N-terminal alanine residues released must be generated by another mechanism. The presence of the CHAP domain in the GBS phage enzyme suggests that the explanation for the increase in N-terminal alanine residues is that they result from endopeptidase activity cleaving between D-Ala of the stem peptides and L-Ala of the interpeptide bridges (see Fig. 4). The endopeptidase of phage φ11 lysin cleaves between the D-Ala of the stem peptide and a glycine residue of the (Gly)5 bridge in the staphylococcal peptidoglycan, but such pentaglycine bridges are not present in GBS peptidoglycan. Instead (L-Ala)2 or L-Ala-L-Ser interpeptide bridges are present. An endopeptidase cleaving at the N-terminus of a D-Ala in GBS peptidoglycan would expose N-terminal L-Ala residues. Presumably the low yield after alanine in cycle 2 (see Table 1) is due to the inability of the Edman degradation to cleave the lysine of the stem peptide that is linked through its ε-amino group. A lysin possessing such a D-alanyl-L-alanine endopeptidase activity has not been described previously.

Several other β-haemolytic streptococci that are lysed by the GBS phage lysin, including those of groups A, C, E and G streptococci, also possess (L-Ala)2 interpeptide bridges (Schleifer & Kandler, 1972). Conversely, Strep. mutans and related organisms, which are not lysed by the enzyme, have been reported to possess L-Thr-L-Ala crossbridges (Schleifer & Kandler, 1972). The GBS phage lysin, which normally cleaves a D-alanyl-L-alanine linkage in β-haemolytic streptococci, may be unable to cleave the D-alanyl-L-threonine linkage in Strept. mutans.

The C1 phage lysin, previously shown to specifically kill streptococci of groups A, C and E, but not streptococci of groups B and G (Nelson et al., 2001), was recently sequenced (Nelson et al., 2003). It showed no significant homology to the GBS phage lysin described in the present report. Although the C1 phage lysin possesses amidase activity (Fischetti et al., 1971; Nelson et al., 2003), it does not possess any known conserved sequence domains.

Our results suggest that the GBS phage lysin, like the staphylococcal phage φ11 lysin, is a bifunctional enzyme possessing two different types of lytic activity. The recent demonstration of a synergistic effect of combining two different types of phage lysins suggests that the presence of two different peptidoglycan-cleaving activities in one enzyme may significantly enhance its ability to lyse bacterial cells (Jado et al., 2003; Loeffler & Fischetti, 2003). Studies designed to exploit the unique bacteriolytic properties of the GBS phage lysin for the prevention of neonatal GBS infections can now be carried out.

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