Group B streptococci (GBS), or Streptococcus agalactiae, are the leading cause of neonatal sepsis and meningitis in the United States and Europe (1). First identified as a pathogen in bovine mastitis, GBS were later found to colonize the human genital and lower gastrointestinal tract, where the organism can be transmitted perinatally to the fetus. In addition to newborn infections, GBS can also cause substantial morbidity and mortality in adults, particularly those who are immunocompromised or elderly (6). GBS are classified into nine serotypes based on differences in the polysaccharide capsule (24). Historically, the most commonly reported serotype associated with diseases is type III; however, recent epidemiological studies found that serotype V now accounts for approximately 30% of nonpregnant adults (11) and 14 to 23% of pregnant women (12). Serotypes V and III now account for approximately 50% of all isolates from pregnant women in the United States and Europe (1). First identified as a pathogen in bovine mastitis, GBS were later found to colonize the human genital and lower gastrointestinal tract, where the organism can be transmitted perinatally to the fetus. In addition to newborn infections, GBS can also cause substantial morbidity and mortality in adults, particularly those who are immunocompromised or elderly (6). GBS are classified into nine serotypes based on differences in the polysaccharide capsule (24). Historically, the most commonly reported serotype associated with diseases is type III; however, recent epidemiological studies found that serotype V now accounts for approximately 30% of nonpregnant adults (11) and 14 to 23% of pregnant women (12). Serotypes V and III now account for approximately 50% of all isolates from pregnant women in the United States and Europe (1).

The efficacy of antibiotic administration during various stages of pregnancy in order to reduce the rate of early-onset GBS infection in neonates has been tested. Hall et al. (10) demonstrated that treatment during pregnancy (antepartum prophylaxis) reduced maternal GBS colonization transiently, but colonization recurred at delivery. For postpartum prophylaxis, universal administration of penicillin to neonates shortly after birth reduced the early-onset GBS infection by 68%, but it was also associated with a 40% increase in overall mortality and therefore is not recommended (2). The most successful approach to block vertical transmission of GBS from mother to infant is intrapartum prophylaxis (4 h prior to delivery) of colonized women. This method was shown to effectively reduce both GBS neonatal colonization and early-onset infection (4). In one study (21), intrapartum prophylaxis reduced the risk of early-onset infection by 56% in neonates born to women who had risk factors for neonatal disease (premature labor or prolonged rupture of membranes). Therefore, intrapartum antibiotic prophylaxis to prevent perinatal GBS disease was issued by the Centers for Disease Control and Prevention (CDC) in 1996 (4) and were revised in 2002 (5).

In the revised CDC recommendations (5), penicillin remains the first-line agent for intrapartum antibiotic prophylaxis. Erythromycin and clindamycin used to be recommended for penicillin-allergic women; however, this is no longer the case due to the significant number of resistant clinic isolates (7). This may suggest a possible occurrence of more antibiotic resistance in the future. Furthermore, recent clinical trials suggest that antibiotic administration to pregnant women may be associated with adverse neonatal problems, such as necrotizing enterocolitis or the increased need for supplementary oxygen (14, 15). Thus, alternative strategies with fewer side effects are necessary to control and prevent GBS neonatal infection, particularly in situations of increased antibiotic resistance.

Bacteriophage lysins are highly evolved cell wall hydrolases that can kill bacteria by digesting the bacterial cell wall, making cells susceptible to osmotic lysis. Previously, a lysin purified from the streptococcal phage C1 was demonstrated to kill group A streptococci in vitro and to prevent streptococcal colonization in mice (23). Following this study, two other lysins (Pal and PlyG) were shown to be able to eradicate Streptococcus pneumoniae (17) and Bacillus anthracis (27), respectively, from in vivo models. To continue this approach, we explore here the possibility of using a lysin from a GBS bacteriophage as an alternative approach for intrapartum prophylaxis. We cloned a lysin gene from the GBS phage NCTC 11261 and
Identification of the lysin genes and construction of an expression vector. Genomic DNA of phage NCTC 11261 was isolated with the Lambda Maxi kit (QIAGEN, Valencia, Calif.). The genomic DNA was partially digested with Tsp509I, and 0.5- to 3-kbp DNA fragments were cloned into expression vector pBAD24 (9). The library was transformed into Escherichia coli and screened for lytic activity as previously described (27), using the indicator strain GBS NCTC 11237 as a bacterial overlay. Alternatively, colonies that grew well on Luria-Bertani (LB) ampicillin plates but had no or poor growth on induction plates (containing ampicillin and arabinose) were isolated and their corresponding plasmids were sequenced. DNA sequences were analyzed with the programs BlastX (National Center for Biotechnology Information, Bethesda, Md.) and MacVector (Accelrys, San Diego, Calif.).

To increase the expression level of plyGBS, it was cloned into a high-expression vector, pET28a (Novagen, Madison, Wis.). Two primers (forward, 5′-GAG GAGTCTCATGGAATTTACAAATATAAAAGC; reverse, 5′-GCTACCC TCGAGTTATACATATCTGTGTAATTTAAG) were designed to amplify pGBS, using genomic DNA of phage NCTC 11261 as the template. The PCR product was end digested with Necl and XhoI and cloned into pET28a to form pCQ67-2, which was subsequently transformed into E. coli strain BL21(DE3) for protein expression. DNA sequence analysis was used to confirm that pCQ67-2 contains no mutation.

GBS overexpression and purification. The E. coli BL21(DE3)pCQ67-2 strain was grown at 37°C overnight in LB broth supplemented with kanamycin (50 μg/ml), subcultured, and grown to an optical density at 600 nm (OD_{600}) of 0.5. Expression of the GBS protein was induced at 30°C for 4 h by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM. Cells were then harvested, resuspended in 50 ml of phosphate-buffered saline plus 500 U of Benzonase (Novagen, Madison, Wis.), and lysed with 10 ml of chloroform for 45 min. After centrifugation, the supernatant was dialyzed against 25 mM Tris- HCl (pH 7.4) at 4°C overnight. The dialyzed lysate was applied to a 40-ml Q-Sepharose column (Amersham Pharmacia, Piscataway, N.J.) and eluted in a linear gradient from 0 to 1 M NaCl in 25 ml Tris- HCl (pH 7.4). Fractions were analyzed as described (23). Acytic activity as previously described (18). In brief, bacterial strains (Table 1), all strains were inoculated overnight, subcultured (1:100), and grown to an OD_{600} of 0.3. Cells were centrifuged and resuspended in a 1/10 volume of phosphate buffer (40 mM, pH 5.0). One hundred-microliter aliquots of the bacterial solution (5 × 10^6 to 5 × 10^7 CFU/ml) were incubated with indicated amounts of GBS lysates at 37°C for 60 min. A spectrophotometer was used to monitor the lytic activity, measured as the drop in OD_{600} per minute (mOD_{600}/min). The initial velocity of this reaction is defined as the rate of lysis. To determine bacterial viability, cells of GBS strain NCTC 11237 from the above assay were centrifuged, diluted, and plated on THY agar plates for cell counts. All experiments were performed in triplicate, and control experiments with the addition of phosphate buffer (pH 5.0) were performed under the same conditions.

In vitro GBS activity. To test the in vitro GBS activity on various bacterial strains (Table 1), all strains were inoculated overnight, subcultured (1:100), and grown to an OD_{600} of 0.3. Cells were centrifuged and resuspended in a 1/10 volume of phosphate buffer (40 mM, pH 5.0). One hundred-microliter aliquots of the bacterial solution (5 × 10^6 to 5 × 10^7 CFU/ml) were incubated with indicated amounts of GBS at 37°C for 60 min. A spectrophotometer was used to monitor the lytic activity, measured as the drop in OD_{600} per minute (mOD_{600}/min). The initial velocity of this reaction is defined as the rate of lysis. To determine bacterial viability, cells of GBS strain NCTC 11237 from the above assay were centrifuged, diluted, and plated on THY agar plates for cell counts. All experiments were performed in triplicate, and control experiments with the addition of phosphate buffer (pH 5.0) were performed under the same conditions.

Microscopy. GBS strain NCTC 11237 was grown in THY broth to an OD_{600} of 0.3. Bacteria were centrifuged and resuspended in 40 mM phosphate buffer (pH 5.0) to an OD_{600} of 1.0. Three hundred microliters of this bacterial suspension was mixed with 300 μl of GBS (final concentration, 10 U/ml) and incubated at 37°C for 15 min. A buffer control was also included for comparison.

In vivo GBS activity on GBS. Six-week-old BALB/c female mice were purchased from Charles River Laboratories (Wilmington, Mass.). Because bacterial colonization in the mouse vaginal cavity is believed to be more efficient at estrus...
RESULTS

Cloning and sequence alignment of PlyGBS. When clones of the GBS phage NCTC 11261 genomic library were screened for a possible lysin gene, no colonies formed clear zones on a GBS NCTC 11237 bacterial overlay. Instead, we identified the partial sequence of a putative lysin gene when sequencing a plasmid from a colony that grew poorly upon induction. Upstream of this partial lysin gene, we discovered a putative holin gene that may have exerted a toxic effect on the cells upon induction (data not shown). The full sequence of the lysin gene (plyGBS) was obtained by sequencing of the phage NCTC 11261 genomic DNA. Similarity searches indicate that, at both N-terminal region has 27% identity to the first 94 amino acids of the endopeptidase domain of staphylococcal phage 187 lysin (Ply187), and the central region of the PlyGBS displays 46% identity to the muramidase domain of pneumococcal phage Cp-1 lysin (Cpl-1). The C terminus of PlyGBS is a putative cell wall binding domain. The domain organization in PlyGBS resembles that of some staphylococcal phage lysins, which have two or more catalytic domains, such as Ply187 containing endopeptidase, amidase, and glucosaminidase domains.

Characterization of PlyGBS. Based on the deduced amino acid sequence, PlyGBS has a theoretical pI value (isoelectric point) of 4.88. With 25 mM Tris-HCl (pH 7.4) as the elution buffer in ion-exchange chromatography, the protein was negatively charged and bound to a positively charged Q-Sepharose anion exchanger. The enzyme was eluted by an NaCl gradient, and the active fractions were pooled and analyzed by SDS-PAGE. As shown in Fig. 2, the major protein band at ~50 kDa correlates with the calculated molecular mass for PlyGBS (49.6 kDa). The final preparation, estimated to be >95% pure by scanning densitometry on the Coomassie-stained SDS-PAGE gel, was used for all subsequent experiments.

The pH optimum of purified PlyGBS was determined to be around 5.0, with an active pH range between 4.0 and 6.0 (data not shown). Gel filtration chromatography of PlyGBS indicates that the protein functions as a monomer (data not shown).
cell wall targets, which contribute to the observed variability of lytic activity.

In addition to GBS, bacterial strains representing a variety of species were analyzed to determine their sensitivity to PlyGBS in vitro (Table 1 and Fig. 4). Of the tested streptococcal strains belonging to different Lancefield groups, PlyGBS had significant lytic activity against group A, C, G, and L streptococci, with little to no activity against group D, E, and N streptococci. This could be explained by the significant similarity between PlyGBS and lysins from *S. pyogenes* (group A) and *S. equi* (group C). We also tested the muralytic activity of PlyGBS against non-Lancefield oral streptococcal species, including *S. gordonii*, *S. oralis*, *S. salivarius*, *S. sobrinus*, and *S. mutans*. Interestingly, PlyGBS had medium to low activity against *S. salivarius*, *S. gordonii*, and *S. mutans* (Fig. 4) but no activity against the other two commensal species tested. No lytic activity was observed with two nonstreptococcal gram-positive species, *Bacillus cereus* and *Staphylococcus aureus*, or two vaginal commensal bacteria, *L. acidophilus* and *L. crispatus*.

Phase-contrast and electron microscopy were used to visualize the lytic effect of lysin on GBS cells. Normally, intact GBS form short chains, as shown in the buffer control (Fig. 5a). After treatment with lysin (Fig. 5b), the cells lost their cytoplasmic contents and became opaque by light microscopy. As seen with other lysins by electron microscopy, a weakness in the cell wall produced by PlyGBS results in extrusions (Fig. 5c) and rupture (Fig. 5d) of the cytoplasmic membrane, which appears to be more localized within the septum region of the dividing cells. Subsequent loss of cytoplasmic contents transforms the cells into empty “cell ghosts” (Fig. 5e).

**PlyGBS activity in vivo.** To determine if PlyGBS was able to remove colonizing GBS from the mouse vagina, two groups of mice were challenged vaginally with 10⁶ CFU of Strr GBS cells. Twenty-four hours later, the vaginal cavities were swabbed to determine the initial colonization rate (0-h samples, pretreatment). The mice were then treated vaginally with either buffer (*n* = 15) or PlyGBS (*n* = 15) and swabbed 2 and 4 h post-treatment. As shown in Fig. 6a, small effect was observed in the buffer control animals. In contrast, animals treated with a single dose of PlyGBS showed a significant reduction (approximately 3-log drop) in the bacterial load at both the 2- and 4-h intervals compared to the level in the buffer control (*P* < 0.0001).

Similarly, two groups of mice were challenged with a total of 10⁶ CFU of Strr GBS delivered orally and nasally to determine if PlyGBS can be used to reduce colonized GBS in the mouse upper respiratory tract. As shown in Fig. 6b, mice treated with a single dose of lysin by the same route exhibited a significant reduction in GBS colonization at both the 2- and 24-h swabbing intervals compared to the level in the buffer control group (*P* < 0.0001).

**DISCUSSION**

In this study, we identified a putative lysin gene from a GBS bacteriophage and the recombinantly expressed lysin PlyGBS was purified using ion-exchange chromatography. We demonstrate that PlyGBS is capable of lysing GBS cells effectively in vitro. Using a mouse model, we conclude that the bacterial
load of GBS colonizing the vagina and oropharynx of the mice could be reduced significantly by a single treatment with PlyGBS. To our knowledge, this is the first reported GBS phage lysin showing in vivo therapeutic efficacy.

GBS primarily colonize the human vaginal tract along with the normal vaginal flora such as *L. acidophilus*, which metabolizes glycogen to lactic acid, resulting in the low pH (4.4 to 5.6) observed in the vaginal tract (3). The low pH and the production of hydrogen peroxide (H$_2$O$_2$) produced by vaginal bacteria are important defenses against pathogens. Given the fact that the optimum pH for PlyGBS (around pH 5.0) is within the range normally found in the human vaginal tract, it is reasonable to assume that the effectiveness observed in the mouse model will translate effectively to the human vagina; however, future trials will be necessary to confirm this.

The introduction of intrapartum antibiotic prophylaxis effectively reduces GBS neonatal colonization and early-onset infection (4). However, there are still situations that may make it more desirable to use PlyGBS treatment than antibiotic therapy. In consideration of antibiotic resistance, PlyGBS provides a better choice than antibiotics because of the inability to identify bacterial mutants resistant to phage lysins (18, 27). Additionally, in situations where GBS screening of pregnant women is not a routine practice, universal administration of PlyGBS before delivery would be preferable. It should be realized that any treatment against GBS during labor must be rapidly effective to prevent disease, and this requirement may provide PlyGBS a significant advantage. Furthermore, because of its specificity for GBS and other pathogens and the lack of activity for two common vaginal commensals, *L. acidophilus* and *L. crispatus*, PlyGBS is not likely to disrupt the vaginal microflora, a significant problem in antimicrobial therapy (19).

In the mouse vaginal model, only one dose of PlyGBS was delivered to each mouse, resulting in a significant reduction of colonization at 2 and 4 h posttreatment (Fig. 6a). When we determined the vaginal GBS colonization status at 24 h posttreatment, the GBS counts rebounded (data not shown), which was previously seen during antibiotic therapy (10), suggesting that multiple doses at predetermined intervals may be necessary for complete control. Because intrapartum antibiotic prophylaxis is the usual treatment 4 h prior to delivery to prevent vertical transmission of GBS from mother to infant and because PlyGBS is effective vaginally in mice up to 4 h posttreatment, PlyGBS may be used as an alternative approach to replace intrapartum antibiotic prophylaxis prior to delivery. Since phage enzymes are proteins, and thus immunogenic, it is possible that antibodies induced through multiple exposures would interfere with the catalytic action of the enzyme, reducing its potency. However, recent evidence suggests that antibodies directed to PlyGBS, like other phage enzymes (18), do not neutralize the catalytic activity of PlyGBS (data not shown).

GBS can be transmitted to the newborn from a colonized mother during or before delivery. Prospective studies of GBS infections in newborns have shown that GBS can colonize four different body sites (ear, anus, throat, and umbilicus) (25). It is likely, but not proven, that colonization of the oropharynx (throat) is the route by which neonatal meningitis is initiated. Because of this and the fact that this site would be the most difficult to safely decontaminate in a newborn, we chose the oropharynx to determine if PlyGBS treatment can be used to disinfect GBS upper respiratory colonization. Our animal experiments showed that PlyGBS, compared to the buffer control, significantly reduced the bacterial counts of GBS in the oropharynx (Fig. 6b). Thus, PlyGBS may be used to reduce GBS vaginal colonization in pregnant women before delivery and may also provide a novel antibacterial agent to safely decontaminate newborns from GBS and thus further reduce colonization at 2 and 4 h posttreatment (Fig. 6a). When we determined the vaginal GBS colonization status at 24 h posttreatment, the GBS counts rebounded (data not shown), which was previously seen during antibiotic therapy (10), suggesting that multiple doses at predetermined intervals may be necessary for complete control. Because intrapartum antibiotic prophylaxis is the usual treatment 4 h prior to delivery to prevent vertical transmission of GBS from mother to infant and because PlyGBS is effective vaginally in mice up to 4 h posttreatment, PlyGBS may be used as an alternative approach to replace intrapartum antibiotic prophylaxis prior to delivery. Since phage enzymes are proteins, and thus immunogenic, it is possible that antibodies induced through multiple exposures would interfere with the catalytic action of the enzyme, reducing its potency. However, recent evidence suggests that antibodies directed to PlyGBS, like other phage enzymes (18), do not neutralize the catalytic activity of PlyGBS (data not shown).

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the rate of GBS-associated neonatal meningitis and sepsis without antibiotics.

Our in vitro experiments indicate that PlyGBS has a lower specific activity than several of the other lysins studied in our laboratory (17, 23, 27). A possible explanation is that, distinctive from streptococci in other Lancefield groups, almost all GBS strains isolated from humans are heavily encapsulated (24) and the capsule may limit the access of PlyGBS to the cell wall. Nonetheless, our data show that, despite the low specific activity compared to other lysins, PlyGBS does provide protection in two different mouse models of GBS colonization. Furthermore, a second-generation PlyGBS could be developed through random mutagenesis (16) or directed evolution (28), which may optimize specific activity, and preliminary experiments suggest that this is indeed possible.

In addition to GBS, PlyGBS has lytic activity on a variety of streptococci in the Lancefield grouping system, including groups A, C, G, and L. This is a second bacteriophage lysin studied in our laboratory that has relative broad specificity (29). While group A streptococci are a primary cause of bacterial pharyngitis, streptococci in groups C and G are also important human pathogens associated with bacterial pharyngitis, arthritis, and bacteremia (13, 26). The relatively extensive activity range of PlyGBS may provide a new agent for controlling multiple streptococcal pathogens. The broad lytic activity of PlyGBS also suggests that these streptococci have a common binding receptor for the enzyme, and comparative genomic analysis may be used to identify this receptor. Given that all tested GBS strains representing various serotypes as well as several other streptococcal groups were sensitive to PlyGBS, we suspect that the binding receptor for PlyGBS is located in the cell wall like other lysins, instead of the capsular polysaccharide.

Our results presented here show the potential of applying PlyGBS to control diseases associated with GBS infection. Future research will focus on improving activity and stability as well as developing an efficient method for drug delivery. Considering that bacteriophages are prevalent in the environment and phage lysins are among the most powerful antimicrobial agents identified in nature, the future development of lysins may provide a novel approach to circumvent resistance to control diseases caused by bacterial pathogens.

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FIG. 6. Control of GBS colonization in mice with PlyGBS. (a) Mice were colonized with GBS vaginally after synchronization with β-estradiol valerate. One day after GBS inoculation, two groups of mice were treated vaginally with either buffer (n = 15) or PlyGBS (n = 15). Each mouse was swabbed vaginally before treatment (0-h samples) and after treatment at 2- to 4-h intervals (2- and 4-h samples). (b) Mice were swabbed vaginally (0 h) and after treatment (2 and 24 h). After transformed into log 10, the colony counts of oropharynx samples were averaged for each time interval in the same group and plotted. Error bars represent the standard error of the mean.


