JOURNAL OF BACTERIOLOGY, Mar. 2006, p. 2096–2105 Vol. 188, No. 6
0021-9193/06/$08.00+0 doi:10.1128/JB.188.6.2096–2105.2006

Abundance of the δ Subunit of RNA Polymerase Is Linked to the Virulence of *Streptococcus agalactiae*

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Received 15 September 2005/Accepted 20 December 2005

Group B streptococcus (GBS) remains a major cause of morbidity and mortality among newborn children. The bacterium is a commensal organism colonizing the rectum and the gastrointestinal and urogenital tracts of adults, but it can be transmitted to neonates by an ascending infection of the maternal genital tract or during parturition. We previously reported that a transposon insertion disrupting *rpoE* resulted in the decreased survival of the mutant in the neonatal rat sepsis model of GBS infection. *rpoE* encodes the δ protein, a subunit of RNA polymerase (RNAP) that has been characterized in *Bacillus* species. In this study, we confirm the association of the δ protein with purified GBS RNAP and show that it is expressed in strains representing all nine serotypes. Flow cytometric analysis of a reporter strain containing a transcriptional fusion of the *rpoE* promoter to *gfp* revealed that, in vitro, this gene is continuously expressed. Analysis of δ expression in the transposon mutant by quantitative Western blotting revealed a 10-fold reduction in relative abundance (which was linked to the attenuation in virulence that was observed for this mutant) compared to that for the wild-type strain. These data suggest that a minimum intracellular concentration of δ is necessary for this organism to cause disease.

Streptococcus agalactiae, or group B streptococcus (GBS), remains a leading cause of pneumonia, sepsis, and meningitis in newborns within the developed world (37). GBS infection can be separated into two distinct syndromes, one occurring among neonates of up to several days old (early onset disease) and the other among infants that are several weeks or months older (late-onset disease). Although the screening of at-risk obstetric patients, followed by the administration of intrapartum antibiotics, has drastically reduced the incidence of early onset disease (41), invasive GBS disease still accounts for significant morbidity and mortality in newborns (27, 43). Increasing numbers of reports of invasive disease among the elderly and in immunocompromised adults have also classified GBS as an important pathogen in these two populations (3). Recognition of the prevalence and severity of human neonatal and adult disease underscores the need for investigating the mechanisms that are important to the pathogenesis of GBS infections.

The GBS bacterium is a commensal organism in adults that colonizes the rectum and the urogenital and gastrointestinal tracts (13). To establish early onset disease, the bacterium must ascend from the acidic environment of the mother's vaginal mucosa to the lung epithelia of the neonate during birth and access the highly oxygenated environment of the blood (for a review, see reference 2). Thus, an important feature that is exhibited by this pathogen is an ability to survive and grow in diverse host environments that differ with respect to the physiological conditions and immune defenses. This ability to adapt likely involves an alteration in the pattern of gene expression. The regulation of gene expression in bacteria primarily occurs at the level of transcription and is controlled through the activity of the transcribing enzyme, RNA polymerase (RNAP). Typically, bacterial RNAP can coexist in two distinct forms: (i) the core enzyme comprised of β and β′ and two identical α subunits and (ii) as a holoenzyme which, in addition to the core, contains a dissociable σ subunit. Functionally, the core enzyme has a low binding affinity for any DNA sequence but is sufficient for transcriptional elongation and termination. The association of a σ factor with the core enzyme drastically increases the affinity for σ-specific promoter sequences, allowing for the initiation of transcription. The constitutively expressed sigma factors σ^{70} and σ^{A} are responsible for initiating the transcription of the majority of bacterial genes in gram-negative and gram-positive bacteria, respectively. However, the induction of competence, adaptation to temperature change, control of sporulation, and survival response to oxidative stress have all been attributed, in part, to the association of specific secondary σ factors with RNAP (14, 15, 28, 31).

A number of accessory proteins are also found in association with RNAP during various stages in transcription. In addition to the core subunits and σ, RNAP purified from *Bacillus subtilis* has also been shown to contain a novel protein designated δ, which is encoded by the *rpoE* gene (1, 24, 26). An interrogation of the published sequence databases suggests that *rpoE* is ubiquitous among gram-positive bacteria.

Activity for δ has thus far been characterized in only *Bacillus* spp. by using in vitro assays. δ has been shown to bind to the core of *Bacillus subtilis* RNAP (25). It is thought to play a role in maintaining transcriptional specificity. Association of δ with RNAP reduces binding to DNA templates containing nonbiologically relevant promoter sites (39) and inhibits transcription

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
S. agalactiae		
A909	Serotype Ia, encapsulated, bloodstream isolate from septic newborn	22
DK14	Serotype Ib, encapsulated	5
DK23	Serotype II, encapsulated	29
COH1	Serotype III, encapsulated, isolated from neonate with septicemia	35
CNCTC 1/82	Serotype IV, encapsulated	33
CNCTC 10/84	Serotype V, encapsulated	44
NT6	Serotype VI, encapsulated	44
87–603	Serotype VII, encapsulated	Pat Ferrieri[b]
JM9	Serotype VIII, encapsulated	Pat Ferrieri
AJ200	A909 Δ*rpoE*, allelic exchange mutant, Km^r	19
AJ8D3	A909 Tn*917*stm::*rpoE*, transposon mutant, Em^r	18
RN114	AJ200 containing pRN021, complemented strain, Sp^r	This study
RN115	AJ200 containing pLZ12, control strain, Sp^r	This study
RS020	A909 bearing pRS*gfpmut3*, Em^r	This study
RS021	A909 bearing $pRSP_{rpoE}$::*gfp*, Em^r	This study
B. subtilis		
MH5636	10× His tag fused to the 3′ end of *rpoC* (β′-subunit of RNAP)	32
E. coli		
XL1-Blue	Host strain for general cloning	Stratagene
MC1061	Host strain for propagation of Em^r plasmids	Stratagene
Origami	IPTG inducible expression strain F-*ompT hsd*S_B(r_B m_B)*gal*λ(DE3)[pLysS Cm^r]	Novagen
Plasmids		
pLZ12	Low-copy streptococcal expression vector, Sp^r	8
pRN021	pLZ12 derivative containing *rpoE* promoter and coding sequence	This study
pDC125	Streptococcal cloning vector, Em^r, Cm^r	4
pRS*gfpmut3*	pDC125 derivative containing *gfpmut3*, Em^r	This study
pBL26	Source of *gfpmut3* allele	Gift from B. Limbago
$pRSP_{rpoE}$–*gfp*	pRS*gfpmut3* derivative containing *gfp* under the control of the *rpoE* promoter	This study
pET32a	*E. coli* T7 expression vector, Ap^r	Novagen
pET32*rpoE*	Vector for over-expressing the δ protein, Ap^r	This study
pET32ck	pET32a derivative with *trx* removed, Ap^r	This study
pET32*rpoD*	Vector for over-expressing σ^A protein, Ap^r	This study
pET32*rpoB*	Vector for over-expressing β protein, Ap^r	This study

[a] Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Ap^r, ampicillin resistant; Sp^r, spectinomycin resistant.
[b] The strain was kindly provided by Pat Ferrieri, University of Minnesota, Minneapolis, MN.

from weak promoters. In addition, depending on the template, δ has been implicated in enhanced promoter melting (20) and may be involved in RNAP recycling (24). These data suggest that δ acts as an allosteric modulator of RNAP conformation and lacks enzymatic activity.

The function of δ in vivo has not been established. We previously reported that a GBS mutant with a transposon insertion adjacent to *rpoE* exhibited decreased survival in a neonatal rat sepsis model (18), suggesting a role for δ in virulence. In this study, we confirm the association of the δ protein with purified GBS RNAP and show that it is widely expressed among the serotypes. Using a reporter assay, we also demonstrate that *rpoE* expression in vitro is growth-phase dependent. An analysis of δ expression in the transposon mutant by quantitative Western blotting revealed a 10-fold reduction in the relative abundance, which was linked to the attenuation in virulence that was observed for this mutant. These data suggest that the abundance of δ may be an important aspect of GBS virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. GBS strains were grown in Todd-Hewitt broth (Difco Laboratories) in 5% CO_2 at 37°C. *Escherichia coli* strain MC1061 served as a host strain for cloning purposes. *E. coli* Origami was used as a host strain for the production of recombinant protein. *E. coli* was grown at 37°C under aeration in Luria broth (LB). When required, antibiotics were added to the medium as follows. For *E. coli*, ampicillin (Ap), 50 μg ml^{-1}, spectinomycin (Sp), 100 μg ml^{-1}, and erythromycin (Em), 500 μg ml^{-1}, were used, and for GBS, 5 μg ml^{-1} Em or 100 μg ml^{-1} Sp was added.

Purification of RNAP. RNAP was purified from GBS strains A909 and AJ200 and *B. subtilis* strain MH5636. For the isolation of RNAP from GBS, the strains were grown to mid-logarithmic phase (optical density at 600 nm [OD_{600}] was 0.4 to 0.6) and harvested by centrifugation. Approximately 5 g of cells (wet weight) were resuspended in protoplast preparation buffer (0.3 M potassium phosphate buffer [pH 7], 40% sucrose) containing endo-*N*-acetylmuramidase (mutanolysin, 0.5 U/μl) (Sigma) and incubated at 37°C for 90 min. Protoplasts were collected by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA [pH 8], 10% glycerol, 1 mM phenylmethylsulfonyl fluoride), and lysed by sonication (Vibra Cell VC 750 sonicator; Sonics). Lysates were clarified by centrifugation at 23,000 × *g* for 20 min, and the undiluted supernatant was applied to a heparin-agarose column (10 ml bed volume) (Sigma). Heparin is a polyanion that mimics DNA in its overall binding properties; thus, it is commonly used as a ligand for the purification of various DNA binding proteins, including DNA-dependent RNA polymerase. The column was washed with 3 volumes of re-equilibration buffer (10 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA [pH 8], 10% glycerol), and proteins were eluted with a linear salt gradient of 0.1 to 1.0 M NaCl in the same buffer (20 ml total volume). Fractions that contained RNAP (based on the presence of the β/β′ subunit doublet) were pooled and concentrated, and the salt concentration was adjusted to 0.1 M NaCl by buffer exchange using an Amicon-Ultra filter device (Millipore). The concentrate was loaded onto a high-pressure liquid chromatography UNO Q1 column (Bio-Rad) that

was equilibrated with pre-equilibration buffer, and proteins were eluted as described above by using an NaCl step gradient (50 ml total volume). Holoenzyme-containing fractions were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and finally loaded on a high-pressure liquid chromatography S-300 column (Amersham Biosciences) in re-equilibration buffer containing 200 mM NaCl. Peak fractions (based on absorbance at 280 nm) were pooled, concentrated, reconstituted in storage buffer (10 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 50% glycerol), and stored at −20°C. RNAP was isolated from *B. subtilis* strain MH5636 as described previously (32).

DNA manipulations. Routine molecular biology techniques for cloning, sequencing, and PCR amplification were performed as previously described (36). Chromosomal DNA was isolated from GBS strains by using the method of Madoff et al. (30). Plasmid DNA was isolated from GBS by using a modified QIAGEN plasmid mini-prep procedure (19). DNA restriction and modification enzymes were used according to the manufacturer's recommendations (New England Biolabs). GBS was transformed by electroporation as described previously (10).

Generation of recombinant RNAP subunits. Recombinant δ was expressed in *E. coli* by using the pET32a expression vector system (Novagen). A DNA fragment containing the entire coding sequence of *rpoE* was PCR amplified by using high-fidelity polymerase and the primers 5′-CATGCCATGGTATATGGATTA GAAAGAGAGGAATC and 5′-TTGCGGCCGCTTTTCTTGCTCGTTTTCC (underlining indicates either an EcoRI or an NotI restriction enzyme site) and A909 genomic DNA as a template. These primers were designed to include an NcoI or NotI restriction enzyme site in order to facilitate cloning into the pET32a plasmid vector. The resulting PCR product was digested with NcoI and NotI, ligated to similarly digested pET32a, and transformed directly into *E. coli* Origami (Novagen). The generation of the expected plasmid pET32*rpoE* was verified by restriction digest analysis and DNA sequencing of plasmids. To induce the expression of the thioredoxin (TRX) hexa-His-tagged–δ fusion protein (TRX-δ), *E. coli* Origami containing pET32*rpoE* was grown to an OD_{600} of 0.6, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM for 3 h. The TRX-δ fusion protein was purified under native conditions by using Ni-CAM HC agarose (Sigma) as the His tag affinity resin. TRX (containing the internal His tag) was cleaved from purified TRX-δ with enterokinase (Stratagene), which was used according to the manufacturer's instructions. Cleaved TRX and uncleaved TRX-δ were removed by further incubation with the Ni-CAM HC resin. The flowthrough containing pure δ was reconstituted in a phosphate-buffered saline solution (PBS; pH 7.0) containing 10% glycerol and stored in aliquots at −80°C until required for use.

Recombinant σ^A and β were generated as His-tagged fusion proteins. For the cloning of *rpoD* (σ^A) and *rpoB* (β), the *trx* coding sequence was first removed from pET32a by PCR, generating pET32ck. *rpoD* was amplified by using the primers 5′-CATGCCATGGCAGAGAAAAAAGGAAATAC and 5′-TTGCGG CCGCATCTTCCATGAAATCTTTAAGTTG (underlining indicates either an NcoI or an NotI restriction enzyme site), while *rpoB* was amplified by using the primers 5′-CGGAATTCTTGGCAGGACATGAAGTTCAG and 5′-TTGCGG CCGCATCTTCTTGAACGACTTCAGA (underlining indicates either an EcoRI or an NotI restriction enzyme site). Amplified products were digested with the appropriate restriction enzymes and ligated to similarly digested pET32ck. *E. coli* clones containing the correct vector were grown and induced as described above. Recombinant α^A and β with carboxyl-terminal His tags were purified under native conditions through incubation with Ni-CAM HC agarose. Eluted protein fractions were reconstituted in PBS and stored as described above.

Preparation of antisera. Antisera against recombinant δ, α^A, and β were generated in female New Zealand White rabbits as a service from Lampire Biological Laboratories (Pipersville, PA). In brief, rabbits were immunized subcutaneously with 500 μg of recombinant protein that was emulsified in complete Freund's adjuvant for the first dose and in incomplete Freund's adjuvant for subsequent doses (days 21 and 42 after initial immunization). Serum was prepared from blood collected approximately 2 weeks after the last dose was given.

Preparation of GBS lysates and Western blot analysis. GBS strains were grown to a designated OD_{600}, pelleted, washed in PBS, and resuspended in lysis buffer (60 mM Tris-HCl [pH 6.8], 10% glycerol). Whole-cell lysates were prepared through the use of a FastPrep FP101 bead beater (Bio 101) by using 2× 45-s bursts at 4°C at full speed, followed by the addition of SDS (final concentration, 3.3% wt/vol). The lysates were boiled for 5 min, and insoluble material was pelleted by centrifugation. The protein content in the supernatant was quantified by using a bicinchoninic acid assay (Pierce). Samples were normalized for protein concentration, mixed with 2× SDS gel loading buffer (36), and heated at 95°C for 5 min prior to SDS-PAGE analysis (36). The proteins were transferred to a nitrocellulose membrane by using a semidry transfer chamber (Bio-Rad) for 25 min at 15 V. The membrane was blocked overnight at 4°C in a 5% (wt/vol) solution of nonfat dehydrated milk in PBS. The blots were incubated at room temperature with a 1:1,000 dilution of rabbit antisera (raised against the designated protein) for 2 h, followed by a 90-min incubation with a 1:3,000 dilution of Alexa Fluor-680 anti-rabbit immunoglobulin G (IgG) (Molecular Probes). Immunoreactive bands were visualized at 700 nm by using a LiCor infrared imager (LI COR Biosciences).

RNA isolation. Total RNA was isolated from strains grown to an OD_{600} of 0.3 by using QIAzol (QIAGEN) according to the manufacturer's instructions, except that GBS cells were lysed through the use of a bead beater as described above. RNA samples were treated with DNase I (Promega) for 60 min at 37°C to remove any contaminating DNA and then purified by using an RNeasy mini kit (QIAGEN). RNA concentration was adjusted to 1 μg/ml, and samples were stored at −80°C until required for use.

Promoter mapping. Rapid amplification of cDNA ends (RACE) was used to identify the 5′ end of the *rpoE* transcript in the wild-type strain A909. First-strand cDNA synthesis was carried out according to the protocol of the 5′-RACE system (version 2.0; Invitrogen) by using 2 μg of total RNA and the *rpoE*-specific primer 5′-CTAAACCTCTTCTTCCTC (GSP1), followed by dCTP tailing of the 5′ end of the cDNA. Tailed cDNA was PCR amplified by using the 5′ RACE-abridged anchor primer (AAP; Invitrogen) and *rpoE*-specific primer 2 5′-CTA AACCTCTTCTTCCTCTTCTTCC (GSP2) with high-fidelity polymerase (Bioline). The product that was generated served as a template for a second PCR by using the nested primers' abridged universal amplification primer (AUAP, Invitrogen) and *rpoE*-specific primer 3 5′-AAAGCATTGACACGTTTCTTCTTA CG (GSP3). The final PCR product was sequenced and aligned to the published genome sequence by using Sequencher version 3.1 software (Gene Codes Corporation) to identify the transcription initiation start site, untranslated region, and promoter elements.

Construction of a complemented strain. To confirm that we had identified the correct promoter, we constructed a complemented strain for testing in our animal infection model. *rpoE* and the promoter were amplified by high-fidelity PCR by using Bio-X-Act DNA polymerase (BioLine) and A909 chromosomal DNA as a template. Restriction enzyme sites for EcoR I were incorporated into the primers to facilitate cloning into pLZ12, a low-copy streptococcal shuttle vector that replicates at 6 to 9 copies per cell in GBS (7). The ligation mixture was transformed into *E. coli* XL1-Blue, and clones containing the correct construct were identified by PCR. Plasmid DNA was isolated from a positive clone and designated pRN021. Electrocompetent AJ200 was transformed with pRN021 (or pLZ12 as a control) and plated on medium containing Sp to select for the plasmid. The complemented strain, AJ200 containing pRN021, was designated RN114, and AJ200, containing the vector pLZ12, was designated RN115.

Animal infection studies. Time-mated, barrier-sustained, female Sprague-Dawley rats were obtained from Charles River Laboratories. Fifty-percent-lethal-dose (LD_{50}) assays were performed by using the neonatal rat sepsis model as previously described (18). All procedures were performed in accordance with the guidelines provided by the Children's Hospital and Regional Medical Center Institutional Animal Care and Use Committee.

Construction of a reporter plasmid. To analyze *rpoE* expression, the native *rpoE* promoter was cloned upstream of the *gfpmut3* allele to allow for monitoring of promoter activity by flow cytometric analysis of green fluorescent protein (GFP) expression. The streptococcus-*E. coli* shuttle vector, pDC125 (4), was used to construct a reporter plasmid. The original *phoZ* and *cat* reporter genes were removed from pDC125 by PCR, and ClaI and NotI sites were added to facilitate the cloning of inserts. A streptococcal ribosomal binding site (GGAGG) (9) was inserted into the vector 7 bp upstream of an ATG start codon that was contained within the cloned ClaI site to optimize the expression of GFP. The *gfpmut3* allele, which has been optimized for bacterial codon usage (6), was PCR amplified by using pBL26 as a template. ClaI and NotI sites were incorporated into the 3′ and 5′ ends of *gfpmut3*, respectively. The resulting PCR product and plasmid were digested with ClaI and NotI and ligated together to generate pRS*gfpmut3*. A BamHI site was introduced into the *gfp* sequence of pRS*gfpmut3* to facilitate the insertion of the *rpoE* promoter fragment. An EcoR I-BamHI fragment, containing the promoter and the first four codons of *rpoE*, was ligated to pRS*gfpmut3* cut with the same enzymes, generating pRSP_{rpoE}-*gfp*. This reporter construct contains an in-frame fusion of the *rpoE* promoter to *gfp*.

Flow cytometry. Flow cytometric analysis was performed with a BD FACSCalibur (Becton Dickinson) equipped with a 488-nm argon laser and FlowJo software version 4.6.2. Overnight cultures of strains containing the reporter construct and the control vector were subcultured into fresh medium and grown to the indicated growth phases. Prior to analysis, cells were fixed at 4°C overnight in 2% (wt/vol) paraformaldehyde in PBS, washed, and finally resuspended in PBS. The PBS used in this study was filtered through a 0.22-μm-pore-size filter (Millipore)

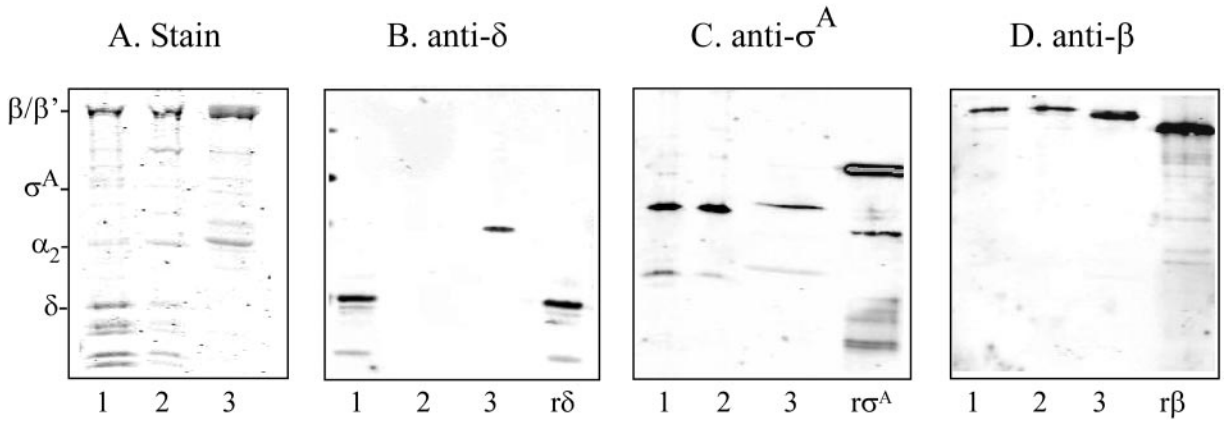

FIG. 1. RNAP purification and subunit analysis. A total of 1 to 2 μg of purified RNAP from GBS strains A909 (1) and AJ200 (2) and *B. subtilis* strain MH5636 (3) were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie blue (A). Subunit identity is shown on the left. Western blot analysis was performed on the RNAP preparations by using anti-δ (B), anti-σ^A (C), or anti-β (D) antisera. Recombinant δ (rδ), σ^A ($r\sigma^A$), and β (rβ) served as positive controls. Immunoreactive bands were detected by using an infrared imager after incubation with a rabbit anti-IgG infrared-labeled secondary antibody.

to minimize fluorescence. A total of 100,000 events were analyzed for each sample at constant parameter settings for each experiment.

Northern hybridization. Total RNA was separated on a 0.8% agarose gel and transferred to a positively charged nylon membrane (Hybond N+, Roche) by alkaline transfer as described previously (36). A probe that encompassed the complete *rpoE* open reading frame was generated by incorporating digoxigenin-11-dUTP into a PCR product by using the protocol provided by the manufacturer (Roche Diagnostics) and the following primer pair: 5′-ATGACAAAA AAACATCTTAAAACG and 5′-TTGCGGCCGCTTTTCTTGCTCGTTTTCC. The hybridization and wash conditions used were as specified by Roche Diagnostics. Gene-specific bands were detected by chemiluminescence using CPSD (Roche Diagnostics) as the alkaline phosphatase substrate.

RESULTS

δ is a subunit of RNA polymerase in GBS. To identify the subunits of RNAP in GBS, native RNAP was purified from the wild-type strain A909 by using a modified affinity and size exclusion chromatography procedure that takes advantage of specific substrate binding and the size of the enzyme. Chromatographic steps included heparin affinity, anion exchange (UNO Q1), and size exclusion (S-300) chromatography. RNAP was isolated from *B. subtilis* MH5636 for comparison. As seen in Fig. 1A, SDS-PAGE analysis of the RNAP preparation from A909 revealed that it consists of subunits that are consistent with the expected sizes for β′ (135 kDa), β (135 kDa), α (34 kDa), σ^A (42 kDa), and δ (21 kDa). This is similar to what we observed for *B. subtilis* MH5636. These results are in agreement with the expected sizes for the gene products of *rpoC*, *rpoB*, *rpoA*, *rpoD*, and *rpoE*, respectively, based on the available GBS genome sequences (12, 40). As a control, we also isolated RNAP from AJ200, a mutant in which *rpoE* has been replaced with a kanamycin cassette by allelic exchange (19). As expected, while we detected subunits corresponding to β′, β, α, and σ, the δ protein was absent.

Western blot analysis was used to further confirm the association of δ with RNAP in GBS. Antiserum raised against purified recombinant GBS δ was used to probe the purified RNAP preparations from A909, AJ200, and *B. subtilis* MH5636. The antiserum reacted with a 21-kDa band corresponding to δ in the RNAP that was isolated from A909 (Fig. 1B). Although there was an approximately 21-kDa protein visible in the Coomassie-stained gel of the RNAP preparation from AJ200 (Fig. 1A), as expected, δ was not detected by Western blot analysis in this preparation (Fig. 1B). The antiserum did not recognize δ in the bacillus RNAP. This observation can be explained by the fact that there is a significant difference in the amino acid composition of δ between GBS and *B. subtilis* (33% identity and 61% similarity). Alternatively, there may be species-specific differences in the tertiary structure of this protein. Although the antisera cross-reacted with a 40-kDa protein in this RNAP preparation, antisera raised against bacillus δ also identified a similarly sized bacillus protein in addition to δ (data not shown). To confirm that we had correctly identified the other subunits of RNAP in GBS, Western blot analysis using antisera directed against σ^A (Fig. 1C) and the β subunit (Fig. 1D) was performed. Both subunits were detected in the RNAP preparations from the GBS strains that we examined. These data suggest that under these conditions, the sigma factor that is associated with RNAP is σ^A. The antisera also reacted with the σ^A and β subunits in RNAP from *B. subtilis* MH5636, which is consistent with the high degree of amino acid sequence homology that the *B. subtilis* σ^A (65% identity and 78% similarity) and β (68% identity and 82% similarity) subunits share with the GBS homologs.

δ is expressed among all serotypes of GBS. Western blot analysis was used to examine whether the expression of δ is limited to strains in serotype 1a. Whole-cell lysates of strains representing all nine serotypes were prepared. The anti-δ antiserum detected an approximately 21-kDa band corresponding to δ in all of the strains that were tested (Fig. 2). These data indicate that the expression of δ is not limited to A909.

***rpoE* is expressed from a σ^A-dependent promoter.** To identify the transcriptional start site and the promoter consensus elements, we mapped the 5′ end of the *rpoE* transcript by 5′-RACE PCR. The positions of the primers and predicted sizes of the products are shown in Fig. 3A. Sequencing of the PCR product that was generated by using the gene-specific GSP3 and AUAP anchor primers identified the transcriptional

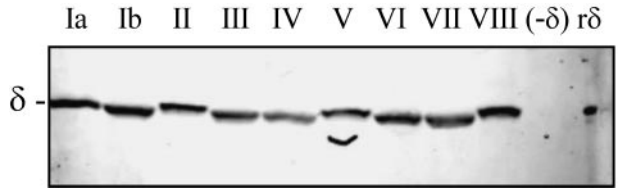

FIG. 2. Analysis of δ expression across the serotypes. Western blot analysis was performed on lysates of strains representing all nine clinically relevant serotypes by using antiserum raised against recombinant δ. The serotype of the strain is indicated above each lane. Ia, A909; Ib, DK14; II, DK23; III, COH1; IV, CNCTC 1/82; V, CNCTC 10/84; VI, NT6; VII, 87–603; VIII, M9; (-δ), AJ200; rδ, recombinant delta protein.

initiation site, which was located ~109 bp upstream of the TTG start codon (Fig. 3C). Comparison of this sequence to the A909 genome sequence identified a putative −10 and −35 promoter region that was located 9 bp upstream of the 5′ untranscribed region. The predicted −35 (TTGACG) and −10 (TAAAAT) elements are consistent with what has been reported for σ^A-dependent promoters in *B. subtilis* (16), suggesting that *rpoE* is transcribed by the RNAP that is associated with σ^A in GBS.

TABLE 2. Lethal dose values for complemented strains in neonatal rat sepsis model of infection

Strain	Log LD_{50} for expt. no.	
	1	2
A909 (wt)	4.91	5.6
RN115 (Δ*rpoE*)	6.99	7.11
RN114 (Δ*rpoE* + *rpoE*)	4.85	5.24
AJ8D3 (A909 Tn*917*::*rpoE*)	7.00	

[a] LD_{50} values were calculated using the method of Reed and Muench (34).

Complementation of virulence in neonatal rat sepsis model. The *rpoE* deletion mutant AJ200 was transformed with a low-copy vector containing the *rpoE* coding sequence and promoter elements that we identified by using the 5′-RACE technique. We compared the virulence of the complemented strain (RN114) to the wild-type strain in the neonatal rat sepsis model. AJ200 containing empty vector (RN115) was used as a control. In two separate experiments, providing *rpoE* expressed from the promoter that we identified on a low-copy vector restored virulence to wild-type levels (Table 2). The log of the LD_{50} for RN114 was similar to that for the wild-type strain A909, while the log of the LD_{50} for RN115 was 1.5 to 2 log

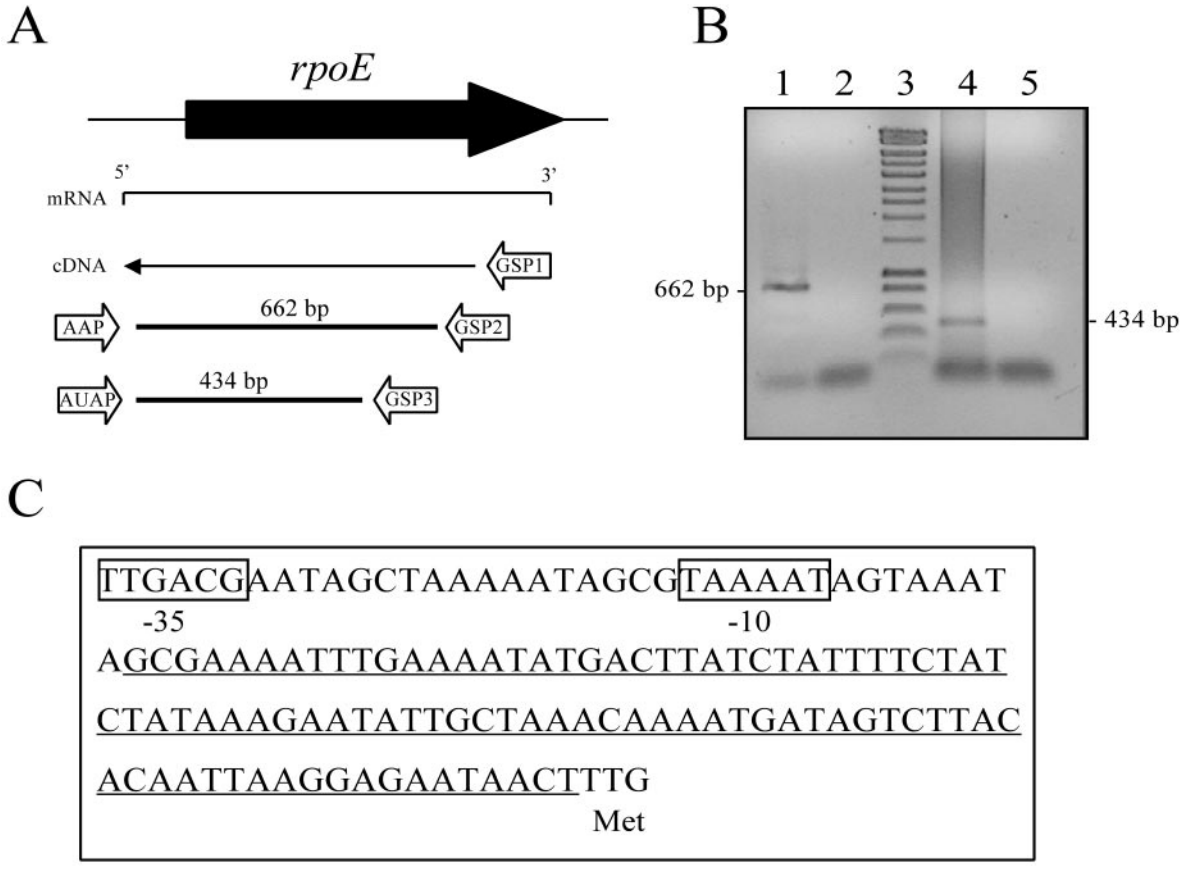

FIG. 3. Identification of the *rpoE* promoter. (A) Schematic diagram of the 5′-RACE PCR strategy, primers, and product sizes. (B) Amplicons were obtained in primary PCR by using primers AAP and GPSP2 (lane 1) and were obtained in nested PCR by using primers AUAP and GSP3 (lane 4). Lanes 2 and 5 contain the respective negative controls for these PCRs, and lane 3 contains a DNA marker. (C) Sequence of *rpoE* promoter and 5′-untranslated region. The predicted −35 and −10 consensus elements are boxed. The 5′-untranslated region is underlined. The methionine start codon (TTG) is also indicated.

units higher than that for the wild type. These data confirmed that we had correctly identified the native *rpoE* promoter. In addition, the virulence of the original transposon mutant AJ8D3 was compared to those of A909 and the complemented strain. The LD_{50} for AJ8D3 was 2 log units higher than those for A909 and RN114 (Table 2), confirming that *rpoE* expression is required for survival in the host.

Analysis of rpoE expression. Once we confirmed that we had identified the native promoter for *rpoE*, we generated a reporter construct to characterize expression during growth in vitro. The expression of *rpoE* was analyzed by measuring the fluorescence of strains containing transcriptional fusions to *gfpmut3*. The promoter region for *rpoE* was cloned upstream of a promoterless *gfpmut3* allele, generating pRS*PrpoE-gfp*. A909 was transformed with the reporter plasmid, generating strain RS021, or empty vector as a control, generating strain RS020. We first confirmed that the expression of GFP from the *rpoE* promoter did not affect growth of the strain. As shown in Fig. 4A, the expression of GFP did not significantly affect the growth of RS021 in laboratory medium relative to the control strain RS020. Flow cytometric analysis of GFP expression in RS021 indicated that *rpoE* was expressed during all stages of growth. GFP fluorescence increased from lag to early logarithmic phase, reached a maximum at the late logarithmic stage, and dropped off during stationary phase (Fig. 4B). These data are consistent with what we have observed by using microarray analysis of *rpoE* expression in A909 during growth in vitro (unpublished data), confirming that the pattern of expression that we observed with the plasmid construct is representative of gene expression occurring from the chromosomal locus.

***rpoE* is transcribed in AJ8D3.** *rpoE* was first identified as being required for virulence of GBS during a signature-tagged transposon mutagenesis (STM) screen utilizing a modified Tn*917* (Tn*917stm*) (18). The transposon mutant AJ8D3 was severely attenuated for virulence and had a single Tn*917stm* insertion adjacent to *rpoE*. Sequencing of genomic DNA isolated from AJ8D3 subsequently revealed that the transposon had inserted between the −10 promoter element and the transcription initiation site (data not shown). To examine whether *rpoE* was expressed in AJ8D3, Northern blot analysis was conducted on total RNA isolated from this mutant and from A909 for comparison. Using a probe that was specific for *rpoE*, we detected a single transcript in RNA from A909 that we estimated to be 550 bp in size, indicating that *rpoE* is transcribed as a monocistronic message (Fig. 5A). In contrast, multiple transcripts were detected in the AJ8D3 RNA. Three primary transcripts were detected (Fig. 5A). However, none were large enough to represent a transcript that was being initiated from the native promoter now located 8.6 kb upstream of the coding sequence for *rpoE*. These data indicate that transcription of *rpoE* in AJ8D3 is being initiated from promoters within the transposon. In addition, the total band intensity of the *rpoE*-specific transcripts detected in AJ8D3 was considerably reduced compared to that of the single transcript detected in A909, suggesting that the strength of the nonnative promoters may be weaker than the native one. Control experiments using a probe for *rpsL* and the same RNA samples demonstrated that equal amounts of total RNA from the isogenic strains were used in the analysis (data not shown).

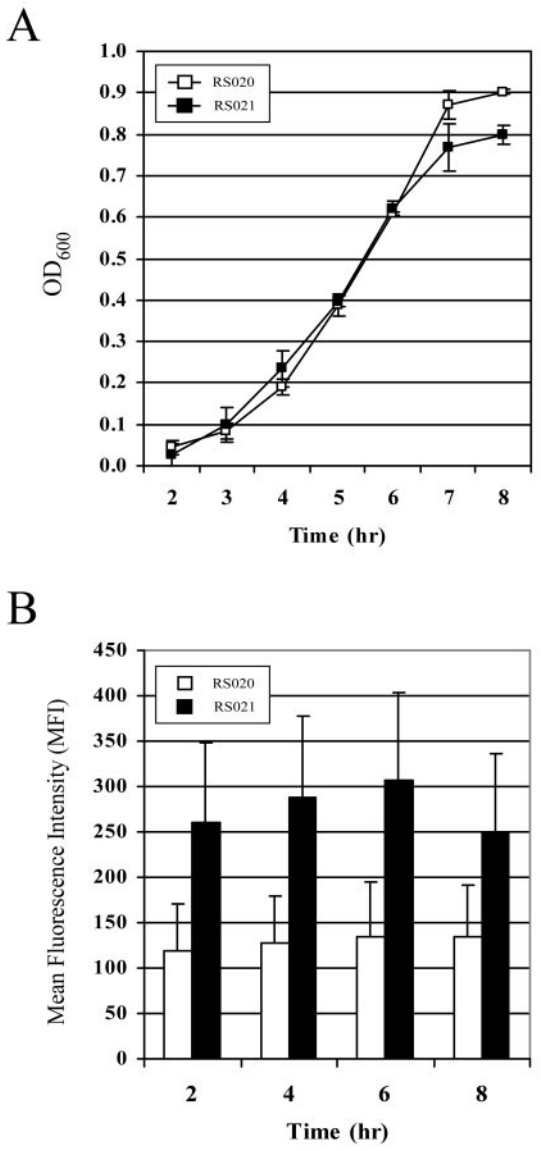

FIG. 4. Analysis of *rpoE* promoter activity using a reporter construct. (A) Growth of RS021 containing the *rpoE* reporter plasmid (■) and the RS020 control strain (□). (B) Flow cytometric analysis of RS021 and RS020. GFP fluorescence is shown as the mean fluorescence intensity (MFI) of the bacterial population, which was calculated by using the FlowJo software version 4.6.2. The data presented are means ± standard error of the means and are representative of three experiments.

The 5′-RACE approach was used to identify the transcription initiation sites for *rpoE* in AJ8D3. Consistent with our Northern analysis, we obtained several amplification products from the 5′-RACE PCR (data not shown). Previous sequencing of Tn*917*, together with transcription studies in *Enterococcus faecalis* transposon mutants, identified three functional promoters (P1, P2, and P3) (Fig. 5C) that were responsible for initiating the transcription of Tn*917* genes (38). The promoter P1 (−35 [TTGATA] and −10 [TATAAT]) is located upstream of the *erm* gene, promoter P2 (−35 [TTAATG] and −10 [TAT AAT]) is immediately upstream of the resolvase gene *tnpR* and

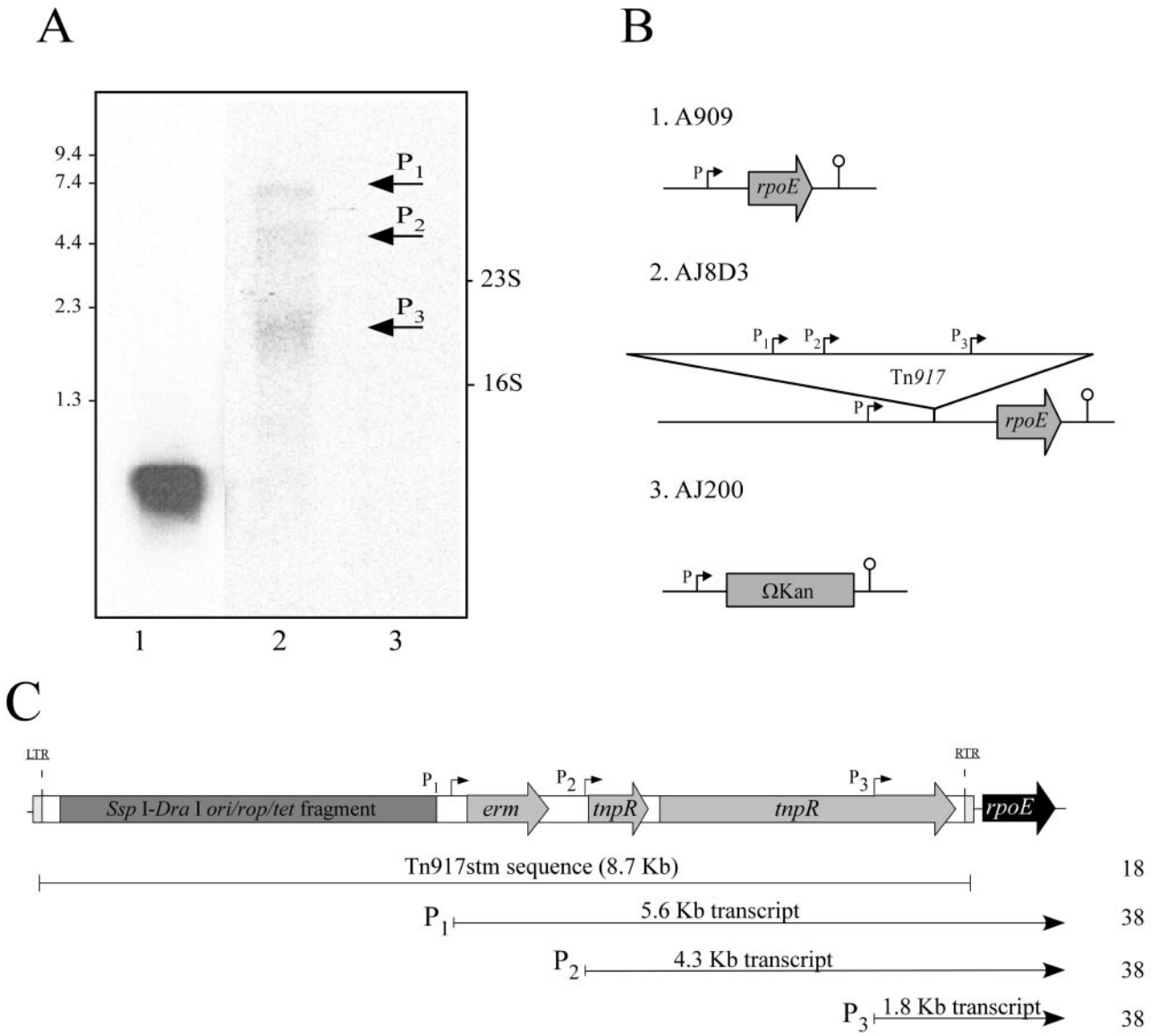

FIG. 5. Transcriptional analysis of *rpoE* in isogenic strains. (A) Northern blot of RNA prepared from A909 (1), AJ8D3(2), and AJ200 (3) using a digoxigenin-labeled DNA probe that was specific for *rpoE*. The positions of size markers (in kilobases) and the 23S and 16S rRNAs are indicated. (B) Schematic diagram of the *rpoE* locus in the isogenic strains. (C) Promoter location within Tn*917stm*. The location of promoter regions within Tn*917stm* are indicated. Predicted transcript sizes that were initiated from these three promoters sites are given in kilobases. Shaded box, pBR322 *ori*/*rop*/*tet* region; gray arrows, erythromycin gene (*erm*), resolvase (*tnpR*), and transposase (*tnpR*); black arrow, *rpoE* gene. LTR, left terminal repeat; RTR, right terminal repeat. Promoter regions within are represented as P1, P2, and P3. Numbers on the right are reference numbers.

promoter P3 (−35 [ATGCCA] and −10 [TATAAA]) is located within the transposase (*tnpA*). Based on the size of the transcripts and the sequence of the 5′-RACE PCR products that we obtained from AJ8D3, it is likely that the transcription of *rpoE* is being initiated from these previously identified promoters in Tn*917*.

Abundance of δ in AJ8D3 is reduced compared to that of the wild-type strain. Since we detected transcription of *rpoE* from nonnative promoters in AJ8D3, we sought to determine whether these messages were translated into protein. We compared the expression of δ in whole-cell lysates from A909 and AJ8D3 during growth in vitro by Western blot analysis using the antiserum directed against δ. In order to estimate the relative quantity of δ, a standard curve of serially diluted recombinant protein was prepared and analyzed in parallel with the lysates. As shown in Fig. 6A, the amount of δ in A909 appeared constant throughout all phases of growth, indicating continual expression. Interestingly, we also detected δ in AJ8D3 at all growth phases tested. However, the amount of δ protein in AJ8D3 was significantly reduced. Comparison of the band intensity to the standard curve (Fig. 6D) indicated that at all stages of growth, there is at least 10-fold less δ present in AJ8D3 compared to that in A909. As expected, no δ was detected in AJ200. As a control to demonstrate equal protein

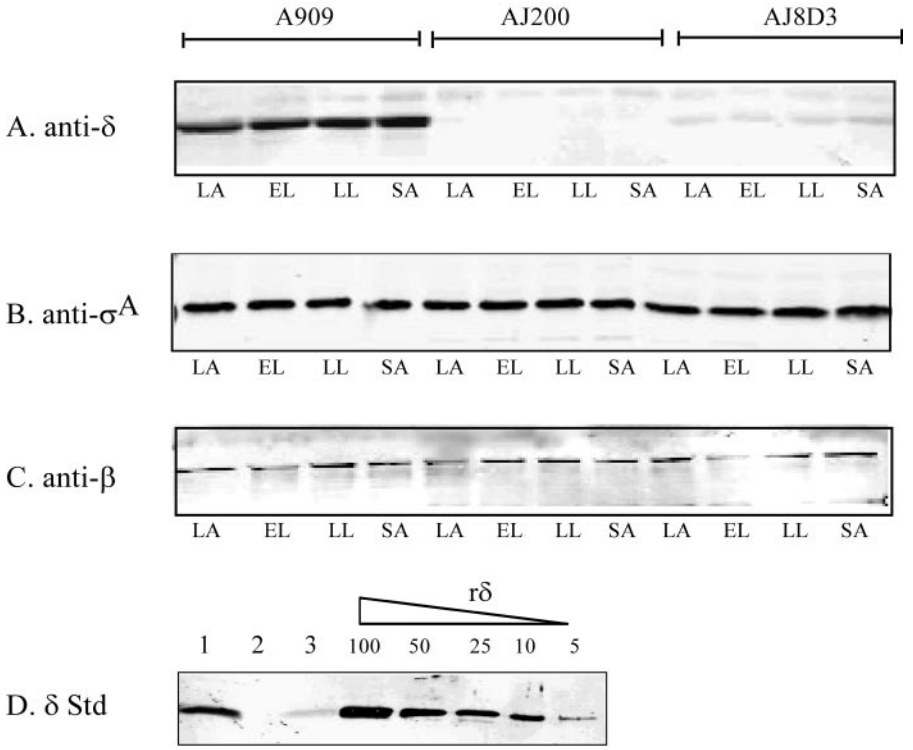

FIG. 6. Immunoblot analysis of δ, σ^A, and β subunits during growth. A909, AJ200, and AJ8D3 were grown to lag (LA) (OD_{600} = 0.1), early log (EL) (OD_{600} = 0.3), late-log (LL) (OD_{600} = 0.3) and stationary (SA) phases (OD_{600} = 1.0). Lysates were normalized for total protein (10 μg) and subjected to SDS-PAGE. Western blots were performed by using antisera directed against δ (A), σ^A (B), and β (C). Protein levels were probed by using polyclonal sera raised against the recombinant protein. (D) Estimation of δ levels. A total of 10 μg of A909 (1), AJ200 (2), and AJ8D3 (3) lysates were loaded together with 100 ng, 50 ng, 25 ng, 10 ng, and 5 ng of purified δ. Blots were probed with antisera directed against δ. Immunoreactive bands were detected by using an infrared imager after incubation with a rabbit anti-IgG infrared-labeled secondary antibody, and band intensity was compared between strains. rδ, recombinant delta protein.

loading between samples, we repeated the analysis by using antisera directed against σ^A (Fig. 6B) and the β subunit (Fig. 6C). Western blotting using anti-σ^A and anti-β antisera indicated that the levels of these two subunits remained constant in the three strains throughout growth. These data demonstrated that the transcription of *rpoE* from the nonnative promoters in Tn*917* results in a reduction in δ protein levels in AJ8D3. This finding provides a likely explanation for the attenuated virulence that was observed for this mutant and suggests that a specific level of δ is required for virulence of GBS.

DISCUSSION

Bacterial RNAP is considered to play a pivotal role in global changes in gene expression. Although the core enzyme is a highly conserved heteromeric structure ($\beta\beta'\alpha_2$), the composition of the holoenzyme can differ considerably among bacterial species and with the environmental conditions. A significant amount of information regarding RNAP composition and the mechanics of transcription has been generated. However, most of this information is based on the analysis of only a few model organisms, including *E. coli* and *B. subtilis*. Our knowledge of RNAP composition in streptococci, particularly in GBS, remains in its infancy. Yet, the elucidation of the composition of the transcriptional machinery is of fundamental importance for understanding the mechanisms that this microbe utilizes to control the transcription process.

In this study, we used a well-documented multistage purification procedure to isolate RNAP from the cytosol of GBS. To our knowledge, this represents the first detailed description of RNAP isolation and subunit composition in GBS. The RNAP that we isolated consisted of subunits corresponding to α, β, β′, and δ, which are consistent with RNAP composition analyses reported for other gram-positive bacterial species (7, 17, 28, 39). The preparation also contained a subunit corresponding to σ^A, the primary sigma factor in gram-positive bacteria. Interestingly, following size exclusion chromatography, the final GBS RNAP preparation also contained a small number of protein bands in addition to the α, β, β′, σ^A, and δ subunits, indicating that other potential RNAP-associated proteins may have been isolated. Analyses of the available genome sequences have identified genes encoding two alternative sigma factors in addition to σ^A (12, 40). Thus, these additional protein bands may represent alternative sigma factors. Alternatively, proteins sharing a similar binding affinities to the various columns used in the chromatography steps may have also copurified, a feature that is indicative of isolating a multisubunit enzyme (17, 39). However, without further analysis, it is impossible to determine whether these proteins represent additional sigma factors, breakdown products of the core, or copurifying contaminating proteins.

The δ protein is a subunit of RNAP found in only gram-positive bacteria. This ~21 kDa protein was originally identified in *B. subtilis* (26) but has also been demonstrated to copurify with RNAP in *Staphylococcus aureus* and *Streptococcus pneumoniae* (7, 28). Here we provide evidence that the δ homolog in GBS also associates with RNAP following purification.

GBS strains are grouped into serotypes on the basis of capsular polysaccharide on the bacterial surface. A number of virulence traits, particularly surface proteins, have been reported to be serotype specific (for a review, see reference 23). Western blot analysis of a panel of strains representing all of the nine serotypes confirmed that δ was expressed in all of the strains tested and not limited to one serotype. Taken together with the observation that an *rpoE* homolog is present in every gram-positive bacterial genome currently sequenced (19), it appears that δ is highly conserved among gram-positive bacteria.

The putative promoter for *rpoE* was identified by using a 5′-RACE PCR approach. The expression of *rpoE* from this promoter on a low-copy plasmid restored the virulence of the deletion mutant to wild-type levels, confirming that we had correctly identified the promoter. Reporter constructs were then used to monitor *rpoE* expression from this promoter during growth in vitro. The expression of *rpoE* was continual throughout growth, but it appeared to reach a maximal level during exponential growth. A similar pattern of expression has been reported for *rpoE* in *Bacillus subtilus* (25), suggesting that this pattern may be conserved among gram-positive bacteria.

δ was initially linked to the virulence of GBS when a mutant with a transposon insertion adjacent to *rpoE* was identified in an STM screen (18). In the current study, we demonstrated that *rpoE* is transcribed in the transposon mutant AJ8D3 but that transcription is initiated from multiple promoters within the transposon and not from the native promoter. Additionally, the overall amount of *rpoE* transcript was substantially reduced compared to that of the wild-type strain. The modified transposon used in the STM screen (Tn*917stm*) contains at least five known promoters. Three promoters were originally reported during the sequencing and analysis of Tn*917* (38), and modification of the transposon for STM introduced two additional promoters (18). The results presented here indicate that the transcription of *rpoE* can occur from these promoters, although this appears to be less efficient than from the native promoter. Western blot analysis demonstrated that this message is translated into δ protein. However, δ is present in reduced abundance of ~10-fold relative to the wild-type strain. We have previously reported that AJ8D3 is as attenuated in the neonatal rat sepsis model as it is in the *rpoE* deletion mutant that does not express any δ (19). Based on these collective observations, we conclude that a critical amount of δ is required within the cell for virulence but that the levels of the protein in AJ8D3 do not reach this threshold. The decreased amount of δ in AJ8D3 is likely a result of the low abundance of the *rpoE* transcript. It is also possible that transcripts that were initiated from the promoters in the transposon are less stable or are translated less efficiently.

It is not yet known what role δ plays in the virulence of GBS. Functional activity for δ has been demonstrated in *Bacillus subtilis* by using in vitro assays (20, 24, 39). The generation of *rpoE* mutants of *Bacillus subtilis* has been reported (21, 25), though they lack a well-defined phenotype. More recently, it has been suggested that *rpoE* may have a direct or indirect role in *Bacillus subtilis* sporulation by affecting the transcription of genes that are required at specific stages (11). A mutation in *rpoE* has also been reported to affect the ability of *S. aureus* to recover from nutrient starvation (42).

If the activities demonstrated for bacillus δ can be extrapolated to GBS, then maintaining the transcriptional specificity and efficient recycling of RNAP would appear to have a profound effect on virulence. These functions may allow this organism to adapt to environmental change more efficiently, making it a more successful pathogen. It seems unlikely that δ has global effects on transcription since *rpoE* is not required for viability and mutants have only a limited number of phenotypic changes (11, 19, 25, 42). In GBS, δ may impact the expression of a subset of genes that are required at a critical stage for survival in our animal model. Our data suggest that the relative abundance of δ is of critical importance for virulence of this organism. Further study is needed to determine how δ influences RNAP activity and whether it affects gene expression in vivo.

ACKNOWLEDGMENTS

We thank J. D. Helmann for generously providing *B. subtilis* strain MH5636 and antiserum directed against bacillus δ as well as Donald Chaffin for technical assistance with the HPLC.

This work was funded by the Streptococcal Initiative of the National Institutes of Health, grant no. BWH 811501/N01-AI-75326 and R01AI52299-01.

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