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Inactivation of the α C protein antigen gene, *bca*, by a novel shuttle/suicide vector results in attenuation of virulence and immunity in group B *Streptococcus*

(*Streptococcus agalactiae*/surface antigens/bacterial pathogenesis/bacterial vaccines)

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ABSTRACT **The α C protein of group B *Streptococcus* (GBS) is a major surface-associated antigen. Although its role in the biology and virulence of GBS has not been defined, it is opsonic and capable of eliciting protective immunity. The α C protein is widely distributed among clinical isolates and is a potential protein carrier and antigen in conjugate vaccines to prevent GBS infections. The structural gene for the α C protein, *bca*, has been cloned and sequenced. The protein encoded by *bca* is related to a class of surface-associated proteins of Gram-positive cocci involved in virulence and immunity. To investigate the potential roles of the α C protein, *bca* null mutants were generated in which the *bca* gene was replaced with a kanamycin resistance cassette via homologous recombination using a novel shuttle/suicide vector. Studies of lethality in neonatal mice showed that the virulence of the *bca* null mutants was attenuated 5- to 7-fold when compared with the isogenic wild-type strain A909. Significant differences in mortality occurred in the first 24 h, suggesting that the role of the α antigen is important in the initial stages of the infection. In contrast to A909, *bca* mutants were no longer killed by polymorphonuclear leukocytes in the presence of α-specific antibodies in an *in vitro* opsonophagocytic assay. In contrast to previous studies, α antigen expression does not appear to play a role in resistance to opsonophagocytosis in the absence of α-specific antibodies. In addition, antibodies to the α C protein did not passively protect neonatal mice from lethal challenge with *bca* mutants, suggesting that these epitopes are uniquely present within the α antigen as expressed from the *bca* gene. Therefore, the α C protein is important in the pathogenesis of GBS infection and is a target for protective immunity in the development of GBS vaccines.**

Group B *Streptococcus* (GBS) is the leading cause of neonatal sepsis and meningitis in the United States. It is also an important pathogen in postpartum endometritis and other serious invasive infections in adults (1–3). On the basis of type-specific capsular polysaccharides, GBS strains are classified into at least nine distinct serotypes. These capsular polysaccharides are the best-studied GBS virulence factors, and antibodies against them are protective in mice to challenge with GBS of the same serotype (4, 5). In the past, efforts have been focused on developing polysaccharide-based vaccines to prevent GBS infections (1). However, the immunogenicity of these capsular components is suboptimal and varies with the specific type of polysaccharide (6, 7). Recently, conjugate protein–polysaccharide vaccines have been developed that elicit a greater quantity of antibodies protective against GBS (8–11).

GBS proteins have also been implicated in bacterial virulence, but their role in pathogenesis of GBS infections is not well understood (12). GBS C proteins are a class of surface-associated proteins identified in many serotypes (13, 14). Two of the C proteins, α and β, have been characterized by their molecular, biochemical, and immunological properties (15–18). The α antigen is the most common C protein found in GBS clinical isolates; it is present on at least 50% of all GBS strains, mainly on serotypes Ia, Ib, and II. Recently, a surface protein called Rib, expressed on serotype III strains, was reported to have properties distinct from but related to those of the α C protein (19). An α-like surface protein is also produced on serotype V strains (20). These proteins may represent a family of related GBS surface antigens.

Antibodies to the α C protein protect mice from lethal challenge with α-expressing GBS strains (18, 21, 22), and these antibodies are opsonic *in vitro* (23, 24). The structural gene for the α C protein, *bca*, has been cloned from the prototype GBS strain A909 (serotype Ia, expressing the α and β C proteins) and reveals an ORF of 3,060 bp (12, 18). Sequence analysis of the *bca* gene demonstrated that the α C protein is related to a class of surface-associated proteins of Gram-positive cocci, including the M proteins of group A *Streptococcus*, thought to have a role in virulence and immunity. One unique feature of the *bca* gene is that it contains nine identical tandem repeats, each 246 bp long. Western immunoblots of both the native and cloned gene products of the α C protein exhibit a regularly laddered pattern of heterogeneous polypeptides. The intervals in the α ladder pattern correlate with the molecular size of the peptide encoded by each of the repeat regions in the *bca* gene (12). The maximum molecular sizes of the α C proteins expressed from different clinical GBS isolates differ from 62,500 to 167,000 Da (23); such size variations correspond to different numbers of the repeats existing within the *bca* gene (12, 18, 24). These heterogeneous laddering patterns have also been found in the Rib protein, which appears similar, if not identical, to the previously described R4 proteins of GBS (19, 25). The molecular size changes in the α protein may provide bacterial cells with diverse antigenic and protective epitopes for evasion of the host's immune defenses (24, 26).

Despite the genotypic and phenotypic diversity in the α C protein and the extensive range of its expression among GBS isolates, it is not known whether the α C protein is directly involved in virulence. In the studies reported here, knockout mutants of GBS strain A909 were developed in which the *bca* gene was replaced with an antibiotic marker by homologous recombination.

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Abbreviations: GBS, group B *Streptococcus*; Ts, thermosensitive; Ia-TT, serotype Ia polysaccharide coupled to tetanus toxoid; sco, single crossover; dco, double crossover; LD_{50}, lethal dose at 50% mortality; PMN, polymorphonuclear leukocyte; cfu, colony forming unit; THB, Todd–Hewitt broth; O/N, overnight.

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The α-antigen-negative mutants were used to examine the function of the α C protein through *in vitro* opsonophagocytic tests and in an animal model involving neonatal mouse lethality determinations. These genetic constructs demonstrate that the α C proteins play a role in both the virulence of and immunity to GBS.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Culture Media, and Antisera. The prototype GBS strain A909 [Ia/C(α^+,β^+)] (21) was used to generate isogenic knockout mutants. *Escherichia coli* strains DH5α and JM83 were used as hosts for molecular cloning. pVE6007 (Cm^r) is a thermosensitive (Ts) plasmid derivative of pWV01 carrying a multicloning site originating from pBluescript (27). pVE6007 replicates at 28°C in a large number of Gram-positive bacteria including streptococci, but replication is blocked at 37°C. To facilitate cloning in *E. coli*, a 935-bp *Dra*I–*Pvu*II restriction fragment containing the origin of replication from plasmid pUC19 was inserted into a *Sac*I site on pVE6007. The resulting plasmid, pJL1055, was used as a shuttle/suicide vector. The pUC19 origin allowed maintenance of the plasmid at 37°C in *E. coli*, the Ts origin maintained the plasmid at 28°C in GBS. A selectable resistance marker from pBRΩKm-2, which contains a cassette with the *aphA-3* kanamycin-resistant element flanked by strong transcription and translation terminators (28), was used to replace the *bca* gene in GBS strain A909.

GBS was grown in Todd–Hewitt broth (THB) (Difco) and on trypticase soy with 5% sheep's blood agar plates (Becton Dickinson). *E. coli* strains were grown in Luria-Bertani broth (29). Final concentrations of antibiotics (Sigma) were chloramphenicol at 10 μg/ml for *E. coli* and GBS and kanamycin at 50 μg/ml for *E. coli* and 500 μg/ml for GBS. Rabbit antisera were prepared to the α C protein (22), the α C protein N-terminal region (30), the β C protein (31), and the serotype Ia polysaccharide coupled to tetanus toxoid (Ia-TT) have been described (32). The mouse mAb 4G8, directed to the repeat region of the α C protein, was also used (22, 30).

Construction of an Integration Plasmid for the *bca* Replacement. DNA fragments containing the flanking regions of the *bca* structural gene were prepared by PCR (33). The N-terminal flanking fragment starts 220 bp upstream of the *bca* gene ATG start codon and contains 1,200 bp. The C-terminal flanking fragment begins immediately downstream of the *bca* gene stop codon and is 750 bp long. Restriction enzyme sites *Eco*RI and *Bam*HI or *Bam*HI and *Sac*II were introduced at the ends of these PCR fragments, respectively (Fig. 1). A 2.1-kb *Bam*HI–*Bam*HI fragment cleaved from pBRΩKm-2, which contains the kanamycin resistance cassette, was ligated between the two *bca*-flanking fragments using *Bam*HI sites, and this tripartite ligation product was cloned into the shuttle/suicide vector pJL1055 at *Eco*RI–*Sac*II sites. The final construct, pJL1147 as shown in Fig. 1, was constructed in *E. coli* and then introduced into GBS A909 by electroporation (ref. 34; P. Framson and C. E. Rubens, personal communication). The structure of pJL1147 was verified by restriction mapping and nucleotide sequencing of the junctions between the various components (data not shown).

Isolation of GBS Homologous Recombinants. Fig. 2 shows the strategy for the recombinational events described below. (*i*) Single crossover (sco) integration into the GBS chromosome. GBS A909 containing pJL1147 was grown overnight (O/N) at 28°C in the presence of chloramphenicol. On day 2, cells were diluted 100-fold and grown at 28°C for an additional 2.5 h. The culture was shifted to 37°C, the nonpermissive temperature for plasmid replication in GBS, and grown for 3 h before plating. Km^rCm^r colonies were selected at 37°C as sco recombinant cells as shown in Fig. 2. (*ii*) Double crossover (dco) integration into the GBS chromosome. A population of sco recombinant cells, in which pJL1147 was integrated into A909 chromosome, were grown for 24 h at 37°C in the presence of chloramphenicol or kanamycin. The culture was diluted $1:10^5$ in medium without antibiotics and grown at 28°C O/N to allow a second crossover recombination event, which would excise the integrated plasmid from the chromosome. To identify cells in which the kanamycin cassette had replaced the *bca* gene on the A909 chromosome, colonies from the O/N culture were replicate plated at 37°C on media containing either chloramphenicol or kanamycin to screen for Km^rCm^s colonies (Fig. 2).

Southern Blot Hybridization. DNA southern blot hybridization (36) was carried out as described (37) or according to the manufacturer's instructions (Amersham). Total DNA was isolated from GBS strains as described (4, 35). DNA probes were made either by PCR on A909 chromosomal DNA templates or by restriction digestion of plasmid DNA. There were three different probes: (*i*) a 535 bp PCR fragment within the *bca* coding region; the PCR primers were 5′-TAACAGTTATGATACTTCACAGAC-3′ and 5′-ACGACTTTCTTCCGTCCACTTAGG-3′; (*ii*) an 860 bp PCR fragment upstream of the *bca* gene; the PCR primers were 5′-CCGGAATTCTTGTTGAGTATAGAATAAGAT-3′ and 5′-CGCGGATCCTTAAATCAAAGGTGGTAACTC-3′; and (*iii*) the 2.1 kb *Bam*HI-*Bam*HI fragment from pBRΩKm-2, which contains the kanamycin resistance cassette. DNA probes were labeled by [α-^{32}P]dCTP with a random primed DNA labeling kit (United States Biochemical) or by a peroxidase complex with an ECL direct nucleic acid labeling kit (Amersham).

Western Immunoblot Analysis. Western blotting experiments were done as described (23). Primary antibodies and secondary alkaline phosphatase-conjugated antibodies (Cappel) were used at a dilution of 1:1,000. Blots were developed with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt/nitroblue tetrazolium phosphatase substrate buffer (Sigma).

ELISA Inhibition. ELISA inhibition was performed as described (23) with purified α or β C protein or with serotype Ia capsular polysaccharide as the coating antigen. Rabbit anti-α, anti-β, or anti-Ia-TT antiserum were used separately. The amount of the α or β C protein present on the surface of GBS cells was quantified using serial dilutions of intact GBS (grown in THB to mid-log phase) as inhibiting antigens and comparing the inhibitory activity to a standard curve generated with purified proteins (26). To measure the polysaccharide level of GBS, organisms were grown in 8 ml THB to an OD_{650} of 0.3 (mid-log phase). Bacterial cells were collected by centrifugation, washed twice with PBS, and treated at 37°C O/N with mutanolysin (Sigma; 4,000 units/ml). Bacterial cells and debris were removed by centrifugation, and supernatants containing the released capsular polysaccharide were used as inhibiting antigens in the ELISA. The amount of capsular polysaccharide released was quantified by comparison to

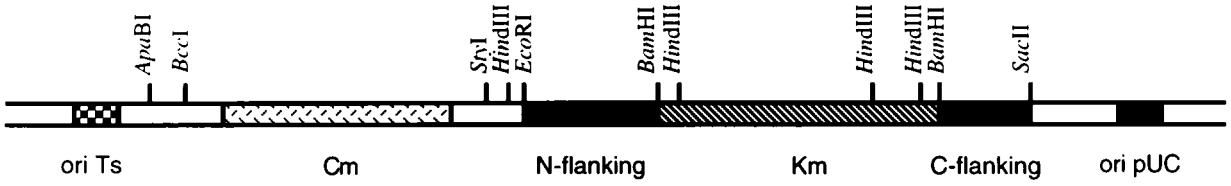

FIG. 1. Construction of the integration plasmid, pJL1147, for allelic replacement of *bca* as described. This construct was prepared to serve as a shuttle/suicide vector for replacing the *bca* structural gene with a selectable kanamycin marker. pJL1147 contains pVE6007, a pUC19 origin of replication, a kanamycin resistance cassette, and the N- and C-terminal flanking regions from *bca*. oriTs, Ts origin of replication; oripUC, pUC19 origin of replication; Cm, chloramphenicol-resistance marker; Km, kanamycin-resistance marker; N-flanking, PCR fragment upstream from the *bca* structural gene; C-flanking, PCR fragment downstream from the *bca* structural gene. The figure is not drawn to scale.

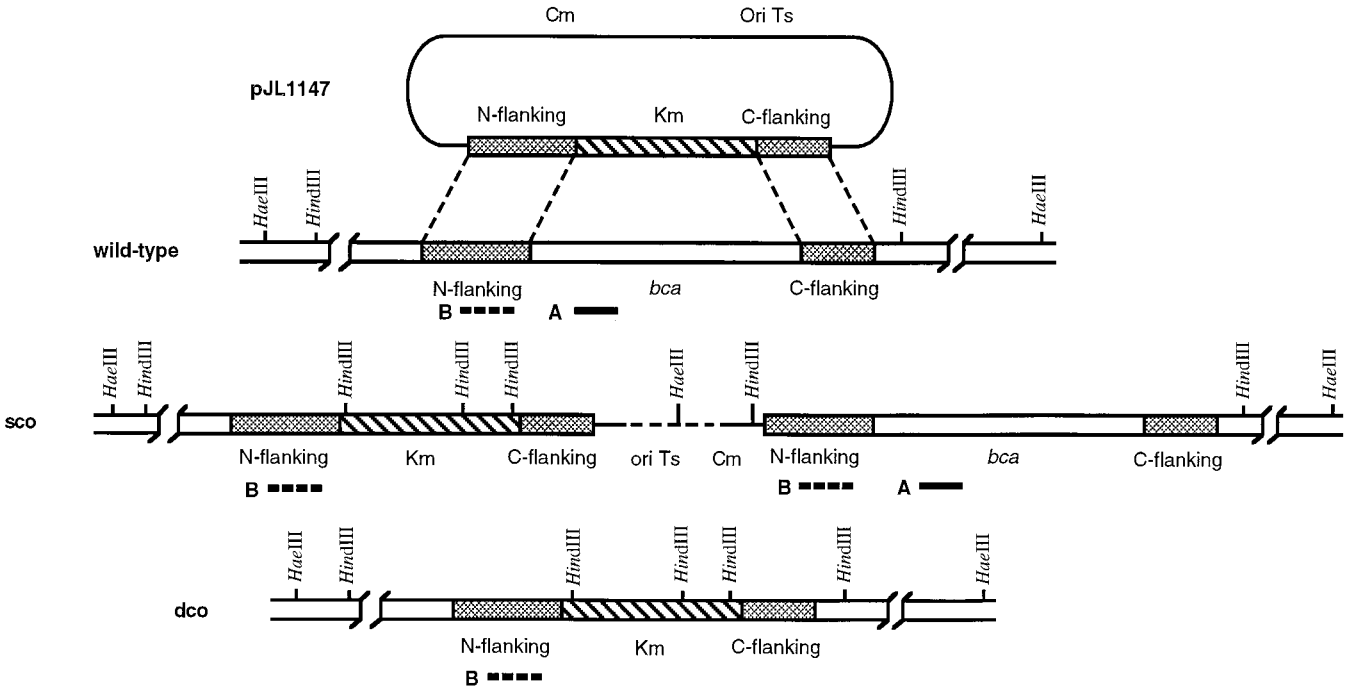

FIG. 2. Isolation of the *bca* gene replacement in GBS through homologous recombination. The shuttle/suicide vector pJL1147 was crossed into the wild-type strain A909. Km^rCm^r clones were selected at 37°C for sco mutants. The first recombination event occurs through homologous sequences in the flanking region of the *bca* gene resulting in JL2050. Interestingly, in all sco mutants mapped the crossover occurred in the N-terminal region. The second recombination event occurs between the C-terminal flanking duplicated regions created in sco mutants lead to excision of the integrated plasmid, thereby creating the dco mutant JL2053. The drawing is not to scale, but the *Hin*dIII and *Hae*III sites used for mapping the mutants are shown; in addition, specific probes for the *bca* structural gene (A) and the N-terminal flanking region (B) used in Southern blots (Fig. 3) are illustrated.

a standard curve, with purified capsular polysaccharide as the inhibitor.

Neonatal Mouse Lethality. Virulence of the GBS α knockout mutants was compared with that of the parent strain A909 in a CD-1 neonatal mouse model (38). For each strain tested, neonatal mice were challenged i.p. within 48 h of birth. GBS was grown in 5 ml THB at 37°C to OD_{650} of 0.3 (mid-log phase). The cultures were serially diluted in THB from 10^{-2} to 10^{-5}, and the size of each bacterial inoculum was quantitated by determining CFU. Twenty-four and 48 h after challenge the surviving mice were counted. The animal experiments were repeated four times. The data for each study was studied independently and compared between experiments. The lethal dose at 50% mortality (LD_{50}) was determined by the method of Reed and Muench (39). Statistical analyses were conducted using version 6.04 of SAS (Cary, NC). Logistic regression was assessed using the computer program PROC.LOGISTIC with terms for log dose to control for dose effects and indicator variables for the different mutant strains. The wild-type control strain A909 was used as a reference, and indicator variables for all the individual experiments were used to determine the differences in experimental conditions between each of the animal studies of lethality.

Mouse Protection Studies. Mouse protection tests were carried out with CD-1 outbred pregnant mice (1 or 2 days before delivery) given 0.5 ml of rabbit antiserum raised to the α C protein (ELISA titer, 1:102,000) i.p. (38). Pregnant control mice were given comparable doses of either rabbit preimmune serum or antiserum raised to Ia-TT. Newborn mouse pups were challenged by i.p. injection with 1,000 times LD_{50} (2×10^5 for the wild-type and 1.5×10^6 for the mutants); after 24 and 48 h, the surviving mice were counted.

Opsonophagocytic Assay. The opsonophagocytic assay, which uses human complement, human polymorphonuclear leukocytes (PMNs), and bacterial cells, was performed with and without rabbit anti-α and anti-Ia-TT antisera (40). In a reaction mixture of 0.5 ml, 3×10^5 PMNs were mixed with 1.5×10^5 cfu GBS cells, 50 μl of absorbed human serum (as a complement source) (41), and 100 μl of preimmune or immune rabbit serum. Bacterial viability were measured immediately after the addition of bacteria to the reaction mixture and compared with viability counts after a 1-h incubation at 37°C. The log decrease in colony-forming unit (cfu) between the two time points was determined.

RESULTS

Isolation of GBS sco and dco Recombinants in the *bca* Gene. Isogenic mutants of GBS strain A909 were made in which the *bca* structural gene was replaced by homologous recombination with a selectable kanamycin resistance marker. As shown in Fig. 1, plasmid pJL1147 has a 1.2-kb DNA fragment from the region upstream of the *bca* coding sequence and a 0.75-kb DNA fragment from the region immediately after the *bca* ORF stop codon. These flanking DNA fragments were chosen to serve as sequences for homologous recombination in GBS with the goal of replacing the entire *bca* structural gene by a kanamycin resistance encoding DNA cassette (Fig. 2).

As described in *Materials and Methods* and shown in Fig. 2, GBS strain A909 containing pJL1147 was grown at 28°C in the presence of chloramphenicol, and then Km^rCm^r colonies were selected at 37°C to obtain strains in which pJL1147 had integrated into the A909 genome by homologous recombination at the *bca* locus resulting in a sco. The frequency of pJL1147 integration into the A909 chromosome, measured as the ratio of the number of Km^rCm^r cells at 37°C to the number of viable cells at 28°C, was about 10^{-2}. Recombinants in which the integrated plasmid had been excised and *bca* replaced by a dco recombination with the kanamycin cassette, were derived by both temperature shifting and changing antibiotic selection as described in *Materials and Methods*. Km^rCm^s recombinants were generated at an unexpectedly high frequency; among the Km^r cells, 90% were Cm^s.

Molecular Analyses of the Recombinants. To characterize the recombinant mutants, one putative sco clone (JL2050) and two independent putative dco clones (JL2053 and JL2054) were studied. Southern blot hybridization was performed to

verify that the sco and dco recombinant mutants had the predicted structural changes in the *bca* locus on the chromosome. Total DNA from the mutants and wild-type A909 was cleaved with restriction enzymes that map immediately surrounding *bca*, and probes specific to *bca* and to the flanking regions were used. As shown in Fig. 3, the probe specific to the *bca* coding region identified a 15-kb *Hae*III fragment in the wild-type mutant and a smaller fragment (11 kb) in the sco mutant because of the expected chromosomal structural change (Fig. 2). No hybridization signal was detected in the dco mutants (Fig. 3*A*). The probe specific to the flanking region upstream of the *bca* coding sequence identified a 5.9-kb *Hin*dIII fragment in the wild-type mutant, two fragments (5.4 kb and 1.8 kb in size) in the sco mutant, and one fragment (1.8 kb) in the dco mutants (Fig. 3*B*). As illustrated in Fig. 2, these results were consistent with those predicted on the basis of genomic mapping studies of the wild-type strain A909 (18, 24, 42). In undigested DNA samples, the hybridized DNA was at the top of the gel (Fig. 3*B*), which suggest that there was no small episomal plasmid DNA in these strains. When the kanamycin cassette from pBRΩKm-2 was used as a probe, the sco and dco recombinant mutants were both found to contain the kanamycin gene on the chromosome; this selectable marker is not present in the wild-type strain (data not shown).

PCR was also used to characterize the recombinant mutants. Primers were chosen that were intragenic either to the *bca* gene or to the *aphA-3* kanamycin resistance gene. PCR performed with chromosomal DNA of the wild type using the α-specific primers yielded fragments of the predicted size; however, as expected, no PCR product corresponding to *bca* was obtained from the dco recombinant mutants (data not shown). Pulsed-field gel electrophoresis (PFGE) was performed on the dco mutants (43). PFGE patterns of the mutants were the same as the wild-type pattern, confirming that the knockout mutants were derived from the parental strain A909 (data not shown).

Expression of the α C Protein from the Recombinants. Expression of the α C protein from the sco and dco recombinant mutants was compared with that of the wild-type strain A909 by immunoblot analysis. Different α-specific antibodies used as probes included rabbit anti-α, rabbit anti-α N-terminal region, and the mAb 4G8, which is directed to the α repeat region. Fig. 4 shows that sco recombinant mutant JL2050 expresses the α C protein with a ladder pattern of heterogeneously sized polypeptides similar to the wild-type (12). However, the overall level of expression of α was lower in the sco cells. As shown in Fig. 4, α C protein expression could not be detected in immunoblots of the dco mutant strains. An ELISA inhibition experiment was performed as a more sensitive method for detection of α C protein expression. The wild-type strain and sco clone inhibited in a dose-dependent fashion, whereas the dco mutants were not inhibitory (data not shown). These findings confirm the DNA blot analysis that homologous recombination events occurred in GBS A909 sco and dco strains; the sco recombination left the entire plasmid pJL1147 inserted into the A909 chromosome with the *bca* gene intact, whereas dco recombination resulted in replacement of the *bca* gene with the kanamycin resistance marker, resulting in an α-deficient (α^-) phenotype.

Phenotypic Characterization of the α C Protein Knockout Mutants. The phenotypic properties of the knockout mutants were compared with those of the wild-type strain A909, including the effect of the mutation on expression of the surface-associated β C protein and type-specific capsular polysaccharide. Immunoblot analysis and ELISA inhibition studies showed that the knockout mutants and the parental strain produced similar levels of these two surface markers (data not shown). Two other GBS phenotypic properties, β-hemolysin (44, 45) and CAMP (Christie–Atkins–Munch-Petersen) factor (46, 47), were also expressed at similar levels in the knockout mutants and the wild-type strain (data not shown). The growth rates of the knockout mutants were identical to that of the wild-type A909, and the kanamycin resistance marker was stably maintained in the chromosome of the knockout mutants in the absence of antibiotic (kanamycin) selection (data not shown).

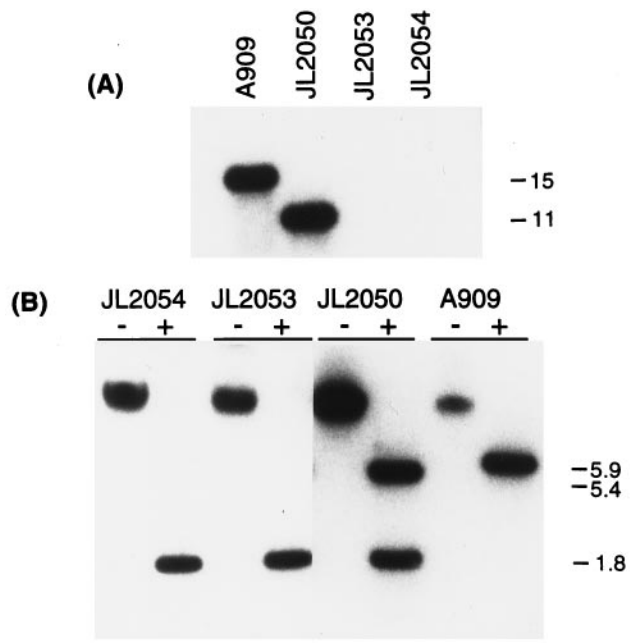

FIG. 3. Detection of homologous recombination in the sco and the dco mutants by Southern blot analysis. DNA was extracted from the wild-type strain A909, the sco strain JL2050, and the dco strains JL2053 and JL2054 and digested with different restriction enzymes; the DNA was then separated by agarose gel electrophoresis, transferred onto nylon membranes, and hybridized with different probes as described. (*A*) Restriction digestion by *Hae*III and hybridization with the probe specific to the *bca* coding region. (*B*) No digestion (−) or restriction digestion (+) by *Hin*dIII and hybridization with the probe specific to the *bca* flanking region. Molecular weight markers are in kb.

Lethality Tests of the α C Protein Knockout Mutants with Neonatal Mice. To study the biological effects of the knockout mutation in the *bca* gene of GBS strain A909, the LD_{50} of the dco knockout mutants (JL2053 and JL2054) was compared with that of the wild-type strain A909 in neonatal mice. The LD_{50} for A909 was 200 cfu/pup, whereas the LD_{50} for the knockout mutants varied between 1,000 and 1,500 cfu/pup ($P < 0.01$–0.03, wild-type vs. mutants) based on logistic regression analyses of four independent sets of animal studies as determined at 24 h. Thus the deletion of the α C protein resulted in a 5- to 7-fold decrease in lethality for neonatal mice, thereby providing strong evidence that α is an independent virulence determinant in GBS. It is of interest that the statistically significant differences in mortality between wild type and mutants all occurred in the first 24 h when these animal studies were analyzed by logistic regression as described in *Materials and Methods*. Comparisons of changes in mortality between 24 and 48 h were not statistically significant (data not shown); this suggests that the role of the α antigen in virulence is more important in the early stages of GBS infection.

Immunologic Analysis of the α C Protein Knockout Mutants. The α C protein serves as an epitope for antibodies protective against GBS in the mouse lethality model. Antibodies raised against the α C protein are protective in mice challenged with α-bearing GBS strains (15, 18, 21, 22). As shown in Table 1, antibodies to the α C protein protected mice from challenge with wild-type A909 (survival rate of 58%) but failed to protect against challenge with the *bca* knockout mutants JL2053 and JL2054 (survival rate of 8%). Antibodies raised to the Ia-TT-conjugated polysaccharide served as a positive control (survival rate of 96%).

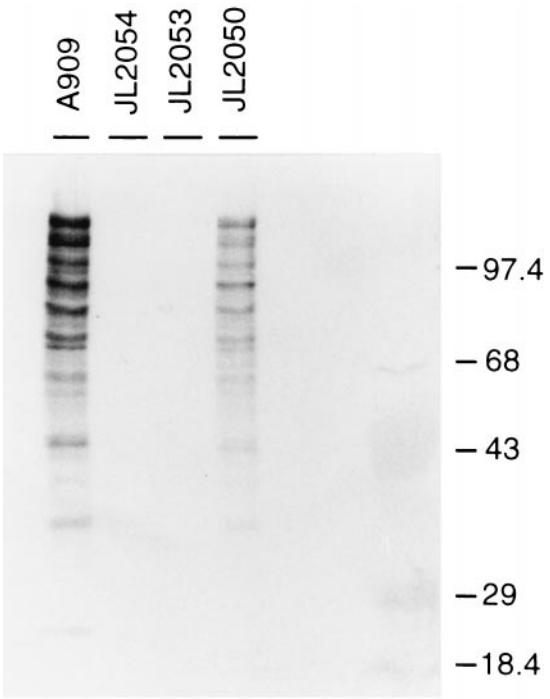

FIG. 4. α C protein expression from the recombinant mutants by immunoblot assay. SDS protein extractions of the wild-type strain A909, the sco strain JL2050, and the dco strains JL2053 and JL2054 are labeled above the lanes. Molecular weight markers are in kDa.

Opsonophagocytic assays compared *in vitro* killing of the knockout mutants in the presence and absence of specific antibodies. As shown in Fig. 5, the mutants were much less susceptible than A909 (a 0.6 $\log_{10}$ cfu decrease) to killing in the presence of the α-specific antibody. The mutants and the wild-type were both killed by antibodies to Ia-TT. However, in the absence of specific antibodies, neither the wild type nor the mutants were readily killed.

DISCUSSION

The α C protein of GBS is a surface-associated structure that has been cloned and sequenced, but its biological function and role in virulence are poorly understood. As an opsonic and protective antigen and with a wide distribution among clinical isolates, the α C protein has been of potential interest as a component of a polysaccharide–protein conjugate vaccine. For example, a vaccine composed of the serotype III polysaccharide and the α C protein could protect against more than 90% of clinically important GBS strains (48). In addition to a possible role in immunity and vaccine development, the α C protein may play a role in bacterial virulence. The α gene, *bca*, is structurally related to other surface proteins of Gram-positive pathogens known to be virulence factors, such as the antiphagocytic M proteins of group A *Streptococcus* (12).

In this study, knockout mutations were generated in the *bca* gene to assess the potential roles of these antigens in virulence. The conjugative transposon Tn916 has been used to study GBS capsular polysaccharide biosynthetic genes (4, 5); however, this technique may be limited by site specificity of the transposon insertion into the chromosome and was not successful for creating insertions in the *bca* gene. We therefore developed a novel shuttle/suicide vector for GBS and used a marker exchange strategy to replace the *bca* coding region with a DNA fragment specifying resistance to kanamycin (a selectable phenotypic marker) through homologous recombination. Our study showed this targeted, site-specific, recombinant DNA technology in GBS, which had been refractory to detailed genetic analysis.

The α C protein knockout mutants lack expression of the α C protein but otherwise appear similar to the wild type with respect to a number of phenotypic properties. These mutants also are very stable and do not lose the kanamycin resistance marker in the absence of antibiotic selection. The effects of the knockout mutants were investigated *in vivo* in neonatal mice models and in an *in vitro* opsonophagocytic assay. In animal studies, the *bca* knockout mutation resulted in an approximately 5- to 7-fold decrease in bacterial lethality. We consistently observed such attenuation of pathogenesis in independent experiments. This model of invasive disease does not permit independent assessment of bacterial cell attachment and/or invasion of host tissue. Because significant differences in mortality occurred within the first 24 h of infection, it will be of interest to test these well-defined *bca* mutants in other models of GBS infection and to further explore the role of the α C protein in pathogenesis.

In the presence of anti-α antibodies, opsonophagocytic killing of the knockout mutants was less than that of the wild type (Fig. 5), thus confirming that the α C protein encodes both immunogenic and opsonic epitopes (18, 21, 23). Knockout of the *bca* gene also led to inactivation of immunity by protective and opsonic antibodies to the α C protein. In previous studies with collections of random clinical GBS isolates, we found that, in the absence of the anti-α antibody, strains expressing the α C protein were more resistant to opsonophagocytic killing than those lacking expression of the α antigen (23). However, in this study we found that the susceptibility of the α C protein knockout mutants to opsonophagocytic killing was essentially the same as that of the wild type (Fig. 5). Because this study compared the isogenic pairs of the wild-type and the mutant strains, our data suggest that the α C protein, unlike the M protein of group A *Streptococcus*, may not be involved in resistance to phagocytosis.

Using an animal model, Madoff *et al.* (24) reported that in the presence of antibodies to the α C protein, strains of GBS passed from adult mice to their neonates undergo changes in the tandem repeats of the α C protein. These escape mutants contain deletions in *bca* specifically within the repeat region. Further studies revealed that these alterations appear to provide a means of antigenic and protective variation for GBS to evade type-specific host immunity (24, 26). The α C protein knockout mutants do not confer any α-specific epitope, so they would escape the host's immunity to GBS surface antigens and could cause GBS infections in an otherwise immune host. This prediction was confirmed in this report by mouse protection studies where antibodies to the α C protein did not passively protect mice from lethal challenge with the mutants (Table 1).

Streptococcus agalactiae has only two known natural reservoirs, humans and cattle, and in both mammals can be either a colonizing organism or a virulent pathogen; the organism is not known to be present in the environment or transmitted through other vectors. The overall biological properties of GBS are not well understood. The range of human disease has been discussed previously; in cattle, GBS are a common cause of bovine mastitis. It is interesting

Table 1. Passive protection of neonatal mice with rabbit antiserum to the C protein

Strain	Antiserum	Number of pups, alive/total	% survival
A909	Anti	7/12	58
	Anti-Ia-TT*	10/10	100
	Preimmune†	2/19	10
JL2053	Anti	0/12	0
	Anti-Ia-TT	8/9	89
	Preimmune	0/18	0
JL2054	Anti	1/12	8
	Anti-Ia-TT	9/9	100
	Preimmune	3/18	17

*Positive control
†Negative control.

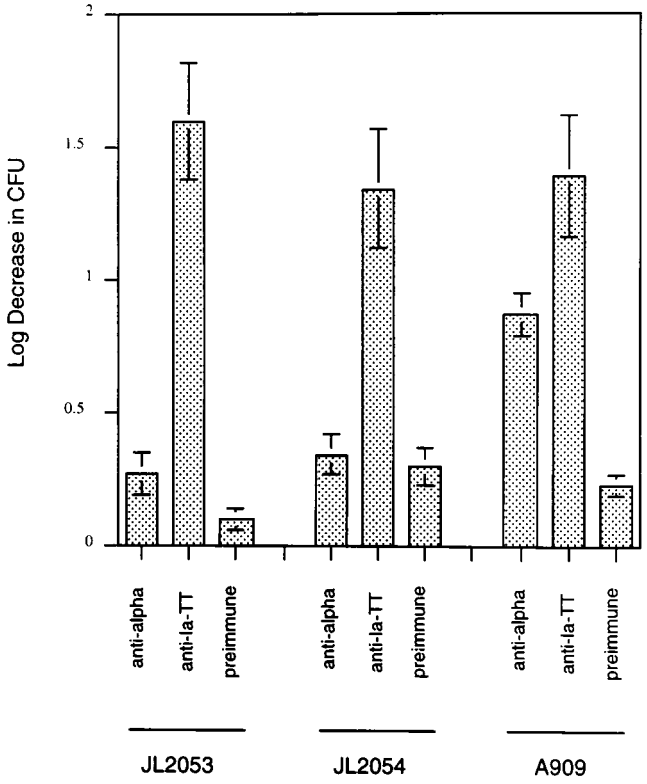

FIG. 5. Opsonophagocytic killing of the α C protein knockout mutants. The mutant and wild-type strains were incubated for 1 h in the presence of leukocytes (human PMNs), preabsorbed human sera (as a source of complement), and rabbit anti-α antiserum. Rabbit anti-Ia-TT antiserum and rabbit preimmune serum were used as controls.

that human and bovine strains have many properties in common that may lead to disease, including type-specific capsular polysaccharides and diverse, surface-associated protein antigens. However, virulence mechanisms, while similar in kind among these isolates, do not share common antigens. For example, antigens associated with pathogenesis in bovine isolates of GBS are not commonly found on virulent human strains. Nonetheless, elucidation of the basic mechanisms underlying antigenic variation and the role of surface proteins among GBS isolates may identify common mechanisms of pathogenesis and lead to the development of vaccines that contain conserved, opsonic, and protective epitopes.

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