

Polymorphisms in the Cell Wall–Spanning Domain of the C Protein β -Antigen in Clinical *Streptococcus agalactiae* Isolates Are Caused by Genetic Instability of Repeating DNA Sequences

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ABSTRACT

The C protein α - and β -antigens are immunodominant components of the surface of *Streptococcus agalactiae*, the most frequent cause of neonatal sepsis. Both proteins are thought to contribute significantly to virulence of *S. agalactiae*. They are mainly expressed by serotypes Ia, Ib, and II. The C protein β -antigen (C β -protein) binds to the Fc portion of human IgA and seems to be of importance in bacterial resistance to mucosal immune defense mechanisms. In this study, PCR analysis of *S. agalactiae* isolates obtained from 189 neonates and 112 pregnant women revealed the presence of the C β -protein gene in 19% and 22% of the isolates, respectively. Size polymorphisms of the PCR products within the gene region encoding the cell wall–spanning domain indicated a high degree of genetic variability. Thirteen different variants of the amplified region were differentiated among the 60 C β -protein–positive isolates by sequence analysis. In all variants, the polymorphisms were caused by

insertions and deletions of repetitive DNA elements that did not alter the open reading frame. Comparison of the C β -protein gene polymorphisms showed a significantly higher rate of isolates carrying deletions >50 bp in serotype Ib than in serotype II isolates ($p = 0.001$); this was also true for neonatal isolates analyzed separately ($p = 0.01$). Neonatal isolates carried a higher rate of large deletions when compared with maternal isolates; this difference, however, did not reach statistical significance ($p = 0.08$). We hypothesize that polymorphisms in the cell wall–spanning domain of the C β -protein are of functional relevance with regard to maternofetal transmission of the pathogen. (*Pediatr Res* 51: 106–111, 2002)

Abbreviations

C β -protein, C protein β -antigen
GBS, group B streptococci, *Streptococcus agalactiae*

Bacterial sepsis is still a leading cause of neonatal morbidity and mortality. Recent data from the United States describe an incidence of 3.5 cases per 1000 live births, with a mortality reaching up to 16% (1, 2). *Streptococcus agalactiae* (GBS) in particular accounts for approximately 1.4 cases of sepsis per 1000 births, and is by far the leading causative agent of neonatal early onset sepsis (2–8). In contrast to the type-specific capsular polysaccharides which are well-defined virulence determinants of GBS (9–12), the role of proteins as factors contributing to pathogenicity is not yet clearly determined. The C proteins are surface-associated immunodominant antigens expressed by most clinical GBS isolates of capsular types Ia, Ib, and II, but are uncommon in serotype III (13–19). The two different C protein antigens α and β are encoded by separate genes, which are independently expressed. Both genes

have been cloned and analyzed at the molecular level (20–22). In addition, another two C protein antigens, the γ and δ antigens, were identified (23). Lancefield *et al.* (24) reported in 1975 that antibodies directed against the C proteins protect mice against a lethal challenge with C protein carrying GBS, indicating that these determinants are involved in both virulence and protective immunity. Further studies revealed that the C proteins contribute to the resistance of opsonization and intracellular killing (25, 26). The C β -protein binds to the Fc portion of human IgA (27, 28), which might be of importance in bacterial resistance to mucosal immune defense mechanisms. By binding IgA to the bacterial surface, GBS may block binding of other opsonizing antibodies, mask other antigens on the cell surface, and inhibit phagocytosis (29–31).

The nucleotide sequences of the C protein α - and β -antigen genes were determined several years ago (20–22). The structure of the α -antigen is characterized by an N-terminal region that is followed by a series of nine tandem repeating units that make up 74% of the mature protein. Each repeating unit is identical and consists of 82 amino acids, which are encoded by

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246 nucleotides (22). The large region consisting of identical repeating units is thought to define protective epitopes. This structure of the protein and its encoding gene may have a role in generating genotypic and phenotypic variability by providing sites for gene rearrangements that create an antigenic diversity of the α -antigen (22, 32, 33). Concerning a similar repeat unit structure of the C β -protein, different and contradictory results have been obtained. Whereas Madoff *et al.* (32) found little variation among the strains analyzed in their study, Brady and Boyle (16) reported heterogeneity of the C β -protein size in clinical isolates. Likewise, Maeland *et al.* (34) demonstrated remarkable variations among the C β -protein gene and the gene products among different GBS strains, whereas Mawn *et al.* (33) did not observe varying sizes of PCR products of the C β -protein gene. However, in contrast to Maeland *et al.* (34) who amplified a region of the gene including a part encoding for each of the IgA-binding domains A and B (nucleotides 1337–1940), Mawn *et al.* (33) analyzed a region downstream, ranging from the nucleotides 2679 to 3270. We have recently described a remarkable heterogeneity in the C β -protein (13) when analyzing clinical isolates with the primers indicated by Mawn *et al.* (33), but all isolates showed identical sizes of the PCR product when the DNA was amplified according to the primers given by Maeland *et al.* (34). The region of the gene amplified by Mawn *et al.* (33) is supposed to belong to a cell wall–spanning domain that has no function in IgA binding. Interestingly, Jerlström *et al.* (21) as well as Hedden *et al.* (20) described an unusual feature of this region as containing proline-rich repeated sequences with a three-residue periodicity. The purpose of the present study was to study the previously described genetic diversity within the cell wall–spanning domain of the C β -protein gene in more detail and to investigate whether individual genotypes are associated with GBS different serotypes and isolates from either neonatal or maternal origin.

METHODS

Bacterial strains. GBS strains were collected from clinical specimens at the University Children's Hospital, Freiburg, Germany, from 1991 through 1999, and included those recently described (13). Bacteria were isolated from blood, meconium, urine, and superficial swab cultures from newborns admitted to the University Children's Hospital, for clinical suspicion of sepsis or for routine screening. GBS isolates from vaginal swabs of pregnant women were randomly collected, 50 of them in 1997, 62 of them in 1999. GBS isolates were identified by characteristic growth on blood agar plates, by β -hemolysis, and by serology grouping, using the latex agglutination test (Streptex, Murex Diagnostics, Dartford, U.K.).

DNA preparation. DNA was isolated as previously described (13). For PCR experiments, precipitated chromosomal DNA was pelleted and adjusted spectrophotometrically to a concentration of 10 ng/ μ L with H₂O.

PCR assay. PCR assays were performed as described in detail previously (13). The isolated bacterial DNA was used as a target. Oligonucleotide primers specific for amplification of the IgA-binding domain of the C β -protein gene were used as

proposed by Maeland *et al.* (34) corresponding to the nucleotides 1337–1360 (5'-AAG GCT ATG AGT GAG AGC TTG GAG-3') and 1917–1940 (5'-CTG CTC TGG TGT TTT AGG AAC TTG-3') of the C β -protein gene sequence (21). These primers amplify a DNA fragment of 604 bp that encodes for a part of each of the IgA-binding domains A and B of the C β -protein gene. For amplification of a DNA fragment corresponding to the region from nucleotide position 2725 to 3287, primers located within the region of the membrane- and cell wall–spanning domain of the C β -protein were designed from published sequence data [(21), GeneBank accession number X59771]. PCR assay was performed with the antisense primer 5'-TTA TCA GCC AAC TCT TTC GTC-3' and the sense primer 5'-CTT AGT ACA CGA TGC ATT CTC-3'. Within this region we had previously observed genetic polymorphisms (13).

DNA sequencing. To determine the nucleotide sequence of amplified DNA, PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and subjected to DNA sequence analysis using the AmpliTaq DNA Polymerase DNA Sequencing Kit (Dye Terminator, Cycle Sequencing Ready Reaction; PerkinElmer Life Science, Boston, MA, U.S.A.). Products from sequencing reactions were analyzed with a sequence apparatus (model 370A, Applied Biosystems, Foster City, CA, U.S.A.).

Serotyping. Serotyping of GBS isolates was performed as described previously (13), using an enzymatic extraction method. Typing was performed with antisera specific for capsular serotypes Ia, Ib, II, III, IV, and V in a slide agglutination test (Denka Seiken, Tokyo, Japan).

Statistical analysis. Fisher's exact test was applied for analysis of prevalence of individual GBS genotypes and DNA polymorphisms among maternal and neonatal isolate populations and for the associations with certain serotypes. Analyses were performed using the SPSS-software package, version 10.0 (SPSS, Chicago, IL, U.S.A.). *P* values <0.05 were considered significant.

RESULTS

Detection of C β -protein gene in isolates of maternal and neonatal origin. One hundred eighty-nine GBS isolates from newborns and 112 GBS isolates of maternal origin were analyzed by the PCR method for the presence of the C β -protein gene. The C β -protein gene was detected in 35 neonatal isolates (19%) and 25 maternal isolates (22%). Clinical characteristics of neonatal strains harboring the C β -protein gene are shown in Table 1. The C β -protein gene–positive isolates were subjected to serotyping, which revealed that the majority of the neonatal isolates belonged to serotype Ib (18 isolates, 51%), followed by serotype II (11 isolates, 31%). Among maternal isolates, nine isolates (35%) belonged to serotype II and 10 isolates (40%) to serotype Ib (Table 2).

Molecular analysis of the cell wall–spanning domain of the C β -protein. To investigate DNA polymorphisms within the cell wall–spanning domain of the C β -protein (Fig. 1), DNA of the GBS isolates were subjected to PCR analysis with the respective primers amplifying fragments of the C β -protein

Table 1. Characteristics of neonatal C β -protein gene-positive GBS strains

Clinical presentation	Source of isolation	Serotype	C β -protein polymorphism	Year of isolation
Sepsis	blood culture	Ia	Original*	1999
Sepsis	cutaneous swab	II	B	1999
Sepsis	meconium	II	B	1999
Sepsis	urine	Ib	A	1998
Sepsis	cutaneous swab	Ia	A	1996
Sepsis	cutaneous swab	Ib	A	1996
Sepsis	cutaneous swab	II	B	1996
Sepsis	cutaneous swab	Ib	B	1995
Sepsis	cutaneous swab	Ib	A	1995
Sepsis	cutaneous swab	Ib	N	1995
Sepsis	cutaneous swab	II	H	1995
Sepsis	cutaneous swab	Ib	A	1995
Sepsis	cutaneous swab	Ib	A	1994
Sepsis	cutaneous swab	Ib	G	1994
Sepsis	cutaneous swab	II	I	1994
Sepsis	cutaneous swab	II	B	1992
Sepsis	blood culture	II	I	1994
Sepsis	blood culture	Ib	A	1993
Sepsis	blood culture	Ib	A	1992
Colonization	cutaneous swab	Ia	B	1999
Colonization	cutaneous swab	Ib	Original*	1999
Colonization	cutaneous swab	III	A	1997
Colonization	cutaneous swab	Ib	A	1997
Colonization	cutaneous swab	II	B	1997
Colonization	cutaneous swab	V	E	1997
Colonization	cutaneous swab	Ib	A	1996
Colonization	cutaneous swab	Ia	K	1996
Colonization	cutaneous swab	Ib	A	1996
Colonization	cutaneous swab	Ib	A	1995
Colonization	cutaneous swab	II	B	1995
Colonization	urine	Ib	G	1993
Colonization	cutaneous swab	Ib	L	1993
Colonization	cutaneous swab	II	Original	1993
Colonization	cutaneous swab	II	M	1993
Colonization	urine	Ib	G	1992

* Mutation of only two nucleotides which do not alter the open reading frame.

Table 2. Distribution of different polymorphisms of C β -protein gene according to serotypes of neonatal or maternal isolates

Polymorphism	No. of isolates (neonatal/maternal) according to serotype					
	Ia (n = 7)	Ib (n = 28)	II (n = 20)	III (n = 1)	V (n = 3)	NT* (n = 1)
Large deletion (>50 bp)	1/1	14/5	3/2	1/0	1/0	0/1
Original or similar	2/2	2/1	7/7	0/0	0/2	0/0
Small insertion	1/0	0/2	0/0	0/0	0/0	0/0
Large insertion (>50 bp)	0/0	2/2	1/0	0/0	0/0	0/0

* NT, nontypable.

gene at positions 2860–3100. As reported earlier, heterogeneity in the size length of the PCR products was observed (Fig. 2). The range of the size variation of the PCR products observed was from 472 to 670 bp. Sequence analysis revealed 13 different subtypes within the amplified region (Table 3). Only two isolates (3%) carried a genetic sequence of this region that was identical to that described earlier by Jerlström *et al.* (21), which was used as a reference for comparative analysis of the different subtype sequences, and which is referred to as the original sequence. In comparison to the original sequence, the majority of GBS isolates carried either small or large DNA deletions, DNA insertions, or a combination of both (Figs. 2 and 3). The two most common DNA polymorphisms were observed in 63% of all isolates. The first

one (type A), which was found in 21 (35%) isolates, comprised two deletions with a length of 72 bp and 18 bp and corresponded to the sequence described by Hedden *et al.* (20). The second most frequently observed genotype was found in 17 (28%) isolates. It carried an 18-bp deletion and was most similar to the originally described sequence data of Jerlström *et al.* (21). The 18-bp deletion of the gene position 3076–3093 occurred separately or in combination with other polymorphisms in 53 strains (88%). Among all isolates investigated, both the largest deletion and largest insertion were 108 bp in size; both the smallest deletion and the smallest insertion were 18 bp in size. All polymorphisms occurred in the region of the gene in which elements are periodically repeated (Fig. 1). All DNA polymorphisms observed were characterized by deletions

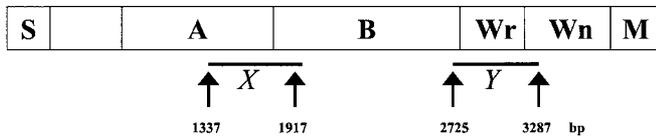


Figure 1. Domain structure of the C β -protein gene [according to Jerlström *et al.* (21)]. Domains of known functions are boxed: S, signal sequence; A and B, IgA-binding domains; Wr, repeated region of the cell wall-spanning domain; Wn, nonrepeated region of the cell wall-spanning domain; M, membrane-spanning domain. The total length of the C β -protein gene is 4200 bp. The region X in the IgA-binding domains was amplified to detect strains harboring the C β -protein. The region Y is located in the cell wall-spanning domain and was analyzed in detail in this study.

or insertions of complete repetitive units. Consequently, the open reading frame was not altered in any genetic subtype analyzed. Because some combinations of different deletions and insertions lead to the same length of the PCR product, several isolates with different polymorphisms were detected only by sequence analysis (Fig. 3 and Table 3). Furthermore, in two isolates that showed the same PCR-product length as the original sequence, a mutation of only two nucleotides was detected, which also did not alter the open reading frame, but led to the exchange of a single amino acid in the protein sequence at position 934, where a leucine is replaced by a threonine.

To test the genetic stability of the described polymorphisms, a selected isolate (serotype Ib, genotype B) was subcultured for a total of 20 passages. Ten different single colonies per generation were analyzed by the PCR assay with the primers given above. No size differences were observed in the amplification products of the 200 different colonies tested, confirming a certain genetic stability of the polymorphisms.

When performing PCR with the primers amplifying a part of the gene region encoding for the IgA-binding domain (34), identical sizes of the PCR products of all GBS isolates were observed, suggesting a conserved genetic structure of this functional domain.

Comparison of defined genetic subtypes with GBS serotypes. Comparison of individual DNA polymorphisms with serotypes revealed that the majority of isolates with large (>50 bp) deletions were associated with the serotype Ib (19 of 28; Table 2). Comparison of the C β -protein gene polymorphisms between serotype Ib and serotype II isolates revealed a higher frequency of rearranged (large deletions) genes in serotype Ib (68 versus 26%; $p = 0.001$). Likewise, when comparing polymorphisms between serotype Ib and serotype II isolates of neonatal origin only, isolates carrying deletions >50 bp were found more frequently in serotype Ib (78 versus 27%; $p = 0.01$). When comparing polymorphisms between isolates of either neonatal or maternal origin, it became evident that 20 of 35 (57%) of the neonatal isolates, but only nine of 25 (36%) maternal isolates, carried large deletions ($p = 0.08$). Within serotype Ib isolates, 14 of 18 (78%) neonatal isolates carried large deletions compared with five of 10 maternal isolates ($p = 0.13$). In contrast, in serotype II isolates seven of nine maternal, and seven of 11 neonatal isolates showed the original nucleotide sequence or a very similar one (Table 2).

DISCUSSION

We have recently described a genetic variability within the cell wall-spanning domain of the C β -protein gene in clinical isolates from newborns with sepsis, healthy newborns, and colonized adult women (13). In the present study the genetic structure of the polymorphisms was further investigated. The C β -protein gene product has a signal sequence at the N terminus, a cell wall- and a cell membrane-spanning C-terminal domain, and two functional domains (A and B) that mediate binding of the Fc portion of human IgA (20, 21, 27) (Fig. 1). As outlined in the original sequence analysis by Jerlstrom *et al.* (21) and Heden *et al.* (20), there is a particular structure of the gene near the C terminus containing repetitive genetic elements with a short periodicity. This region was designated as XPZ motif region by Heden *et al.* (20) and Wr region by Jerlstrom *et al.* (21). The region is characterized by proline-rich stretches of amino acids with every third amino acid being a proline. Regions with a high proline content are also found in the cell wall-spanning region of several surface proteins from Gram-positive cocci, in which the proline-rich sequences are located close to the membrane anchors (21). In the C β -protein of GBS isolates, the proline-rich region corresponds to the cell wall-spanning domain. In our collection of clinical GBS isolates we could identify significant genetic polymorphisms within this proline-rich domain. The majority of isolates had deletions, some had insertions, and others a combination of both. None of these polymorphisms altered the open reading frame, but changed considerably the number of repeats within the cell wall-spanning domain. This diversity might correspond at the protein level to the observation of Maeland *et al.* (35), who described two GBS strains that expressed C β -proteins of different sizes. The following evidence from that study supports this hypothesis. First, the sizes of these proteins were smaller

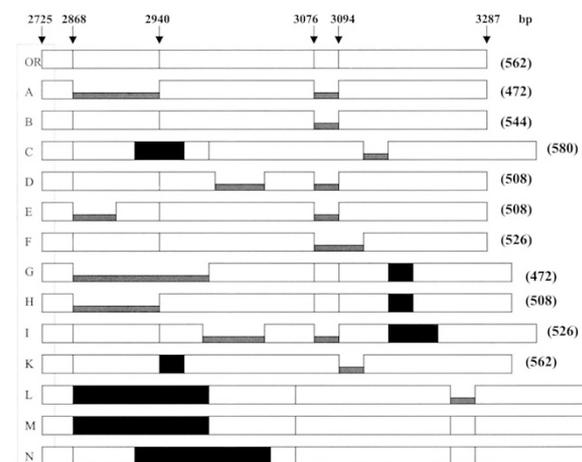


Figure 2. Schematic representation of deletions and insertions in the C β -protein gene. In the first line a schematic model of the original gene sequence (OR) is shown (21). The open boxes represent the unaltered parts of the original sequence. Noteworthy, the box size does not match exactly the length of the nucleotide sequence. The arrows indicate the region of the gene where the majority of deletions and insertions were observed; the exact positions are given in Figure 3. Low gray-shaded boxes indicate deletions in individual variants. The insertions are indicated by black boxes. At the right of each line the size of the amplified PCR product is shown.

Table 3. Detailed description of DNA polymorphisms within cell wall-spanning domain encoding region of *Cβ*-protein gene

Polymorphism	Deletion with gene position and size	Insertion with gene position and size	No. of isolates
A	del 1: pos. 2868–2939, –72 bp del 6: pos. 3076–3093, –18 bp		21
B	del 6: pos. 3076–3093, –18 bp		17
C	del 6: pos. 3076–3093, –18 bp	Ins 2: pos. 2911, + 36 bp	3
D	del 5: pos. 2983–3018, –36 bp del 6: pos. 3076–3093, –18 bp		1
E	del 2: pos. 2868–2903, –36 bp del 6: pos. 3076–3093, –18 bp		1
F	del 7: pos. 3076–3111, –36 bp		1
G	del 3: pos. 2875–2982, –108 bp	Ins 5: pos. 3130, + 18 bp	3
H	del 1: pos. 2868–2939, –72 bp	Ins 5: pos. 3130, + 18 bp	1
I	del 4: pos. 2965–3018, –54 bp del 6: pos. 3076–3093, –18 bp	Ins 6: pos. 3130, + 36 bp	2
K	del 6: pos. 3076–3093, –18 bp	Ins 4: pos. 2940, + 18 bp	1
L	del 6: pos. 3076–3093, –18 bp	Ins 1: pos. 2875, + 108 bp	2
M	–	Ins 1: pos. 2875, + 108 bp	1
N	–	Ins 3: pos. 2911, + 108 bp	2

**Figure 3.** Results of sequence analysis of the *Cβ*-protein gene variants. Deletions and insertions as compared with the original *Cβ*-protein gene sequence (21) are marked. The exact nucleotide position of each variant is indicated.

than that calculated from the original sequence data: the sizes of 94 kD and 84 kD, respectively, were well below the estimate of 120 kD for the original protein. Second, the proteins were not anchored at the surface of the bacteria, inasmuch as the isolates could not be stained with a fluorescent antibody directed against the *Cβ*-protein. Third, the proteins were not able to bind the antibody directed against the β -antigen in Western blot analysis. Nevertheless, the proteins behaved normally in terms of IgA binding, and they were secreted during bacterial growth. It was speculated by Maeland *et al.* (34) that the

Cβ-protein was rearranged at the C-terminal end, and that this was because of deletions in the proline-rich region. Likewise, other authors had also demonstrated that surface-anchoring of the *Cβ*-protein varies among GBS isolates (16). Therefore, there is indirect evidence to believe that the genetic variability of the proline-rich cell wall-spanning domain that is described in this study corresponds to the previously reported phenotypic observations. One might speculate that large deletions in the cell wall-spanning domain of the *Cβ*-protein gene may lead to a loss of fixation or attachment of the protein to the bacterial

cell wall, which will result in the release of the IgA-binding domain from the bacterial surface. Blocking the binding activity of mucosal immunoglobulins not on the bacterial surface, but apart, might protect the pathogen from opsonization and killing. This hypothesis, however, remains to be proven by functional analysis.

Recently, we have demonstrated that five distinct subtypes of the C β -protein gene-positive strains can be differentiated by PCR analysis of the gene region encoding cell wall-spanning (13). The sequence analysis performed in this study revealed that there is a greater genetic heterogeneity than expected previously. Some strains had larger deletions together with small insertions, which resulted in a very similar PCR product size to that of other strains with smaller deletions. Surprisingly, only a minority of C β -protein gene-positive GBS isolates (two isolates, 3%) contained the original genetic sequence that was described by Jerlström *et al.* (21), whereas 18 isolates (33%) revealed a sequence with minor deletions of 18 and 36 bp. The majority of C β -protein gene-positive isolates, however, showed deletions of up to 108 bp (29 isolates, 48%), and a minority of eight isolates (13%), insertions of up to 108 bp. These findings reflect a considerable genetic variability within this region, which according to the limited evidence from our subculturing experiments remains fairly stable.

In summary, we describe a significant heterogeneity of the C β -protein gene among clinical *S. agalactiae* isolates on the genetic level. The polymorphisms occur in the proline-rich region of the C β -protein, which corresponds to the cell wall-spanning domain. They are supposed to be of functional impact in terms of an altered shedding behavior of the proteins from the bacterial surface.

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