Identification of novel *cps* locus polymorphisms in nontypable group B *Streptococcus*

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Group B *Streptococcus* (GBS) is an important pathogen responsible for a variety of diseases in newborns and the elderly. A clinical GBS isolate is considered nontypable (NT) when serological methods fail to identify it as one of nine known GBS serotypes. Eight clinical isolates (designated A1–A4, B1–B4) showed PFGE profiles similar to that of a GBS serotype V strain expressing R1, R4 surface proteins. These unique isolates were further characterized by immunologic and genetic methods. Rabbit sera to isolates A1 and A2 reacted weakly with concentrated HCl extracts of A1–A4 isolates, but not with those of B1–B4 isolates. In addition, a type V capsular polysaccharide (CPS) inhibition ELISA revealed that cell wall extracts from isolates A1–A4, but not from B1–B4, expressed low but measurable amounts of type V CPS. Molecular serotyping with PCR analysis showed that all eight isolates contained a type V-specific CPS gene (*cpsO*) and harboured the gene encoding the surface protein Alp3. Multilocus sequence typing identified isolate A1 as belonging to a new sequence type (ST) designated ST-173, whereas the other seven isolates keyed to ST-1. Sequencing of the 18 genes (17 736 bp) in the *cps* locus showed that each NT isolate harboured one to three unique polymorphisms, and also identified an IS1381 element in *cpsE* of the B4 isolate. Collectively, genetic and immunologic analyses revealed that these NT isolates expressing R1, R4 proteins have a genetic profile consistent with that of type V, an emergent, antigenically diverse and increasingly prevalent GBS serotype.

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INTRODUCTION

Streptococcus agalactiae or group B *Streptococcus* (GBS) is the most common cause of neonatal sepsis and meningitis, and is increasingly recognized as an important cause of invasive disease in non-pregnant adults and immunocompromised individuals. It is an opportunistic pathogen, and can be isolated from the genitourinary tracts of about 35 % of healthy adult women (Bliss *et al.*, 2002). Although the use of intrapartum antibiotic prophylaxis has considerably reduced the incidence of neonatal GBS disease, vaccination offers the best long-term solution to prevention of disease, and, towards that end, considerable progress has been made in the development of GBS vaccines (Maione *et al.*, 2005; Paoletti & Kasper, 2003; Ramaswamy *et al.*, 2004).

Abbreviations: CPS, capsular polysaccharide; GBS, Group B *Streptococcus*; MLST, multilocus sequence typing; NT, nontypable; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ359707–DQ359714.

Capsular polysaccharides (CPSs) are major virulence factors and the main components of glycoconjugate vaccines. In addition, these surface carbohydrates provide useful epidemiologic markers, as GBS isolates are serotyped on the basis of their CPS antigens; nine distinct serotypes (Ia, Ib, and II–VIII) have thus far been described. The prevalence of GBS serotypes fluctuates over time and by geographic location (Le Thomas-Bories *et al.*, 2001). Although serotype III strains are responsible for the majority of cases of neonatal meningitis worldwide, serotype V strains are isolated with increased frequency from all age groups within a population, and an estimated 24–31 % of the cases of invasive GBS disease in non-pregnant adults in the USA are caused by this serotype (Blumberg *et al.*, 1996; Farley, 2001; Le Thomas-Bories *et al.*, 2001).

Isolates without identifiable CPS by the standard Lancefield serotyping method are considered nontypable (NT). NT isolates may account for up to 4 % of all GBS isolates (Ferrieri *et al.*, 1997), but account for up to 12 % in collections studied in some laboratories (Palacios *et al.*, 1997). The extent of sequence variation in the *cps* genes of clinical

isolates within a serotype, and among different serotypes, has not been well studied. It has been observed that closely and distantly related clones of GBS may share the genes encoding a specific CPS, suggesting that capsule switching may occur in GBS (Davies *et al.*, 2004).

Most NT isolates express surface proteins: alpha and beta components of the C protein or one or more of the R protein antigens, Rib, and Alp1–4 proteins (Benson *et al.*, 2002; Kong *et al.*, 2002b). These isolates can be genotyped and distinguished by PFGE and by the expressed surface-protein antigens (Ferrieri *et al.*, 1997; Ferrieri & Flores, 1997; Sellin *et al.*, 2000). The C proteins were recognized by Lancefield as protective determinants found in GBS of serotypes Ia and Ib (reviewed by Lindahl *et al.*, 2005). One of the C proteins, the beta C protein, binds to human IgA and is found mainly in serotype Ib GBS. The other C protein, the alpha C protein, has been recognized to be the prototype for a family of proteins (alpha-like proteins) that is found in most GBS and includes Rib and Alp1–4. The protein Rib is most frequently associated with type II and III GBS, while Alp1 is found frequently in serotype Ia GBS, and Alp3 most frequently in serotype V. R proteins (R1–4) have been defined immunologically on the basis of cross-reactivity among streptococcal strains, but are less well defined at the genetic level (Flores & Ferrieri, 1989), except that the gene for the R4 protein has been cloned and is identical to the Rib gene (Smith *et al.*, 2004). The other R proteins appear to be closely related or identical to members of the Alp family. While certain surface proteins tend to be associated with certain capsular types, there is no genetic linkage, and numerous exceptions occur (Tettelin *et al.*, 2005).

Herein we performed immunologic and genetic analysis of eight clinical isolates that were NT, but expressed R1, R4 proteins associated with serotype V. We hypothesized that these isolates might harbour genetic changes in the *cps* locus that lead to an NT phenotype. Sequencing of the 18 genes in the *cps* locus revealed unique mutations in each of the eight GBS isolates.

METHODS

Bacterial isolates. Eight colonizing (vaginal or rectal) GBS isolates designated A1–A4 and B1–B4 were collected as part of an active GBS surveillance study between January 1994 and January 1996. They did not express any definable CPS by routine double-immunodiffusion in an agarose serotyping assay. Isolates A1–A4 and B1–B3 were isolated from pregnant and non-pregnant women in Seattle, WA, USA, and isolate B4 was a vaginal isolate recovered from a woman in Pittsburgh, PA, USA. These isolates expressed R1, R4 surface proteins and had a PFGE banding profile identical to that of a prototype V/R1, R4 isolate. A prototype V strain (SS1169) was used as a control for several experiments. In addition, four more serotype V clinical isolates were used in comparative experiments. Two of these, 17 and 19, were invasive isolates from Houston, TX, USA (collected in 2001 and 2003, respectively), 26 was a vaginal isolate from Pittsburgh (collected in 2002), and 10 was a vaginal isolate from Minneapolis, MN, USA (collected in 1981). All GBS isolates

were grown at 37 °C in Todd–Hewitt broth (THB; Difco Laboratories) and modified with 2% glucose and 1.5% sodium phosphate for experiments designed to upregulate CPS production.

Double-immunodiffusion reactions. Five millilitres of 7 h cultures were inoculated into 50 ml modified THB and incubated overnight at 37 °C. Cells were extracted with hot 0.2 M HCl, concentrated 5 × in a Microcon YM-10 centrifugal filter device (Amicon) and serotyped by double immunodiffusion in agarose using type V mono-specific antisera (Slotved *et al.*, 2002). Isolates A1 and A2 were used to immunize rabbits designated R-41 and R-43, respectively. Antisera obtained from the two rabbits, along with hyperimmune rabbit antiserum to prototype serotype V, were used in double-immunodiffusion reactions in agarose (Ferrieri & Flores, 1997). These reactions were also used to identify surface-protein antigens (Flores & Ferrieri, 1989).

Inhibition ELISA. An inhibition ELISA was performed to assess the relative amounts of CPS produced by the eight isolates. All eight isolates were grown in modified THB to an OD₆₅₀ of 0.3. The CPSs were extracted with mutanolysin (Ferrieri & Flores, 1997). Briefly, the harvested cell pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0) and then resuspended in 750 µl of 40% sucrose in 50 mM sodium phosphate buffer (pH 7.0). To each sample, 250 µl mutanolysin (1 mg ml⁻¹ in 50 mM sodium phosphate buffer, pH 7.0) was added, followed by incubation at 37 °C overnight with end-over-end mixing. Protoplasts and cell debris were removed by centrifugation, and the supernatants containing CPS were transferred to fresh tubes and stored at –20 °C. The ELISA plates were coated with type V CPS–human serum albumin conjugate, as described previously (Baker *et al.*, 2004). Mutanolysin-extracted CPS was added as an inhibitor, and threefold serial dilutions were made with PBS. Rabbit serum (diluted 1:200 000) containing antibodies raised against type V–tetanus toxoid conjugated vaccine was added. After incubation, a goat anti-rabbit IgG–alkaline phosphatase conjugated secondary antibody (diluted 1:2000) was added to the plates, and the reaction plates were read in a Biotek microtitre plate reader (Biotek Instruments). The experiments were done in duplicate for all the isolates, and the results from the ELISA plates were averaged. Relative percentage inhibition of each preparation was determined by comparison to that obtained with purified type V CPS, which resulted in complete (100%) inhibition. Percentage inhibition was calculated as $[(A_{\text{no inhibitor}} - A_{\text{test}})/A_{\text{no inhibitor}}] \times 100$. The concentration of type V CPS in each preparation was determined by multiplying the amount of purified type V CPS that resulted in 60% inhibition (linear portion of the standard curve) by the dilution of the extract that resulted in the same degree of inhibition.

PFGE. A rapid PFGE assay was performed on isolates according to a published protocol (Benson & Ferrieri, 2001; Fasola *et al.*, 1993; Tenover *et al.*, 1995). Briefly, bacteria were embedded in agarose plugs and treated with mutanolysin and proteinase K to lyse the cell wall and precipitate the proteins. The DNA was digested *in situ* with *Sma*I restriction enzyme, and the resulting fragments were resolved by PFGE. The PFGE profiles of the isolates were compared visually to the band profiles of the prototype isolate using the criteria of Tenover *et al.* (1995).

Multilocus sequence typing (MLST). Chromosomal DNA was extracted from cells using Qiagen genomic-tip kits. PCR reactions of the seven housekeeping genes *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt* were performed using primers described previously (Jones *et al.*, 2003). The PCR products were then purified using Montage PCR centrifugal filter devices (Millipore). The amplicons were sequenced on both strands of DNA with internal nested primers using ABI Prism BigDye Terminator reaction mix. The reaction products were separated and detected with an ABI Prism 3700 DNA analyser (Applied Biosystems). A consensus sequence was generated using the MacVector program (Accelrys) for the seven gene fragments. This sequence was then uploaded to the GBS database at <http://pubmlst>.

org/sagalactiae/ to obtain an allele designation. The combination of alleles at the seven loci provided a sequence type (ST) for each isolate (Jones *et al.*, 2003; Maiden *et al.*, 1998).

Molecular serotyping. PCRs were performed with new sets of primers (L. C. Madoff, unpublished results) along with primer combinations shown elsewhere to amplify regions of CPS locus thought to be specific for the nine serotypes (Kong *et al.*, 2002a). DNA extracts from the prototype strains of the nine serotypes were used as positive controls. In addition, the *cfb* (CAMP factor) gene was used as a control for GBS DNA.

Surface-protein genes. PCRs were performed with primer combinations shown elsewhere to amplify regions of the *bca* (C alpha protein), *bac* (C beta protein), *rib* (Rib protein), *alp1* (C alpha-like protein) and *alp3* (C alpha-like protein) genes (Kong *et al.*, 2002b). In addition, the *cfb* gene was used as a control for GBS DNA. DNA from the prototype strains was used as a positive control for the presence of the surface-protein genes.

CPS locus sequencing. All eight NT isolates plus the four serotype V clinical isolates were sequenced for polymorphisms in the CPS locus. The 18 genes (SAG1158–SAG1175) found in the CPS locus were amplified by PCR (Takara LA PCR kit, Chemicon International) using standard conditions, and the resulting amplicons were treated with shrimp alkaline phosphatase (Amersham Biosciences) and exonuclease I (Amersham Biosciences). The purified PCR products were then sequenced on both strands using the BigDye terminator cycle sequencing kit (Applied Biosystems) according to standard protocols. A total of 80 sequencing primers were used to cover the entire CPS locus at 500 bp intervals. For strains B2 and B3, strain-specific primers were designed to generate sequence data in regions where initial primers did not anneal. The sequencing reactions were run on 3730XL automated DNA sequencers (Applied Biosystems). The data from the sequencing instrument was assembled using TIGR assembler software (Pop & Kosack, 2004; Sutton *et al.*, 1995). The edited and assembled sequence was then compared with the sequence from strain 2603V/R deposited in GenBank (accession no. AE009948) using MacVector software.

RESULTS

Immunoprecipitin reactions

Sera from rabbits immunized with GBS isolates A1 and A2 reacted with HCl extracts from isolates A1, A2, A3 and A4, but not that from isolates B1, B2, B3 and B4 (Fig. 1). In addition, concentrated HCl extracts from isolates A1–A4, but not isolates B1–B4, reacted, albeit weakly, with type V CPS-specific antibody. These data suggest that isolates A1–A4 were atypical type V, whereas isolates B1–B4 were nontypable. All eight isolates expressed R1 and R4 surface-localized proteins, as determined by double-immunodiffusion reactions with specific antisera (Flores & Ferrieri, 1989). The results suggested that A1–A4 isolates were poorly encapsulated or had a modified type V CPS, whereas the NT isolates B1–B4 were acapsular, expressed an altered capsule, or had a small amount of CPS that was not detectable in a double-immunodiffusion reaction.

Inhibition ELISA

An inhibition ELISA was performed to determine the relative amounts of type V CPS produced by each isolate. In this

Fig. 1. Double-immunodiffusion reactions with rabbit antiserum produced against the prototype serotype V isolate (A) and reactions of atypical serotype V isolates with antisera from rabbit 41 and rabbit 43 (B). In panels IA and IIB, the central well, c, contains antiserum raised against the prototype serotype V isolate. The peripheral six wells in panel IA contain 4 × concentrated HCl extracts from the following isolates: 1, prototype V; 2, A1; 3, A2; 4, prototype V; 5, A3; 6, A4. Panel IIB contains 4 × concentrated HCl extracts from the following isolates: 1, prototype V; 2, B1; 3, B2; 4, prototype V; 5, B3; 6, B4. In panel (B), the central well, c, contains serum from rabbit 41 containing antibodies raised against isolate A1 (IB) and from rabbit 43 containing antibodies raised against isolate A2 (IIB). The peripheral six wells in panel IB contain 5 × concentrated HCl extracts from the following isolates: 1, A1; 2, A2; 3, A3; 4, A1; 5, prototype V; 6, A4. The peripheral six wells in panel IIB contain 5 × concentrated HCl extracts from the following isolates: 1, A2; 2, A1; 3, A3; 4, A2; 5, prototype V; 6, A4. The prototype serotype V extracts in all panels are unconcentrated.

assay, 0.03–0.05 $\mu\text{g ml}^{-1}$ of purified type V CPS inhibited binding of type V CPS-specific antibody by 60 %. The concentration of type V CPS in the mutanolysin extracts was approximately 1.0 $\mu\text{g ml}^{-1}$ for isolates A1, A2 and A3, and 73 $\mu\text{g ml}^{-1}$ for isolate A4. The relatively high amount of type V CPS expressed by isolate A4, as measured by inhibition ELISA, is consistent with the strong immunoprecipitin reaction seen in Fig. 1, panel IA. In contrast, extracts prepared from isolates B1–B4 each contained <0.05 $\mu\text{g ml}^{-1}$ of type V CPS.

Clonality of isolates

All eight isolates expressed R1, R4 proteins, and their PFGE band profile patterns showed that they were all similar and belonged to DNA profile group 4 (Fig. 2). This banding pattern was similar to that of a prototype V/R1, R4 isolate when matched with the PFGE database. Three of these isolates (B2–B4) had a one-band difference and were assigned to profile group 4a.

Fig. 2. *Smal* macrorestriction analysis by PFGE of the eight isolates. Numbers at the top of the gel are lane designations. Lane 1, lambda DNA marker with size in kb shown on left. Lanes (the protein profile of each isolate is shown after the CPS designation in parentheses): 2–5, isolates B1–B4 (NT/R1, R4); 6, A1 (V/R1, R4); 7, A3 (V/R1, R4); 8, A4 (V/R1, R4); 9, A2 (V/R1, R4); 10, prototype VII (none); 11, Denmark VII/c(α + β); 12, PFGE control lb/c(α + β). The restriction profile number of each isolate is shown at the bottom of the gel.

An MLST assay was performed on all eight isolates to determine if the strains could be further distinguished genotypically (Maiden *et al.*, 1998; Urwin & Maiden, 2003). Isolate A1 had a new *adhP* allele, and hence a new ST was allocated (Table 1). The other seven isolates (A2–A4, B1–B4) were all ST-1. These molecular typing results suggest that these eight isolates are genotypically similar.

Molecular serotyping

To gain further insight into the genetic makeup of these eight isolates, we performed molecular serotyping to identify the presence of CPS genes. All eight isolates had a positive PCR band for the *cfb* gene (which encodes the CAMP factor) and type V-specific (*cpsO*) CPS gene. No PCR bands were detected for the other serotype-specific *cps* genes.

Surface-protein genes

Previous studies (Ferrieri & Flores, 1997; Kong *et al.*, 2002b) have shown strong associations between capsule serotypes and surface-protein genes. All eight isolates were found to have *alp3*, which is significantly associated with GBS type V (Kong *et al.*, 2002a).

CPS locus sequencing

About 17.7 kb of chromosomal DNA from each of the eight isolates was sequenced for polymorphisms in the CPS genes. Four additional serotype V isolates were sequenced to identify the diversity of polymorphisms in the *cps* locus and also to serve as a control sequence for interpretation of the sequence data from the atypical V and NT/R1, R4 isolates. The data from the sequencing reactions were used to build a contig and were then compared with the published 2603V/R

Table 1. Immunologic and genetic analysis of the eight isolates

Type V CPS expression was determined by inhibition ELISA. Isolate A1 had a novel *adhP* allele designated 38, and its ST is a single-locus variant of ST-1.

Isolate	Accession no.	Type V CPS ($\mu\text{g ml}^{-1}$)	CPS/protein profile	PFGE profile	MLST	Molecular serotype	Protein genes
A1	DQ359711	~1	V/R1, R4	4	173	<i>cpsO</i>	<i>alp3</i>
A2	DQ359710	~1	V/R1, R4	4	1	<i>cpsO</i>	<i>alp3</i>
A3	DQ359713	~1	V/R1, R4	4	1	<i>cpsO</i>	<i>alp3</i>
A4	DQ359707	73	V/R1, R4	4	1	<i>cpsO</i>	<i>alp3</i>
B1	DQ359709	<0.05	NT/R1, R4	4	1	<i>cpsO</i>	<i>alp3</i>
B2	DQ359714	<0.05	NT/R1, R4	4a	1	<i>cpsO</i>	<i>alp3</i>
B3	DQ359712	<0.05	NT/R1, R4	4a	1	<i>cpsO</i>	<i>alp3</i>
B4	DQ359708	<0.05	NT/R1, R4	4a	1	<i>cpsO</i>	<i>alp3</i>

genome sequence. Sequencing results for the four atypical V isolates (A1–A4) and the nonserotypable isolates (B1–B3) indicated that all the genes (SAG1158–SAG1175) were present without major deletions or insertions.

Isolate B4 had an 861 bp insert at the third base of codon 373 in *cpsE* (Fig. 3). This insert was identified as an insertion sequence, IS1381. The insertion sequence contained a 20 bp terminal inverted repeat. The transcriptional direction of the two ORFs encoding putative transposases in the insertion sequence was reversed compared with that of *cpsE*.

All the isolates were carefully scanned for single nucleotide polymorphisms at the CPS locus. The atypical V/R1, R4 isolates, as well as the NT/R1, R4 isolates, had 28 common polymorphisms distributed across 10 CPS genes (Table 2); 12 of these mutations were synonymous changes, 13 were nonsynonymous changes, two were insertion mutations, and one was a single substitution mutation in the *cpsD*–*cpsE* intergenic region. In addition, each isolate (A1–A4, B1–B4) showed one to three unique mutations (Table 3). The 28 common polymorphisms found in the eight isolates were also found in the three control typable V isolates whose ST was ST-1, suggesting a strong linkage disequilibrium between the polymorphisms found in the *cps* locus and the ST of the isolates. Another typable V isolate with an ST-7 profile shared most of the polymorphisms with 2603V/R (ST-19) strain, except for two polymorphisms (Table 2). All 12 isolates, independent of ST, had a 9 bp insertion at codon 217 of *cpsD*.

DISCUSSION

In the course of routine characterization of clinical GBS isolates, we identified eight strains that expressed R1, R4 proteins and shared PFGE profiles with a GBS serotype V strain, but were nontypable. We tested the hypothesis that these isolates harboured genetic changes in the GBS *cps*

locus that result in an NT phenotype. These isolates were epidemiologically distinct and were NT by the routine double-immunodiffusion typing assay in agarose. Isolates A1–A4 gave a very weak reaction of identity with type V antiserum when their HCl extracts were concentrated five-fold, whereas isolates B1–B4 gave no reaction of identity even with concentrated extracts. This finding suggested that isolates A1–A4 were micro-encapsulated or had a modified type V CPS, and that isolates B1–B4 were acapsular or expressed very low amounts of type V CPS. It is also plausible that a novel serotype could exist among these latter isolates.

PFGE showed that all eight isolates had a profile similar to that of a GBS type V containing R1 and R4 proteins (Ferrieri et al., 1997). Previous studies have shown an association of isolates with specific CPS serotypes with certain protein profiles, e.g. alpha C protein with type Ia, alpha and beta C proteins with type Ib, Rib protein with type III, and R1, R4 proteins with type V (Ferrieri & Flores, 1997; Flores & Ferrieri, 1989; Kong et al., 2002b). Serotype V isolates have been found to fall into a limited number of profile groups, and serotype V isolates producing R1, R4 proteins tend to belong to profile group 4. Also, NT isolates with the R1, R4 protein profile tend to belong to PFGE group 4, suggesting that there is restricted genetic diversity among isolates producing R1, R4 proteins (Ferrieri et al., 1997). The PFGE profile group 4 is identical to PFGE subtype O described elsewhere (Elliott et al., 1998). The recent increase in the recovery of isolates with the NT/R1, R4 profile from patients may indicate a periodic shift of this clonal complex with improved genetic fitness. This periodic shift may also account for the observed restriction in genetic variation among NT/R1, R4 isolates. Future surveillance data will assess the extent of clonal divergence that may arise from this group of isolates. All eight isolates cluster into a single clonal group and belong to the ST-1 complex, which includes the newly identified ST-173 (isolate A1), a single-locus variant of ST-1.

Fig. 3. Schematic of the *cps* genes sequenced. Isolate B4 has an IS1381 insert with two ORFs encoding transposases oriented in a direction opposite to the *cps* operon transcript. The DNA-sequenced regions of the eight isolates have been deposited in GenBank with accession numbers DQ359707–DQ359714.

Table 2. Mutations common among the isolates and the relationship between MLST and *cps* locus polymorphisms

Isolates A2–A4 and B1–B4 were ST-1. Isolate A1 was ST-173, a single-locus variant of ST-1. Three additional serotype V isolates of ST-1 and a fourth isolate of ST-7 were sequenced. The isolate with ST-7 shared most of the polymorphisms with the 2603V/R strain, except for the polymorphisms in the two genes denoted by an asterisk. The identification of nucleotide and corresponding amino acid changes was based on the 2603V/R genome sequence. All 12 isolates had the 9 bp insert in *cpsD*. AA, single-letter amino acid designation; ups, upstream nucleotide sequence from the start site of the gene.

Gene	Codon	Sequence		AA	
		2603V/R ST-19	A1–B4 ST-1, ST-173	2603V/R	A1–B4
<i>neuA</i>	367	CTG	TTG	L	L
	261	ACC	ATC	T	I
	194	AGG	AAG	R	K
	189	AGC	ATC	S	I
	25	TCT	GCA	S	A
<i>neuD</i>	180	TCA	TCG	S	S
	128	AAT	AAC	N	N
<i>cpsL</i>	401	ATT	GTT	I	V
<i>cpsK</i> *	12	TTA	CTA	L	L
<i>cpsO</i>	53	TTT	TTA	F	L
<i>cpsE</i>	34	AAT	AAC	N	N
	17	ACA	ACC	T	T
<i>cpsE</i> ups	10	C	T		
<i>cpsD</i> *	217	GAT	9 bp insert		
	176	ACC	ACT	T	T
	52	GCT	ACT	A	T
	43	GTT	TTT	V	F
<i>cpsC</i>	110	TTG	CTG	L	L
	33	GCA	GCG	A	A
<i>cpsB</i>	165	TCC	TCT	S	S
	25	TTA	TTG	L	L
<i>cpsA</i>	421	AAC	AAT	N	N
	181	GAT	AAT	D	N
	179	AGT	AAT	S	N
	158	AAA	GAA	K	E
	64	GTA	GCA	V	A
	8	CAC	CAA	H	Q
<i>cpsA</i> ups	84		A insert		

GBS isolates of both human and bovine origin belong to the ST-1 complex (Bisharat *et al.*, 2004). STs can be grouped into lineages or clonal complexes by the BURST program, and isolates of the ST-1 complex are grouped as BURST lineage 1 (Bisharat *et al.*, 2004; Jones *et al.*, 2003). The typing assays indicate that the four atypical V/R1, R4 isolates and the four NT/R1, R4 isolates are genetically similar, but that they differ in the amount of type V CPS produced and/or may have a modified type V CPS.

The *cpsO* gene in the *cps* locus specifically associated with serotype V was present in all eight isolates. Although the presence of *cpsO* does not indicate whether the gene product is expressed, the expected size of the amplicon obtained (642 bp, data not shown) rules out large insertions or

deletions in the gene fragment that could potentially result in an NT phenotype. This result was corroborated by the CPS locus sequencing data. PCR analysis of the surface-protein genes identified the presence of *alp3* in all eight isolates. Our results did not identify the *rib* gene in any of the eight isolates, although all of them produced R1, R4 proteins. This finding concurs with the recent observation that isolates co-expressing the R1 and R4 proteins show a negative reaction in both PCR and Southern hybridization assays with an *r4* gene probe (Smith *et al.*, 2004). The strong association between the presence of *alp3* and the co-expression of R1, R4 proteins suggests that *alp3* encodes a protein that reacts immunologically with both R1 and R4 antisera. Recent studies involving nucleotide and amino acid sequencing of the R4 protein indicate that the proteins

Table 3. Mutations unique to each isolate

The nucleotide and amino acid changes in the isolates were based on the genome sequence of the 2603V/R strain. Isolate B4 had an 861 bp insert at the third base of codon 373 in *cpsE*. This insert was identical to IS1381. AA, single letter amino acid designation; STOP, termination mutation; del, deletion of the nucleotide.

Isolate	Gene	Codon	Sequence		AA	
			2603V/R	Isolate	2603V/R	Isolate
A1	<i>cpsG</i>	103	CAT	TAT	H	Y
	<i>cpsA</i>	82	TTA	TTT	L	F
A2	<i>cpsG</i>	84	GGA	A del		
	<i>cpsA</i>	95	GGA	GGC	G	G
A3	<i>cpsG</i>	84	GGA	A del		
	<i>cpsA</i>	463	TAT	AAT	Y	N
		412	TCC	TCA	S	S
A4	<i>cpsM</i>	82	GAT	GGT	D	G
B1	<i>cpsJ</i>	178	CCA	CTA	P	L
	<i>cpsH</i>	269	GGA	TGA	G	STOP
	<i>cpsF</i>	12	CAT	TAT	H	Y
B2	<i>cpsE</i>	362	CGA	TCA	R	S
B3	<i>cpsG</i>	84	GGA	A del		
	<i>cpsA</i>	466	TCA	TTA	S	L
B4	<i>cpsE</i>	373	TAT	861 bp insert		

R4 and Rib are identical and encoded by the *r4/rib* gene (Smith *et al.*, 2004). The gene that encodes the R1 protein has not been identified, and further study of the R1 and Alp family of proteins at the genetic level is warranted.

All 18 genes in the CPS locus were sequenced to find the genetic changes that would explain the phenotypic differences between the four atypical and the four NT isolates. Except for isolate B4, no major insertions or deletions were found in the isolates. Isolate B4 had an IS1381 insertion in the C terminus of *cpsE*. Originally identified in a *Streptococcus pneumoniae* R6 strain (Sanchez-Beato *et al.*, 1997), IS1381 has been reported in GBS isolates of both human and bovine origin (Dmitriev *et al.*, 2004; Shackleina *et al.*, 2004; Tamura *et al.*, 2000), although this is the first observation of the disruption by IS1381 of a gene in the GBS *cps* locus. *cpsE* is thought to encode a glycosyltransferase in type Ia GBS and is proposed to have a role as the initiating enzyme in the biosynthesis of polysaccharide repeating units (Cieslewicz *et al.*, 2001). Indeed, a *cpsE* knockout mutant in a serotype III strain has reduced CPS in both cell wall and protoplast fractions when compared with the wild-type parent strain (Chaffin *et al.*, 2005). This finding is consistent with the reduced or disrupted expression of CPS due to apparent loss of the function of glycosyltransferase by isolate B4. Sequencing analysis also identified a 9 bp repetitive sequence in the C terminus of *cpsD*. All isolates used in this study, except for the 2603V/R isolate, had this repetitive sequence. This finding extends and corroborates the finding elsewhere

(Kong *et al.*, 2002a) that all serotype V isolates have this repetitive sequence.

Sequencing analysis also showed the distribution of 28 polymorphisms across 10 CPS genes found in common in all eight isolates. The three typable isolates with the ST-1 profile (BURST lineage 1) had the same 28 polymorphisms, suggesting a linkage disequilibrium between the ST and the polymorphisms, and indicative of the clonal structure of these isolates. Furthermore, another typable isolate with ST-7 (BURST lineage 3) and a 2603V/R isolate (single-locus variant of ST-19, BURST lineage 6) had different sets of polymorphisms (Jones *et al.*, 2003). The presence of these common sets of polymorphisms in both typable and NT isolates suggests that these polymorphisms are not involved in the NT phenotype.

In addition to the 28 common polymorphisms seen, one to three unique mutations were observed in seven of the eight isolates. Other than the IS1381 insertion, no unique single-nucleotide polymorphisms were found in isolate B4. Isolate B1 had a termination mutation in *cpsH*, a gene known to encode a polymerase in both serotypes Ia and III (Cieslewicz *et al.*, 2001). This isolate also had a nonsynonymous mutation in two other genes, *cpsJ* and *cpsF*, whose products, CpsJ and CpsF, are annotated to be a glycosyltransferase and a polysaccharide biosynthetic protein, respectively, in the sequenced genome of 2603V/R (Tettelin *et al.*, 2002). A combination of these three mutations may account for the NT phenotype in this isolate. Four isolates (A1–A3, B3) had two mutations, one each in the *cpsG* and *cpsA* genes. *cpsA* is thought to encode a transcriptional regulator (Cieslewicz *et al.*, 2001), and *cpsG* is thought to encode a β -1,4-galactose transferase in both serotypes Ia and III (Cieslewicz *et al.*, 2005). Three of the isolates (A2, A3 and B3) had a single-nucleotide deletion in codon 84 of *cpsG*, resulting in a frame-shift mutation that potentially results in loss of function of this glycosyltransferase. This truncation mutation, along with the other nonsynonymous changes found, may account for the NT phenotype in these isolates. Isolates A4 and B2 have a single nonsynonymous substitution in the *cpsM* and *cpsE* genes, respectively.

In conclusion, we have identified new genetic correlates for the NT phenotype in GBS. Knowledge of the composition and function of the various *cps* genes in different serotypes is warranted, as it provides insight into the dynamics of capsule biosynthesis, surface-protein expression, and the evolution of GBS serotypes.

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