A unique serine-rich repeat protein (Srr-2) and novel surface antigen (ϵ) associated with a virulent lineage of serotype III *Streptococcus agalactiae*

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Group B streptococci (GBS) are pathogens of both neonates and adults, with serotype III strains in particular being associated with invasive disease and meningitis. In this study, a novel GBS surface antigen, ϵ , was found to be co-expressed with the previously reported δ antigen on an identical subset of serotype III GBS. Expression of δ/ϵ on the surface of serotype III GBS was shown to distinguish the restriction digest pattern (RDP) III-3 and multilocus sequence typing (ST)-17 lineage. ϵ -Specific antibodies were reactive with a unique, high-molecular-mass, serine-rich repeat protein (Srr-2) found exclusively in RDP III-3 strains. The gene encoding Srr-2 was located within a putative accessory secretory locus that included *secY2* and *secA2* homologues and had a genetic organization similar to that of the *secY2/A2* locus of staphylococci. In contrast, serotype III δ/ϵ -negative strains and strains representative of serotypes Ia, Ib, Ic and II shared a common Srr-encoding gene, *srr-1*, and an organization of the *secY2/A2* locus similar to that of previously reported serotype Ic, δ/ϵ -negative serotype III and serotype V GBS strains. Representative serotype III δ/ϵ -positive strains had LD₅₀ values 3–4 logs less than those of serotype III δ/ϵ -negative strains in a neonatal mouse model of infection. These results indicate that the RDP III-3/ST-17 lineage expresses Srr-2 and is highly virulent in an *in vivo* model of neonatal sepsis.

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INTRODUCTION

Lancefield group B streptococci (GBS) are a major cause of neonatal sepsis (Baker & Edwards, 2001) and are also important pathogens in adults, particularly those with underlying chronic medical conditions (Farley, 2001). There are two main clinical forms of neonatal GBS disease, early- and late-onset disease (Ferrieri, 1985; Baker & Edwards, 2001). Early-onset disease accounts for 80 % of all GBS infections. These infections occur within the first week of life and are vertically transmitted from mother to infant. Each of the nine

serotypes of GBS has been isolated from infants suffering from early-onset disease; however, serotypes III and V cause the majority of these infections. Late-onset disease occurs from 1 week to 3 months after birth. Serotype III GBS is responsible for ~90 % of these infections as well as 90 % of cases of meningitis, irrespective of the time of onset of disease (Baker & Edwards, 2001). Serotype III organisms are also the second most common serotype identified in invasive adult infections (Tyrrell *et al.*, 2000).

A number of surface antigens associated with GBS have been identified. Wilkinson and Eagon first described the c-protein in 1971 (Wilkinson & Eagon, 1971). It was subsequently discovered that c-protein typing serum recognizes at least four antigenic moieties, α , β (Wilkinson & Eagon, 1971; Johnson & Ferrieri, 1984; Bevanger, 1985), γ and δ (Brady *et al.*, 1988; Chun *et al.*, 1991). The last two markers were identified by residual seroreactivity after exhaustive adsorption of the c-protein typing reagent with strains

Abbreviations: GBS, group B streptococci; HRP, horseradish peroxidase; MLST, multilocus sequence typing; RDP, restriction digest pattern; RIA, radioimmunoassay; Srr, serine-rich repeat; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *secY2/A2* loci from GBS strains 874391 and J48 reported in this paper are AY669067 and DQ174691, respectively.

known to express α and β . Genes encoding the α and β proteins have been cloned (Cleat & Timmis, 1987; Michel *et al.*, 1991) and the proteins characterized (Russell-Jones & Gotschlich, 1984; Michel *et al.*, 1991; Gravekamp *et al.*, 1996; Maeland *et al.*, 1997; Ustinovitch *et al.*, 1999; Areschoug *et al.*, 2002; Auperin *et al.*, 2005). α and β are expressed predominantly by serotypes Ia, Ib and II. The γ antigen is an amino-terminus variant of the α protein (L. C. Madoff, personal communication). The genetic basis for expression of the δ antigen is unknown. It is associated almost exclusively with serotype III strains (Chun *et al.*, 1991). The δ antigen is immunologically distinct from protein Rib, another GBS antigen associated primarily with serotype III organisms (Stalhammar-Carlemalm *et al.*, 1993).

High-molecular-mass streptococcal and staphylococcal serine-rich repeat (Srr) proteins are encoded within operons containing a dedicated secretion system (Wu *et al.*, 1998; Bensing & Sullam, 2002; Stephenson *et al.*, 2002; Takamatsu *et al.*, 2004b; Siboo *et al.*, 2005). Many of these highly glycosylated, surface-localized proteins have adhesive functions. In the present study, an antigenic marker, designated ε , was identified that is co-expressed with the δ antigen on all δ -positive serotype III GBS strains. Cell surface expression of this novel immunological reactivity correlates with the presence of a unique ORF, *srr-2*, contained within a genetic locus that includes homologues of *secA2* and *secY2* (Bensing & Sullam, 2002).

In addition to antigenic differences, serotype III GBS strains also have been classified into four distinct lineages, designated RDP types III-1, -2, -3 and -4, based on *Hind*III and *Sse*83871 restriction digest patterns (RDPs) of chromosomal DNA (Takahashi *et al.*, 1998). A multilocus sequence typing (MLST) system has also been developed that categorizes GBS strains based on the combination of alleles of seven housekeeping genes (Jones *et al.*, 2003). Comparison of these two typing methods has shown that RDP III-3 strains correspond to the ST-17 clonal complex lineage. Over 90 % of serotype III invasive neonatal isolates have been identified as RDP III-3, suggesting that this may represent a more virulent lineage (Takahashi *et al.*, 1998). Several investigators have now also reported that ST-17 lineage strains are significantly associated with invasive disease in neonates (Jones *et al.*, 2003; Luan *et al.*, 2005; Bisharat *et al.*, 2004). In this report, it is shown that ε represents a unique, cell surface-localized, antigenic marker that, in association with the previously identified δ antigen, distinguishes RDP III-3/ST-17 strains from other GBS. In addition, δ/ε -positive RDP III-3 strains that contain *srr-2* in the context of the variant *secY2/A2* locus are markedly more virulent and demonstrate significantly lower LD₅₀ values in a murine neonatal sepsis model than serotype III GBS that contain *srr-1*.

METHODS

Bacterial strains and growth conditions. GBS strains used in this study are listed in Table 1. Strains DL797, DL769, DL771, DL686, DL775, DL1104, DL785 and DL700 were clinical isolates kindly provided by Dr Daniel Lim from the University of South

Florida, Tampa, FL, USA. GBS strains representing RDP III types 1–4 were isolated from vaginal swabs, blood or cerebrospinal fluid, as described previously (Takahashi *et al.*, 1998). Strains COH1 and COH1-13 were kindly provided by Dr Craig Rubens of the University of Washington and Children's Hospital and Regional Medical Center, Seattle, WA, USA. Strain COH1 was isolated from a septic infant (Martin *et al.*, 1988). Strain COH1-13 is an acapsular derivative of COH1 (Rubens & Heggen, 1988). Strain NEM316 (serotype III, RDP III-1) is an isolate from an infected infant (Glaser *et al.*, 2002), and was obtained from the American Type Culture Collection (ATCC 12403). All streptococci were grown to late-exponential phase in Todd-Hewitt broth (THB) (Becton Dickinson) at 37 °C without shaking. *Escherichia coli* strain TOP10 was grown in Luria-Bertani (LB) broth at 37 °C with shaking.

MLST. Internal fragments (400–500 bp) of seven different housekeeping genes were amplified by PCR and sequenced as described by Jones *et al.* (2003). Sequences were assembled and analysed with the Staden suite of computer programs (Staden, 1996). For each locus, every different sequence was assigned an allele number. Each isolate was assigned a sequence type (ST) based on the seven integers constituting its allelic profile. The sequences of all alleles and the composition of the STs identified in this study are available at <http://pubmlst.org/sagalactiae>.

Generation of polyclonal anti-J48 whole-cell antiserum.

Rabbit antiserum was produced in a New Zealand white rabbit against stationary-phase cells of GBS strain J48, killed by treatment at 4 °C overnight with 4 % formalin in 0.85 % saline. Density was adjusted to OD₆₆₀ = 0.4 in 0.85 % saline and the rabbit was immunized intravenously with 0.5 ml whole cell suspension three times per week for 1 week, then with 1 ml three times per week for 3 weeks, rested for 3 weeks, and finally with 1 ml three times per week for 2 weeks. The rabbit was rested for 2 weeks, exsanguinated by cardiac puncture under anaesthesia and serum was stored at –20 °C. Immunization was conducted in accordance with the University of Florida Institutional Animal Care and Use Committee.

Identification of ε by two-stage radioimmunoassay (RIA).

A two-stage RIA was performed as described previously (Brady *et al.*, 1988) using anti-J48 antiserum adsorbed as described below (1:1000). All assays were performed in duplicate with less than 5 % variation observed between replicate samples. Reactivity of each strain with normal rabbit antiserum was tested as a negative control.

Generation of ε -specific antiserum.

To remove antibodies reactive with known GBS antigens, including the group B carbohydrate, serotype III capsular polysaccharide, protein Rib and the δ antigen, the anti-J48 whole-cell antiserum was exhaustively adsorbed with GBS strain DL700 (serotype III, Rib-positive) followed by strain A909 (serotype Ic/ $\alpha\beta\gamma\delta$). Adsorption was performed at a ratio of 100 μ l antiserum to 10⁹ c.f.u. PBS-washed cells with rotation end-over-end at 4 °C for 1 h, and repeated until reactivity against the adsorbing strain, as evaluated by ELISA, was eliminated. The resulting adsorbed polyclonal rabbit antiserum was reactive with strain J48, but not with strain DL700 or A909. The residual antigenic reactivity was designated ε and this adsorbed reagent was used to detect the ε antigen in subsequent experiments.

ELISA to evaluate ε expression. Costar High Binding plates (Corning) were coated with $\sim 10^7$ c.f.u. of representative RDP III-1, -2, -3 and -4 GBS strains, blocked with PBS containing 0.03 % Tween 20, reacted with adsorbed anti- ε -specific antiserum (1:100) followed by affinity-purified, horseradish peroxidase (HRP)-labelled, goat anti-rabbit secondary antibody (1:1000) (MP Biomedicals), developed with 0.4 mg ml⁻¹ o-phenylenediamine (OPD) in 0.1 M citric acid/0.2 M sodium phosphate buffer, pH 4.5, with 0.03 % hydrogen peroxide, and absorbance was read at 450 nm. Control

Table 1. Bacterial strains

Strain	Serotype	Surface antigens*	ϵ	RDP type	MLST	Reference
A909	Ic	$\alpha\beta\gamma\delta$	—	Ia-3,4 (lac)	7	Michel <i>et al.</i> (1991)
ss618	Ib	$\alpha\beta\gamma$	—	Ib	6	Seifert <i>et al.</i> (2003)
9B200	II	None	—	II	22	Seifert <i>et al.</i> (2003)
J48	III	δ	+	III-3	17	Seifert <i>et al.</i> (2003)
J52	III	δ	+	III-3	17	Seifert <i>et al.</i> (2003)
PEH	III	δ	+	III-3	17	Seifert <i>et al.</i> (2003)
COH1	III	δ	+	III-3	17	Martin <i>et al.</i> (1988)
DL797	III	δ	+	III-3	17	This study
DL769	III	δ	+	III-3	31	This study
874391	III	δ	+	III-3	17	Adderson <i>et al.</i> (2003)
DL785	III	δ	+	ND	ND	This study
DL686	III	None	—	III-1	23	Seifert <i>et al.</i> (2003)
DL775	III	None	—	III-2	19	This study
DL1104	III	None	—	III-4	1	This study
DL700	III	None	—	III-2	19	Seifert <i>et al.</i> (2003)
NEM316	III	None	—	III-1	23	Glaser <i>et al.</i> (2002); Jones <i>et al.</i> (2003)
865043	III	None	—	III-2	19	Takahashi <i>et al.</i> (1998)
i10	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
i27	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
i43	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
610522	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
861503	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
590444	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
872306	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
i01	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
i25	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
610525	III	ND	+	III-3	17	Jones <i>et al.</i> (2003)
843118	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
C44	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
870917	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
530119	III	ND	—	III-2	21	Jones <i>et al.</i> (2003)
C65	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
872809	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
C45	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
C17	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
873036	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
871952	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
854325	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
840397	III	ND	—	III-2	19	Jones <i>et al.</i> (2003)
884062	III	ND	—	III-1	ND	This study
880557	III	ND	—	III-1	23	Jones <i>et al.</i> (2003)
870071	III	ND	—	III-4	1	Jones <i>et al.</i> (2003)
852529	III	ND	—	III-4	ND	This study
871474	III	ND	—	III-4	ND	This study

ND, Not determined.

*Surface antigens reactive with c-protein typing serum.

wells were incubated with secondary antibody alone. GBS were considered positive for ϵ expression if reactivity with the test antiserum was at least twofold greater than that of the background control. All assays were performed in duplicate with less than 5% variation observed between replicate samples.

Extraction of ϵ , anion-exchange chromatography, competition ELISA and Western immunoblot analysis. Cellular extracts were prepared by sonicating 4 g (wet weight) GBS strains J48 or DL700 in 4 ml 20 mM Tris/HCl, pH 8.0, with 4 g P-0080 glass beads (Potter's Industries) three times for 30 s each on ice with a

Sonic 300 Dismembrator (ARTEK Systems). Glass beads and bacterial debris were removed by centrifugation at 10 000 *g* for 5 min followed by centrifugation in a Beckman L7-55 ultracentrifuge (Beckman Coulter) at 100 000 *g* for 2 h to remove wall-membrane complexes. Supernatants were filtered through 0.2 µm pore-size Acrodiscs (Gelman Sciences) and diluted 1:3 in 20 mM Tris/HCl, pH 8.0. Extracts were loaded onto a UNO-Q1 (Bio-Rad) anion-exchange column and bound molecules were eluted with 0.1 M NaCl in 20 mM Tris/HCl, pH 8.0, in 0.05 M NaCl stepwise increments. A competition ELISA was used to identify the ϵ antigen in the column fractions. Microtitre plates were coated with $\sim 10^7$ c.f.u. per well of strain J48 whole cells. Test wells were incubated with 100 µl individual fractions and 100 µl adsorbed anti- ϵ -specific rabbit antiserum (1:100), followed by HRP-labelled goat anti-rabbit conjugate (1:1000) (MP Biomedicals) and development with OPD as described above. Percentage inhibition of anti- ϵ binding was determined by comparing absorbance values of test wells with control wells containing column buffer only. Column fraction 14 from strain J48 that contained ϵ as determined by competition ELISA, and the corresponding fraction from strain DL700 (serotype III), were evaluated by 7.5% SDS-PAGE and Western immunoblotting (Protran nitrocellulose; Schleicher & Schuell) with anti- ϵ -specific antiserum (1:100) followed by HRP-conjugated goat anti-rabbit IgG (1:1000) and development with ECL detection reagents (Amersham Biosciences-GE Healthcare).

Elution of ϵ -containing material from SDS-polyacrylamide gels and composition analysis. One millilitre (1.6 mg ml^{-1}) of sonic extracts of strains J48 and DL700 was separated on multiple 1.5 mm thick 7.5% preparatory SDS-polyacrylamide gels ($8.6 \times 6.8 \text{ cm}$). A 2 mm gel-slice corresponding to the region predicted to contain the ϵ antigen, determined by comparative Western immunoblot analysis of the strains J48 and DL700 anion exchange column fractions, was excised from each gel, finely minced with a razor blade and eluted with 500 µl 50 mM ammonium bicarbonate, pH 7.4, and 1% SDS for 2 h with rocking at 37 °C. Eluted materials were evaluated by Western immunoblots reacted with anti- ϵ (1:100), anti- δ (Brady *et al.*, 1988) (1:500) or anti-GBS serotype III (Accurate Chemical and Scientific Corporation) (1:1000) antisera, followed by HRP-conjugated goat anti-rabbit IgG (H and L chains) (1:1000) and development with ECL detection reagents. The eluted materials were also tested for the presence of group B carbohydrate by latex agglutination (Phadabact, Boule Diagnostics, Huddinge, Sweden). Material present in samples from each strain was analysed for amino acid composition by the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville, FL, USA, and for carbohydrate content by the Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA.

Cloning, expression and Western immunoblot analysis of recombinant Srr-2. Previously, a genomic phage library of GBS strain 874391 (Bohnsack *et al.*, 2002) had been screened with the RDP type III-3 specific probe, DY-3 (Bohnsack *et al.*, 2001). An 8816 bp genomic DNA fragment that hybridized with this probe was purified and cloned (Adderson *et al.*, 2003). The clone included an ORF of 3.6 kb predicted to encode a serine-rich protein that was subsequently designated *srr-2* for serine-rich repeat variant 2 to differentiate it from the *srr-1* ORF (gbs1529) identified in the GBS NEM316 genomic sequence (Glaser *et al.*, 2002). The *srr-2* gene from the RDP type III-3 GBS strain J48 was PCR-amplified from genomic DNA (Nagano *et al.*, 1991) using primers KS51 (5'-tcacg-caaagttcagtgtaaaa-3') and KS53 (5'-cgcagatttagtagctctaa-3') and ligated to the pBAD-TOPO expression vector (Invitrogen), resulting in pKS60. Expression of recombinant Srr-2 was induced by addition of 10-fold increasing amounts of L-arabinose (0.0002–2%) to mid-exponential phase *E. coli* TOP10 cells ($\text{OD}_{600} = 0.5$) harbouring pKS60. After 4 h, cells were harvested and boiled in SDS/sample

buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 0.02% bromophenol blue) and samples analysed by Western immunoblotting. One membrane was incubated with mouse anti-V5 monoclonal antibody (Invitrogen) (1:5000) followed by HRP-conjugated goat anti-mouse IgG (H and L chain specific) (1:1000) and developed with 4-chloro-1-naphthol solution [7 ml PBS, 1 ml methanol containing 3 mg 4-chloro-1-naphthol (Sigma) with 0.03% hydrogen peroxide]. A replicate membrane was incubated with anti- ϵ specific antiserum (1:100) followed by HRP-conjugated goat anti-rabbit IgG (1:1000) and developed with ECL detection reagents.

Southern analysis of *srr* genes. DNA hybridization was performed by the method of Southern (Sambrook *et al.*, 1989). Chromosomal DNA was isolated as described by Nagano *et al.* (1991) and further purified on CsCl gradients. Genomic DNA was digested with *EcoRV* (Promega), separated by electrophoresis on replicate 0.7% agarose gels, and transferred to nylon membranes (Roche Applied Science) by capillary blotting. One membrane was probed with *srr-1* that had been PCR-amplified from strain DL700 genomic DNA using primers KS13 (5'-atgtcccaaaagacttttggc-3') and KS14 (5'-cgtcccaaaagggttgaccagtc-3') derived from the genomic sequence of *srr-1* (gbs1529) from strain NEM316 (Glaser *et al.*, 2002). The other membrane was probed with *srr-2* that had been PCR-amplified from strain J48 genomic DNA as described in the previous section. Both probes were labelled during PCR-amplification with DIG-11-dUTP (Roche Applied Science). Hybridization was at 42 °C for 16 h and the blots were developed according to the manufacturer's instructions. Negative and positive controls included pPC185 (Kremer *et al.*, 2001) and unlabelled PCR-amplified *srr-1* or *srr-2*, respectively.

DNA sequencing. Chromosomal DNA from strain J48 was prepared as described in the previous section and primer walking was used to obtain sequences of genes upstream and downstream of *srr-2*. DNA sequencing was performed by the DNA Sequencing Facility at Iowa State University (Ames, IA, USA) and the DNA Sequencing Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida, Gainesville, FL, USA. Sequences were compiled at the University of Florida and analysed with MacVector version 8 (Accelrys) to find and translate ORFs. Translated ORFs were subjected to BLASTp analysis (<http://www.ncbi.nlm.nih.gov>) to identify protein homologues.

Evaluation of LD₅₀. A neonatal mouse model of infection (Rodewald *et al.*, 1992) was used to compare the LD₅₀ values of serotype III/ $\delta\epsilon$ -positive and serotype III/ $\delta\epsilon$ -negative strains. Offspring (8–14) from one dam of timed-pregnant, CD-1 virus antibody-free female mice (Charles River Laboratories) were each inoculated intraperitoneally 24–48 h after birth with a given challenge dose (10^2 – 10^{10} c.f.u.) diluted in 100 µl THB. Deaths were recorded for 72 h post-inoculation. LD₅₀ values were determined by the Reed-Muench method (Reed & Muench, 1938). The LD₅₀ values were grouped according to δ/ϵ expression and compared using a two-sample *t* test of log-transformed data. Animal experiments were conducted in accordance with the University of Florida Institutional Animal Care and Use Committee.

RESULTS

Identificaton of ϵ antigen

The δ antigen was initially recognized on the surface of a subset of serotype III strains following exhaustive adsorption of anti-c-protein typing antiserum with non-serotype III strains of GBS that expressed α , β and γ antigens (Brady *et al.*, 1988). The GBS strains analysed in this study and their surface expression of the α , β , γ and δ antigens are indicated in Table 1. In the current study, formalin-fixed

serotype III/ δ -positive strain J48 whole cells were used as an immunogen with the goal of generating a polyclonal reagent containing anti- δ antibodies, but lacking anti- α , β and γ antibodies. The antiserum was exhaustively adsorbed with the serotype III/ δ -negative GBS strain DL700 to eliminate antibodies against other cell-surface-localized, non- δ antigens, including protein Rib, the Group B carbohydrate and the serotype III capsular polysaccharide. To evaluate the relationship between the δ antigen present on serotype III GBS and that originally detected on GBS serotype Ic strain A909, the immunizing strain used to generate the c-protein typing serum, the anti-J48 δ -reactive reagent described above was further adsorbed with A909 whole cells and reacted with serotype III/ δ -positive and III/ δ -negative strains, as well as with strain A909, using a two-stage RIA (Fig. 1). Prior to adsorption with A909, the anti-J48-derived δ -reactive antiserum reacted with A909 and those serotype III strains previously identified as δ -positive, thus replicating the results obtained using the original δ -specific reagent derived from the anti-c-protein antiserum. However, following adsorption of the strain J48-derived anti- δ -reactive reagent with A909, residual reactivity remained with all of the serotype III δ -positive strains, even though reactivity with the δ -positive A909 strain had been completely eliminated. This indicates that an additional antigen is co-expressed with δ on serotype III strains and that this antigen is absent from strain A909. The newly identified antigen was designated ε , and the anti-J48 antiserum that had been exhaustively adsorbed with strains DL700 and A909 was used as the ε -specific antiserum in subsequent experiments. Over 30 serotype III/ δ -positive strains have been tested and found to express ε . To date, we have found no serotype III strain that expresses ε in the absence of δ or vice versa (data not shown).

Fig. 1. Identification of ε antigen by RIA. GBS were reacted with anti-J48 antiserum adsorbed with DL700 (white bars) or DL700 and A909 (solid bars). Lanes 1–9 correspond to GBS strains A909, J48, PEH, DL797, DL769, DL771, DL686, DL775 and DL700.

Extraction and partial characterization of the ε antigen

Sonic extracts of GBS strains J48 and DL700 were prepared and subjected to anion-exchange chromatography in parallel. A competition ELISA was used to identify ε in individual anion-exchange column fractions. Material contained in fractions 13–17 (eluted at 0.2 M NaCl) from the J48 sample inhibited anti- ε -specific antiserum binding to J48 whole cells to a greater degree than other column fractions or any column fraction from strain DL700, indicating the enrichment of ε in these fractions (Fig. 2A). These fractions also contained other surface antigens, including the δ antigen and serotype III polysaccharides, identified by competition ELISA and Western immunoblotting, as well as the group B carbohydrate identified by latex agglutination (data not shown). Material from strain J48 column fraction 14 and the corresponding column fraction from the negative control strain DL700 were analysed by SDS-PAGE and Western immunoblotting with anti- ε antiserum. While SDS-PAGE profiles of fractions 14 derived from J48 and DL700 appeared identical (data not shown), a prominent band migrating at >250 kDa was identified by Western immunoblotting of the J48 sample (Fig. 2B, lane 2, arrow) but not the DL700 sample (Fig. 2B, lane 1). This band did not stain with Coomassie brilliant blue, silver, or colloidal gold protein stains (data not shown). To gain some insight into the nature of the ε antigen, material in the >250 kDa molecular mass range was eluted in parallel from SDS-polyacrylamide preparatory gels of both the strain J48 and DL700 sonic extracts. The material eluted from the J48, but not the DL700, gel inhibited anti- ε reactivity with J48 whole cells by competition ELISA, confirming the presence of ε in strain J48 and its absence in DL700 (data not shown). Anti- ε antiserum reacted with the high-molecular-mass gel-eluted material from strain J48 but not from DL700 (Fig. 2C, panel a). Neither anti- δ nor anti-serotype III antisera reacted with the material eluted from SDS-polyacrylamide gels from either strain (Fig. 2C, panels b and c); however, the gel-purified material from each strain still tested positive by latex agglutination for the group B, but not A, C, D or G group antigens (data not shown). Carbohydrate composition analysis of the ~ 250 kDa eluted material from both J48 and DL700 demonstrated the presence of rhamnose, mannose, galactose and glucose in a molar ratio of 4:1:1:6. The group B antigen is composed of rhamnose, N-acetylglucosamine, galactose and glucitol (Michon *et al.*, 1988); therefore, it is likely that a component of the group antigen was present in the samples from both strains. Amino acid composition of gel-eluted materials from both J48 and DL700 indicated the presence of either a glycine- or serine-rich protein (data not shown).

Evaluation of ε expression by RDP III type 1–4 strains and correlation with MLST type

Serotype III GBS are categorized into four distinct lineages based on *Hind*III and *Sse*83871 RDPs of chromosomal DNA (Takahashi *et al.*, 1998). To determine if there was a

Fig. 2. Detection of ϵ by competition ELISA and Western immunoblotting. (A) Column fractions from anion-exchange chromatography of strain J48 (◆) and DL700 (○) sonic extracts were tested for inhibition of binding of anti- ϵ -reactive antiserum to J48 whole cells by competition ELISA. Stepwise elution of bound material with NaCl is indicated. (B) Western blot of column fraction 14 from strain DL700 (lane 1) or J48 (lane 2) reacted with anti- ϵ -reactive antiserum. (C) Western blot of >250 kDa material eluted from strain DL700 (lane 1) or J48 (lane 2) reacted with anti- ϵ (a), anti- δ (b) or anti-serotype III (c) antisera. Migration of molecular mass markers is indicated in kDa.

correlation between ϵ expression and RDP type, a panel of previously typed GBS strains representing RDP III-1, -2, -3 and -4 (Takahashi *et al.*, 1998; Jones *et al.*, 2003) was tested by ELISA using anti- ϵ antiserum. All RDP III-1 ($n=4$), III-2 ($n=15$) and III-4 ($n=4$) strains tested were negative for ϵ expression, while all RDP III-3 strains ($n=17$) were ϵ -positive. These results indicate that among serotype III GBS, ϵ expression represents an antigenic marker for the RDP III-3 lineage. As stated above, to date we have found no serotype III strain that expresses ϵ without δ or vice versa. No non-serotype III GBS have been identified that express ϵ and strain A909 represents the only known exception of δ expression by a non-serotype III strain.

Because of the reported association of RDP III-3 GBS with ST-17 (Jones *et al.*, 2003), additional MLST of serotype III ϵ -positive and ϵ -negative strains was performed. These results are summarized in Table 1. As expected, all ϵ -positive RDP III-3 strains ($n=17$) either were ST-17 ($n=16$) or were of the ST-17 clonal complex lineage, ST-31 ($n=1$), which differs from ST-17 at only one allele (Davies *et al.*, 2004). None of the non-RDP III-3 strains tested ($n=23$) was found to be ST-17 or to belong to the ST-17 clonal complex.

Identification of a novel GBS Srr protein

Bohnsack *et al.* (2002) previously used genomic subtractive hybridization to identify DNA sequences unique to RDP III-3 strains. One resulting clone from the RDP III-3 GBS strain 874391 included a 3618 bp ORF, designated herein as *srr-2*, predicted to encode a high-molecular-mass protein (125 kDa) containing a highly repetitive serine- (36 %) and aspartate-rich (12 %) region. The predicted protein, Srr-2, has a signal peptide and LPXTG motif common to this

family of cell-wall-anchored proteins (Fischetti *et al.*, 1990). Srr-2 has only limited homology (<20 % identity) with the predicted Srr protein identified in the sequenced genomes of GBS strains NEM316 (serotype III, RDP III-1), A909 (serotype Ic) and 2603V/R (serotype V) [Glaser *et al.* (2002), GenBank accession no. NC_004128; Tettelin *et al.* (2002)], and designated herein as Srr-1.

Schematic representations of GBS Srr-1 and Srr-2, and serine-rich proteins from other streptococci, are shown in Fig. 3. Srrs from GBS and other streptococci are relatively large, with ~50 aa signal sequences, a small N-terminal Srr region (RI) located between two non-repeat regions (N), a large Srr region (RII) that comprises a substantial portion of the entire protein, and an LPXTG-wall-anchor region (A) near the C terminus. The predicted secondary structure of the Srr proteins from GBS is similar to that of Srr proteins from other streptococcal species (Wu *et al.*, 1998; Bensing & Sullam, 2002; Takahashi *et al.*, 2002). The amino acids that alternate with serine in the repeat regions of Srr proteins vary among individual proteins. Srr-1s from the three sequenced GBS strains share 89–100 % identity prior to the RII region, with only a length difference and minor substitutions observed among the RII sequences themselves. In contrast, Srr-1 and Srr-2 sequences demonstrate no more than 36 % identity prior to the RII region. The predominant amino acid residues that alternate with serine within RII of Srr-1 are alanine, threonine and methionine, whereas in Srr-2 they are valine, isoleucine, threonine, asparagine, glutamic acid and serine. The predicted amino acid sequences of Srr-2 from strains J48 and 874391 are nearly identical to one another except that the RII region from 874391 contains an additional 100 aa.

Fig. 3. Schematic representation of streptococcal Srr proteins. Srr-1 is from GBS strains NEM316, 2603V/R and A909; Srr-2 is from GBS strains J48 and 874391; Hsa and GspB are from *S. gordonii* strains Challis and M99 (Bensing & Sullam, 2002; Takahashi *et al.*, 2002), respectively; and Fap1 is from *S. parasanguinis* strain FW213 (Wu *et al.*, 1998). S, signal sequence; N, non-repeat region; RI, small repeat region I; RII, large repeat region II; A, cell wall sorting signal; (X)S, di-peptide repeat motif. The predominant amino acid residues that alternate with serine within the RII regions are indicated in parentheses. Bar, 100 aa.

Identification of *srr-2* in ϵ -positive GBS

Southern hybridization was used to determine the relationship between ϵ expression and the presence of either *srr-1* or *srr-2* in representative GBS strains. DIG-labelled *srr-1* PCR-amplified from strain DL700 genomic DNA hybridized with all ϵ -negative strains (Fig. 4, Panel A, lanes 1–3, 9 and 10), while labelled *srr-2* PCR-amplified from strain J48 genomic DNA hybridized with all ϵ -positive strains (Fig. 4, Panel B, lanes 4–8). This suggests that ϵ -positive RDP III-3 strains possess *srr-2*. Conversely, non-RDP III-3 strains that do not express ϵ possess *srr-1*.

Comparison of *secY2/A2* loci in GBS strains

The sequenced genomes from GBS strains NEM316, A909 and 2603V/R indicate that *srr-1* is located in a putative operon similar to that of *Streptococcus gordonii*, named *secY2/A2*, due to the presence of the genes *secA2* and *secY2* which are dedicated to the export of the Srr protein GspB, and which bear homology to *secA* and *secY* (Bensing & Sullam, 2002). To determine whether *srr-2* from strain J48 was located in a similar *secY2/A2* locus, genes upstream and downstream of *srr-2* were sequenced and analysed for the presence of ORFs. The homology of translated amino

Fig. 4. Detection of *srr-1* and *srr-2* by Southern hybridization. DIG-labelled *srr-1* PCR-amplified from strain DL700 (A) and *srr-2* from strain J48 (B) were reacted with *EcoRV*-digested chromosomal DNA from GBS strains A909, ss618, 9B200, J48, J52, PEH, COH1, COH1-13, DL700 and DL1104 (lanes 1–10); pPC185 (Kremer *et al.*, 2001) (negative control; lane 11) and PCR-amplified *srr-1* or *srr-2* (positive control; lane 12).

acid sequences was compared to that of other proteins entered into the GenBank database using the BLASTP program (<http://www.ncbi.nlm.nih.gov:80/blast/>) (Fig. 5). In strain J48, a *secY2* gene homologue is located immediately downstream of *srr-2* and is followed by *asp1-3*, *secA2*, *gtfA* and *gtfB*, gene homologues similar to those contained within the *secY2/A2* loci of other streptococcal and staphylococcal species (Takamatsu *et al.*, 2004a, b). Interestingly, the *srr-2*-containing locus of strain J48 has an organization more similar to that of staphylococci, in that the *secY2* gene lies only 113 nt downstream of the *srr-2* gene. In GBS strains NEM316 (Glaser *et al.*, 2002), A909 (Tettelin *et al.*, 2005) and 2603V/R (Tettelin *et al.*, 2002), *srr-1* and *secY2* are separated by approximately 12 kb of DNA that includes a homologue of streptococcal *nss* and four to five putative glycosyltransferase (*gly*) genes. In strain 2603V/R, a vestige of a transposase gene replaces *gly3*. In strain J48, *nss* and a single *gly* gene are located at the 3' end of the locus and lie downstream of *gtfA* and *gtfB*.

Anti- ϵ antibody reactivity with recombinant Srr-2

To determine if Srr-2 itself was recognized by the anti- ϵ antiserum, *srr-2* was PCR-amplified from strain J48 and cloned into the pBAD-TOPO expression vector. Expression of Srr-2 was induced in *E. coli* with increasing concentrations of arabinose, and cell lysates were analysed by Western immunoblotting for the presence of the recombinant protein, using a monoclonal antibody directed against the V5 epitope tag. A reactive band was detected at >250 kDa

(Fig. 6, Panel A, arrow) that was also recognized by the anti- ϵ -specific antiserum (Fig. 6, Panel B). Several smaller molecular mass bands were also reactive with the anti- ϵ reagent. These presumably represent Srr-2 breakdown products or incompletely translated recombinant protein. Minimal background reactivity was detected in the non-induced, negative control sample (Fig. 6, lane 6). The apparent molecular mass of >250 kDa is larger than the size predicted for recombinant Srr-2 (125 kDa); however, when the protein was analysed in the presence of 9 M urea, Srr-2 migrated at the predicted size (data not shown). This suggests that the protein may exist as a dimer in the absence of strongly denaturing conditions. This abnormal migration may also be due to the highly repetitive nature of the protein.

Determination of LD₉₀ for serotype III δ/ϵ -positive and δ/ϵ -negative GBS strains

A large majority (91 %) of serotype III invasive neonatal isolates are of the RDP III-3 type (Takahashi *et al.*, 1998), and Brady *et al.* (1996) found that serotype III GBS isolated from infants with GBS sepsis expressed δ/ϵ (83.3 %, $n=18$) more frequently than serotype III isolates from colonized, but healthy, newborns (58.8 %, $n=34$). LD₉₀ experiments were conducted to evaluate the lethality of *srr-2*-containing, δ/ϵ -positive, RDP III-3 GBS compared to δ/ϵ -negative, non-RDP III-3 serotype III strains using a neonatal mouse model (Rodewald *et al.*, 1992). LD₉₀ values were notably partitioned into two distinct groups (Fig. 7). RDP III-3,

Fig. 5. Schematic representation of GBS *secY2/A2* loci containing *srr-1* and *srr-2*. Sequences for GBS strains NEM316, A909 and 2603V/R were retrieved from the NCBI website (<http://www.ncbi.nlm.nih.gov>) using BLASTP. Chromosomal DNA from strain J48 was sequenced as part of this study. ORFs were identified, translated and analysed for homologies to genes within other streptococcal and staphylococcal *secY2/A2* loci using the MacVector version 8 program (Accelrys). The gene designations *srr*, *secY2*, *secA2*, *asp1-3*, *nss* and *gly* (Takamatsu *et al.*, 2004a), and *gtfA* and *gtfB* (previously *gtf* and *orf4*) (Takamatsu *et al.*, 2004b) were used to conform to those previously described within the *secY2/A2* locus of *S. gordonii* M99. The two small red arrows in the 2603V/R schematic represent the putative OrfA- and OrfB-encoded transposases of strain IS1381 (*tnp*) (Takamatsu *et al.*, 2004a).

Fig. 6. Western immunoblotting of recombinant Srr-2. Mouse anti-V5 (A) or rabbit anti- ϵ -specific (B) antibodies were reacted with cell lysates of *E. coli* harbouring pKS60 containing PCR-amplified *srr-2* from strain J48. Lanes 1–5, induction of recombinant protein expression with 10-fold decreasing amounts of L-arabinose from 2% (lane 1) to 0.0002% (lane 5); lane 6, uninduced control. Migration of 250, 150, 100, 75, 50 and 37 kDa molecular mass markers is indicated.

δ/ϵ -positive strains demonstrated LD₉₀ values between 4.6×10^2 and 8×10^3 c.f.u., while δ/ϵ -negative strains demonstrated significantly higher values between 9.8×10^6 and 6×10^8 c.f.u. ($P < 0.0005$). This striking difference in LD₉₀ values supports the hypothesis that δ/ϵ -positive RDP III-3 organisms are more virulent than other serotype III GBS strains in neonates.

DISCUSSION

In this study, a novel immunological reactivity, designated ϵ , was detected on serotype III GBS that expressed the previously identified δ antigen (Brady *et al.*, 1988). Analysis of ≥ 250 kDa material reactive with anti- ϵ antiserum,

present in a sonic extract and separated by anion-exchange chromatography, suggests a protein rich in either glycine or serine. The additional detection of rhamnose, mannose, galactose and glucose is consistent with ϵ belonging to a family of high molecular mass, serine-rich, glycoprotein adhesins described for other streptococci and staphylococci (Wu *et al.*, 1998; Wu & Fives-Taylor, 1999; Takahashi *et al.*, 2002; Bensing *et al.*, 2004; Siboo *et al.*, 2005). Examination of the sequenced genomes of three GBS strains revealed no putative glycine-rich proteins, but did demonstrate the presence of an ORF predicted to encode a protein composed of $\sim 32\%$ serine. In addition, examination of a previously described clone (Bohnsack *et al.*, 2001), identified using a probe generated by subtractive hybridization of a GBS RDP III-3 strain with an RDP III-2 strain, revealed the presence of an ORF encoding a different Srr protein. This ORF was designated *srr-2* to distinguish it from *srr-1* contained within the three known GBS genomic sequences. While similar in architecture, comparison of *srr-1* and *srr-2* showed substantial differences in their primary sequences. In addition, the number of putative glycosyltransferase genes and organization of the *secY2/A2* loci containing *srr-1* and *srr-2* were different.

Streptococcus agalactiae is known to harbour at least five mobile genetic elements, a factor presumably involved in the genomic and phenotypic plasticity of this organism (Héry-Arnaud *et al.*, 2005). Analysis of eight GBS genomes, encompassing five serotypes, has revealed a core genome composed of 1811 genes ($\sim 80\%$ of each genome) and a variable genome of at least 765 additional genes that are not present in all strains (Maione *et al.*, 2005; Tettelin *et al.*, 2005). In this age of comparative genomics, our results highlight that certain variable genes are associated with more highly virulent organisms and underscore that antigenic characterization is a useful tool, in conjunction with genetic techniques, to identify and group strains with distinguishing characteristics. Interestingly, comparison of the eight sequenced GBS genomes revealed a strain-specific locus within the serotype III GBS strain COH-1 predicted to encode SecA and SecY subunits, three glycosyltransferases and a highly repetitive serine-rich, cell-wall-anchored protein (Tettelin *et al.*, 2005). Strain COH-1 was evaluated as part of the current study and is a δ/ϵ -positive, RDP III-3, ST-17 strain.

An absolute correlation was demonstrated between δ/ϵ expression, RDP III-3 type, ST-17 clonal complex lineage and the presence of *srr-2*. A murine model of neonatal sepsis was employed to determine the LD₉₀ values for serotype III GBS strains that contain *srr-2* compared to *srr-1*. Notably, the LD₉₀ values of the former group were several orders of magnitude less than the latter group. These results indicate that, in this model system, *srr-2*-containing serotype III GBS are significantly more virulent than *srr-1*-containing organisms, and that the cell surface expression of δ/ϵ readily identifies this lineage. Results are consistent with epidemiological observations, as well as with the previous identification

Fig. 7. Comparison of LD₉₀ values. Strains DL700, DL785, 865043 and NEM316 are serotype III, δ/ϵ -negative strains, and 874391, COH1, J52 and J48 are RDP III-3 serotype III, δ/ϵ -positive strains ($P < 0.0005$ for two sample *t* test of log-transformed data).

of a high-virulence clone of serotype III GBS associated with invasive neonatal disease (Musser *et al.*, 1989) that was subsequently identified as an RDP III-3 strain (Fleming *et al.*, 2004). Several studies have reported an association of the GBS ST-17 lineage with invasive disease in neonates (Jones *et al.*, 2003; Luan *et al.*, 2005; Bisharat *et al.*, 2004), although Davies *et al.* (2004) did not detect an association of MLST type with invasive versus colonizing serotype III GBS isolates. An evaluation of MLST types among 158 isolates collected from neonates and adults strongly suggests that the ST-17 lineage is particularly well adapted to neonates (Luan *et al.*, 2004). Furthermore, Bisharat *et al.* (2004) have reported that ST-17 complex strains are hyperinvasive in neonates and represent a discrete clonal lineage that has recently arisen from a strain of bovine origin. Our results support the hypothesis that δ/ϵ -positive, RDP III-3, ST-17 complex GBS strains represent the same clonal lineage and that GBS strains within this lineage are significantly more lethal than other serotype III organisms in an animal model of neonatal sepsis. Whether pathogenesis is linked directly to the expression of *srr-2*, or other genes within the *srr-2*-containing locus, is the focus of ongoing work and will require the construction and evaluation of isogenic mutants.

The functions of the GBS Srr proteins are as yet unknown. Other streptococcal Srr proteins have been reported to function as adhesins (Wu *et al.*, 1998; Bensing & Sullam, 2002; Takahashi *et al.*, 2002). Fap1 from *Streptococcus parasanguinis* (formerly *parasanguis*) binds to saliva-coated hydroxyapatite (Wu *et al.*, 1998; Wu & Fives-Taylor, 1999), and a Fap1-like protein, SrpA of *Streptococcus cristatus*, is associated with adhesion to heterologous bacterial species from plaque biofilm (Handley *et al.*, 2005). *S. gordonii* GspB mediates binding to human platelets (Bensing & Sullam, 2002). *S. gordonii* strain DL1 contains a gene (*hsa*) with a deduced amino acid sequence similar to that reported for GspB. The encoded protein, Hsa, mediates binding to α -2,3-linked sialoglycoconjugates and bacterial haemagglutinating activity (Takahashi *et al.*, 2002). It is, as yet, unknown whether the Srr proteins of GBS also act as adhesins and, if so, whether Srr-1 and Srr-2 bind to different substrates.

The streptococcal Srr proteins that have been characterized to date are high-molecular-mass glycoproteins (Wu *et al.*, 1998; Chen *et al.*, 2002; Takahashi *et al.*, 2002; Takamatsu *et al.*, 2004a). GspB from *S. gordonii* is glycosylated with *N*-acetylglucosamine, glucose and a minor amount of *N*-acetylgalactosamine (Bensing *et al.*, 2004). Nss, Gly, GtfA and GtfB function intracellularly and are required for the correct glycosylation of GspB, although the specific enzymic functions of these proteins are unknown (Takamatsu *et al.*, 2004a, b). Homologues of genes encoding these proteins are present within the *secY2/A2* loci from both *srr-1*- and *srr-2*-containing GBS. In *S. parasanguinis*, Fap1 is associated with rhamnose, glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine (Stephenson *et al.*, 2002). Hsa, the sialic acid-binding adhesin from *S. gordonii*, has also been reported to be glycosylated, although the sugars associated

with Hsa have not been determined (Takahashi *et al.*, 2004). The cloned product of GBS, *srr-2*, was recognized by polyclonal anti- ϵ antiserum, indicating that this antigenic reactivity consists, at least in part, of a protein component. Anti- ϵ reactivity with GBS J48 whole cells, or with antigen contained within J48 cellular extracts, was destroyed by treatment with proteinase K (data not shown). In addition, reactivity was also notably diminished by treatment with sodium metaperiodate (data not shown). These results are consistent with the complete native streptococcal ϵ antigen being a glycoprotein with *srr-2* encoding the protein backbone. Like the highly glycosylated native Fap-1 protein from *S. parasanguinis* (Wu *et al.*, 1998), the GBS-derived ϵ antigen could not be visualized by conventional protein staining, while this was not the case for the recombinant product. More extensive biochemical analysis of native GBS Srr proteins, as well as mutagenesis studies, will help to clarify the molecular basis for GBS cell surface ϵ expression, whether the anti- ϵ antibodies differentiate between protein and/or carbohydrate epitopes, and the contribution of putative glucosyltransferase (GTF) and other accessory genes within the variant GBS *secY2/A2* loci to this antigenic reactivity.

Although a long-sought goal and the focus of promising research (Law *et al.*, 2005; Lindahl *et al.*, 2005; Maione *et al.*, 2005), a GBS vaccine is not yet available. A reduction in the incidence of early-onset neonatal GBS infections has been achieved by intrapartum treatment of colonized women with high-dose intravenous antibiotics (Schrag *et al.*, 2000; Puopolo *et al.*, 2005), but this approach currently does not take into consideration potential differences in relative risk associated with different lineages of GBS. As many as 30 % of pregnant women are colonized with GBS, although fewer than 1 % of colonized women give birth to infants who develop invasive GBS infections (Boyer *et al.*, 1983; Baker & Edwards, 2001). The results presented in this report have obvious clinical relevance. Serotype III organisms account for a substantial proportion of invasive GBS disease, including meningitis and early- and late-onset neonatal sepsis. As serotype III GBS that contain *srr-2* are significantly more virulent than *srr-1*-containing strains, and the *srr-2*-containing RDP III-3/ST-17 lineage can readily be identified by the cell surface expression of the antigenic marker ϵ , a rapid diagnostic test to detect ϵ on clinical isolates promises to be a useful tool to identify those patients, particularly pregnant women, who may be specifically targeted for prophylactic antibiotic therapy during labour and delivery.

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