Impact of *lgt* mutation on lipoprotein biosynthesis and *in vitro* phenotypes of *Streptococcus agalactiae*

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Although *Streptococcus agalactiae*, the group B *Streptococcus*, is a leading cause of invasive neonatal disease worldwide the molecular basis of its virulence is still poorly understood. To investigate the role of lipoproteins in the physiology and interaction of this pathogen with host cells, we generated a mutant *S. agalactiae* strain (A909ΔLgt) deficient in the Lgt enzyme and thus unable to lipidate lipoprotein precursors (pro-lipoproteins). The loss of pro-lipoprotein lipidation did not affect the viability of *S. agalactiae* or its growth in several different media, including cation-depleted media. The processing of two well-characterized lipoproteins, but not a non-lipoprotein, was clearly shown to be aberrant in A909ΔLgt. The mutant strain was shown to be more sensitive to oxidative stress *in vitro* although the molecular basis of this increased sensitivity was not apparent. The inactivation of Lgt also resulted in changes to the bacterial cell envelope, as demonstrated by reduced retention of both the group B carbohydrate and the polysaccharide capsule and a statistically significant reduction ($P=0.0079$) in A909ΔLgt adherence to human endothelial cells of fetal origin. These data confirm that failure to process lipoproteins correctly has pleiotropic effects that may be of significance to *S. agalactiae* colonization and pathogenesis.

INTRODUCTION

Streptococcus agalactiae, the group B *Streptococcus* (GBS), is a leading cause of invasive neonatal disease worldwide (Henneke & Berner, 2006; Johri *et al.* 2006). Although prenatal screening and intra-partum antibiotic prophylaxis has been successful in reducing the burden of GBS disease, significant levels of GBS disease remain. For example, in the UK its incidence is 0.72 cases per 1000 live births (Heath *et al.*, 2004). Consequently there is a need for greater understanding of the molecular basis of GBS pathogenicity in order to inform novel strategies for prophylaxis (notably vaccine development) and/or therapy in several different population groups. Such studies should be greatly facilitated by the availability of three complete and five draft genome sequences for GBS strains (Tettelin *et al.*, 2002, 2005; Glaser *et al.*, 2002).

Bacterial lipoproteins (Lpp) are an important family of proteins that are anchored to cell membranes, where they carry out diverse functions (Braun & Wu, 1994; Sutcliffe & Russell, 1995; Sutcliffe & Harrington, 2002; Hutchings

et al., 2009). Lipoprotein biosynthesis depends on the synthesis and translocation of precursor Lpp (prolipoproteins; pro-Lpp) with type II signal peptides. Following translocation, typically by the Sec pathway, pro-Lpp are lipid modified by addition of diacylglyceride to a cysteine residue within the Lpp signal peptide, a reaction carried out by the prolipoprotein diacylglyceryl transferase (Lgt; Braun & Wu, 1994; Hutchings *et al.*, 2009). The signal peptide is then removed by a lipoprotein-specific signal peptidase (Lsp; Braun & Wu, 1994; Hutchings *et al.*, 2009), leaving the lipid-modified cysteine as the N-terminus of the mature lipoprotein. These two steps are apparently sufficient for anchoring of Lpp to the extracytoplasmic face of the plasma membrane in Gram-positive bacteria (Sutcliffe & Harrington, 2002; Hutchings *et al.*, 2009).

The conserved features of Lpp signal peptides have allowed the development of bioinformatic strategies for the identification of putative Lpp encoded in bacterial genomes (Sutcliffe & Harrington, 2002; Rahman *et al.*, 2008). We have previously analysed the genomes of two GBS strains (Sutcliffe & Harrington, 2004) and determined that putative Lpp represent about 2% of the GBS proteome. Many of these putative Lpp have predicted roles that are likely to affect the ability of GBS to survive *in vivo*. For example, more than 20 solute-binding proteins for the import of diverse substrates by ABC transport systems have been identified (Sutcliffe & Harrington,

Abbreviations: GBS, group B *Streptococcus*; HUVEC-C, human umbilical vein endothelial cell line; Lgt, prolipoprotein diacylglyceryl transferase; Lpp, lipoproteins.

A supplementary figure showing the strategy for generating allelic replacement mutants of *S. agalactiae* A909 is available with the online version of this paper.

2004). In addition, some putative Lpp, including the previously characterized adhesin Lmb (Spellerberg *et al.*, 1999; Tenenbaum *et al.*, 2007), may have direct roles in host colonization. Moreover, Lpp are apparently crucial to the recognition of GBS by Toll-like receptor 2 (Henneke *et al.* 2008).

As Lpp are likely to make an important contribution to the physiology and virulence of GBS we have investigated Lpp biosynthesis in this important pathogen.

METHODS

Bacterial strains and growth media. *S. agalactiae* A909 and its derivatives were cultured aerobically in Todd–Hewitt Broth (THB; Oxoid) or anaerobically on Granada medium (Biomedics) at 37 °C. For some experiments, media with depleted metal ion content were prepared by incubating THB with Chelex-100 resin as described previously (Bray *et al.*, 2009). Antibiotics were added to growth media as appropriate to allow selection of antibiotic-resistant GBS strains.

Generation of Lgt-deficient (A909ΔLgt) and capsule-deficient (A909ΔCPSE) strains of *S. agalactiae* A909 by allelic replacement. Lgt-deficient and CPSE-deficient mutants of *S. agalactiae* A909 were generated by allelic replacement. Copies of the *lgt* and *cpsE* genes containing in-frame deletions were constructed by PCR using primers based on the A909 genome sequence (Table 1; see also Supplementary Fig. S1, available with the online version of this paper). For the *lgt* gene these primer pairs amplified 5' and 3' DNA fragments, leaving 354 bp of the central portion of the *lgt* gene unamplified. For the *cpsE* primers, these primer pairs amplified 5' and 3' DNA fragments, leaving 741 bp of the *cpsE* gene unamplified. To combine the upstream and downstream fragments, one primer of each downstream primer pair (SALGTTOM3U/CPSEBEV3U) contained a 5' sequence which was complementary to the 3' sequence of one of the upstream primers (SALGTTOM2L/CPSEBEV2L). The terminal

primers were also engineered to contain cleavage sites for *ApaI* and *SacII* restriction enzymes.

First-round PCR fragments were amplified using chromosomal A909 DNA as template and *Pfu* DNA polymerase. These DNA fragments were purified and combined to serve as the template for the second round of PCR, using only the terminal primers (SALGTTOM1U/SALGTTOM4L or CPSEBEV1U/CPSEBEV4L) in order to produce a single contiguous DNA fragment for each gene, each of which contained an in-frame deletion. Second-round PCR products were A-tailed with *Taq* polymerase and ligated into the pGEM-T cloning vector (Promega). The cloned fragments were then excised from the resulting recombinant pGEM-T vectors, using *ApaI* and *SacII*, and subcloned into pG⁺host9:ISS1 (Maguin *et al.*, 1996) to generate the plasmids pBBALgt and pBBACPSE.

Transformants containing either pBBALgt or pBBACPSE were subjected to two rounds of homologous recombination as previously described (Biswas *et al.*, 1993; Maguin *et al.*, 1996). The presence of mutant alleles in the chromosome of putative mutants was determined by amplifying the allele present (using *Pfu* DNA polymerase) and sequencing the PCR products.

Radiolabelling of lipoproteins using [¹⁴C]palmitic acid.

Radiolabelling was carried out as described by Stoll *et al.* (2005). Briefly, [¹⁴C]palmitic acid (3.32 μCi ml⁻¹; 123 kBq ml⁻¹) was added to early-exponential-phase GBS cultures and growth continued until the cultures reached late exponential phase. Cells were harvested and washed three times in PBS. Soluble cell extracts were separated on 12 % SDS-polyacrylamide gels. After Coomassie blue staining, gels were soaked in Amplify solution (Amersham) for 30 min before being dried. Dried gels were exposed to Kodak X-OMAT LS X-ray film (–80 °C, 2 weeks) before being developed.

Investigation of lipoprotein processing in *S. agalactiae* strains.

Overnight cultures of GBS strains were diluted 1 in 20 in fresh THB and grown to mid-exponential phase. Cells were harvested and cell fractions were separated as previously described (Low *et al.*, 2003). Briefly, cell pellets were washed thoroughly in PBS, resuspended in

Table 1. Oligonucleotide primers and plasmids used in this study

Primer or plasmid	Sequence (5'–3') or description	Reference
Primers*		
SALGTTOM1U	<u>GGGCCC</u> TTGATTATTATGCACCAGAGCG	
SALGTTOM2L	GGTAATTAACCCCTCCGTAAATAG	
SALGTTOM3U	CTATTTACGGAGGGTTAATTACCGAAGGGATGAGGACAGATAGC	
SALGTTOM4L	<u>CCGCGG</u> TTCTTACTAGCGATGCTGGC	
CPSEBEV1U	<u>GGGCCC</u> TTGCAATTACCTCTGTAGGG	
CPSEBEV2L	GTCAAACCTGCAGAAAAATAAAC	
CPSEBEV3U	<i>GGTTATTTTCTGCAAGTTTGACCACGGGTGCTATTATAGGTTTG</i>	
CPSEBEV4L	<u>CCGCGG</u> CTCCTAGCATCTTCTTTATCAAAG	
Plasmids		
pG ⁺ host9:ISS1	Replication-thermosensitive derivative of pWV01 containing cloned ISS1 sequence	Maguin <i>et al.</i> (1996)
pBBALgt	pG ⁺ host9:ISS1† containing in-frame deleted <i>lgt</i> gene of <i>S. agalactiae</i>	This study
pBBΔcpsE	pG ⁺ host9:ISS1† containing in-frame deleted <i>cpsE</i> gene of <i>S. agalactiae</i>	This study
pGEM-T	T-A cloning vector	Promega

*Underlined sequences represent engineered restriction sites for *ApaI* (GGGCCC) and *SacII* (CCGCGG). The italicized sequences in SALGTTOM3U and CPSEBEV3U are the reverse and complementary sequences to the SALGTTOM2L and CPSEBEV2L primer sequences, respectively, and provide the overlapping sequences for the overlap-deletion PCR strategy.

†The ISS1 sequence is completely removed during the cloning strategy.

200 µl lysozyme solution (0.2 mg ml⁻¹ in PBS) and incubated at 37 °C for 20 min. The suspensions were then vortexed with 200 mg glass beads (acid washed, 425–600 µm; Sigma) for 2 min. The beads were allowed to settle under gravity and the supernatant centrifuged (800 g, 1 min) to pellet any unlysed cells. The supernatant was recovered and centrifuged (13 000 g, 30 min, 4 °C) to generate a lower, membrane-enriched fraction and an upper phase which contained the cytoplasmic and lysozyme-released (i.e. cell-wall-associated) fractions. Fractions were analysed on 10 % SDS-polyacrylamide gels and stained with Coomassie blue or Western blotted as described by Hamilton *et al.* (2000). Blotted membranes were probed with anti-MtuA antibody (Smith *et al.*, 2003; diluted 1/1000), anti-SAG0808 antibody (Tettelin *et al.*, 2002; diluted 1/1000) or anti-Sip antibody (Brodeur *et al.*, 2000; diluted 1/500).

Sensitivity of *S. agalactiae* strains to oxidative stress and aminopterin. The sensitivity of GBS strains to challenge with hydrogen peroxide was assessed using the method of Johnston *et al.* (2004). Manganese co-factored superoxide dismutase activity was assessed by the zymographic method of Janulczyk *et al.* (2003). Aminopterin was added to mid- to late-exponential-phase GBS cultures at final concentrations of 0, 0.1, 1.0, 10 or 100 nM. The OD₆₀₀ of each culture was taken immediately pre- and post-addition. The cultures were incubated at 37 °C for a further 2 h and the OD₆₀₀ monitored. All experiments were performed in triplicate.

Quantification of streptococcal Lancefield group B carbohydrate by latex agglutination. A Streptococcal Latex Grouping kit (Oxoid) was used, following the manufacturer's instructions. Standardized aliquots of washed GBS cells were resuspended in 200 µl extraction buffer (Oxoid). After incubation at 37 °C for 10 min the extract was serially diluted and 5 µl of the dilutions was mixed with 20 µl latex bead suspension on non-absorbent card. The time taken for visible agglutination to start to occur was recorded. All experiments were performed in triplicate.

Investigation of capsular polysaccharide expression. Capsular polysaccharide in GBS strains was detected with rabbit anti-CPS (Ia) antiserum, using the semiquantitative method of Chaffin *et al.* (2000).

Gold labelling of GBS strains was carried out using the protocol of Dixon *et al.* (2001). Rabbit anti-CPS (Ia) antiserum was used as primary antibody and goat anti-rabbit IgG conjugated with 10 nm colloidal gold particles (Sigma-Aldrich; diluted 1/10 in PBS-Tween) as secondary antibody.

Adherence to and invasion of the immortalized Human Umbilical Vein Endothelial Cell cell line (HUVEC-C) by *S. agalactiae* strains. HUVEC-C cells (ATCC; CRL-1730) were grown in Medium 199 (Sigma, UK) with 10 % (v/v) fetal calf serum at 37 °C in a 5 % CO₂ atmosphere. Prior to adherence/invasion experiments being carried out, HUVEC-C cells were seeded into 12-well plates and grown to confluence (approx. 2 × 10⁵ cells per well) as determined by visual inspection using an inverted microscope. Adhesion and invasion were determined by the method of Rubens *et al.* (1992). Overnight GBS cultures were diluted into fresh THB and incubated at 37 °C to mid-exponential phase. Standardized aliquots of cells were harvested, washed three times in sterile PBS and resuspended in cell culture growth medium without antibiotics to give a cell density of 2 × 10⁵ c.f.u. ml⁻¹. Triplicate wells containing confluent HUVEC-C cells were inoculated with 2 × 10⁵ c.f.u. of bacterial cell suspensions (i.e. a 1:1 ratio of bacteria to HUVEC-C cells) in duplicate 12-well plates. The plates were centrifuged (800 g, 10 min) to bring the bacteria into contact with the HUVEC-C monolayers and then incubated for 2 h at 37 °C in a 5 % CO₂ atmosphere. The culture medium was removed from the wells and the HUVEC-C cells were washed three times with sterile PBS. To measure bacterial adhesion

plus invasion the HUVEC-C cells from the first plate were detached by the addition of 0.4 ml trypsin-EDTA solution and incubation at 37 °C for 4 min before being gently resuspended. The detached cells were vortexed for 1 min to ensure complete disruption of HUVEC-C clumps and 1.2 ml 0.025 % (w/v) Triton X-100 was added to lyse the HUVEC-C cells before further vortexing. Each suspension was serially diluted, plated in triplicate onto Todd-Hewitt agar and incubated overnight at 37 °C to determine bacterial counts. To measure bacterial invasion, 1 ml culture medium containing 5 µg penicillin G and 100 µg gentamicin was added to the wells of the duplicate 12-well plate and incubated for 2 h at 37 °C to kill any extracellular bacteria. HUVEC-C monolayers were then washed, detached, lysed and plated on agar as described above. Bacterial colony counts were used to calculate the mean number of bacteria that had invaded the HUVEC-C cells for each strain. This number was then subtracted from the mean colony counts obtained for the equivalent wells in the corresponding adherence plus invasion assay to give a mean number of bacteria that had adhered to the HUVEC-C cells for each strain. Each mean was expressed as a percentage of the original bacterial inoculum added to each HUVEC-C monolayer.

RESULTS AND DISCUSSION

Construction of an Lgt-deficient strain of GBS A909

Bioinformatic analysis of the GBS genomes identified a single candidate Lgt protein (PFAM family PF01790), annotated as SAK_0863 in the genome of strain A909, which exhibits 123/254 (48 %) amino acid sequence identity to the characterized Lgt of *Bacillus subtilis* (Leskelä *et al.*, 1999). An overlap-deletion PCR strategy was used to construct an in-frame 354 nt deletion within *lgt* and an unmarked A909 strain carrying a mutant *lgt* allele (strain A909ΔLgt) was obtained by allelic replacement (Fig. S1). The deletion within *lgt* was confirmed by amplification of an appropriately truncated PCR product from A909ΔLgt in comparison to the wild-type A909 and DNA sequencing of the truncated amplification product (data not shown). This deletion removes a section of SAK_0863 (from Gly₉₇ to Ile₂₁₄) which contains many of the conserved residues predicted to be needed for Lgt function (our unpublished observations) and should result in the production of a truncated Lgt protein with a significantly altered topology. The generation of a viable Lgt-deficient GBS mutant is consistent with previous reports indicating that loss of Lgt does not significantly affect *in vitro* viability of Gram-positive bacteria (Leskelä *et al.*, 1999; Petit *et al.*, 2001; Stoll *et al.* 2005; Hamilton *et al.*, 2006; Baumgärtner *et al.*, 2007), including GBS (Henneke *et al.*, 2008).

To verify that Lpp biosynthesis had been abrogated in A909ΔLgt, the parent and mutant strains were grown in the presence of [¹⁴C]palmitic acid. Extracted proteins were separated by SDS-PAGE and analysed by autoradiography (Fig. 1). As expected, multiple selectively radiolabelled Lpp bands, predominantly within the 25–37 kDa size range, were clearly detected in the parent strain whereas these bands were not evident in the extracts from strain

Fig. 1. Lipoprotein profiles of *S. agalactiae* A909 and A909ΔLgt. Lipoproteins in GBS strains were labelled by the addition of [14 C]palmitic acid to the growth medium. Labelled proteins were separated by SDS-PAGE and visualized by autoradiography. Lane 1, parent strain (P); lane 2, A909ΔLgt (ΔLgt). Radiolabelled palmitic acid incorporated in membrane lipids can be seen at the bottom of each lane.

A909ΔLgt. Thus, direct biochemical evidence was obtained to support the prediction that SAK_0863 is the GBS Lgt protein and that this activity had been successfully inactivated in strain A909ΔLgt. This phenotype is identical to that of a recently described GBS strain NEM316 *lgt* deletion mutant (Henneke *et al.* 2008).

Effect of Lgt inactivation on lipoprotein processing

Western blotting was used to characterize the effects of *lgt* mutation on specific lipoproteins (Fig. 2). MtsA (SAK_1556) is an experimentally verified Lpp in GBS (Bray *et al.*, 2009; Henneke *et al.*, 2008). Cellular fractions were prepared from strains A909 and A909ΔLgt and analysed by Western blotting with a polyclonal antibody that cross-reacts with MtsA (Bray *et al.*, 2009). In the parent strain a single cross-reacting band of ~34 kDa was seen in all cellular fractions (Fig. 2a, lanes 2, 4 and 6). In contrast, the failure to lipidate MtsA in strain A909ΔLgt resulted in the appearance of two cross-reacting bands in each fraction (Fig. 2a, lanes 3, 5 and 7). One of the two bands appeared to be about the same molecular mass as the mature protein observed in the parent strain ('mature-like' protein) whilst the higher molecular mass band is presumed to be a non-lipidated MtsA pro-Lpp with an uncleaved signal peptide. The 'mature-like' protein probably represents non-lipidated pro-Lpp which has been proteolytically processed by cleavage of some or all of its lipoprotein signal peptide. 'Mature-like' products from Lpp precursors have been observed previously in *lgt* mutants (Bubeck Wardenburg *et al.* 2006; Hamilton *et al.*, 2006), and in some Gram-positive bacteria Lsp can process both lipidated and unlipidated Lpp precursors (Baumgärtner *et al.*, 2007; Henneke *et al.*, 2008; Denham *et al.*, 2009). Almost identical results were obtained for the putative maturase lipoprotein SAK_0932 (Fig. 2b). In both cases additional cross-reacting bands of 50–75 kDa were seen in the membrane-enriched and cell-wall-associated

Fig. 2. Aberrant processing of lipoproteins in *S. agalactiae* A909ΔLgt. Localization of the MtsA lipoprotein (a), the putative maturase lipoprotein (b) and the non-lipoprotein Sip protein (c) was investigated by Western blotting with specific antisera. In each panel, lane 1 contains molecular mass markers and other lanes contain proteins recovered from the cytoplasmic/peptidoglycan fraction (C/P), the membrane-enriched fraction (Memb) and the whole-cell proteins (Cells) as indicated. Lanes 2, 4 and 6 contain proteins extracted from the A909 parent strain (P). Lanes 3, 5 and 7 contain proteins extracted from the A909ΔLgt mutant (M). All sample lanes contain 50 µg total protein.

fractions of the Lgt mutant but not the parent strain (Fig. 2b, lanes 3 and 5). As these bands were detected with specific antibodies in the mutant strain only it is possible that they represent aggregated forms of the pro-Lpp. There were no differences in the processing of the non-lipoprotein Sip (Brodeur *et al.*, 2000) in the A909ΔLgt mutant (Fig. 2c), confirming that aberrant processing is restricted to lipoproteins.

The data reported here for MtsA processing are generally consistent with the observations of Henneke *et al.* (2008) for the same protein (ScaA) in an Lgt-deficient mutant of GBS strain NEM316. While those authors did not observe the build-up of the higher molecular mass form of ScaA (which we attribute to the non-lipidated pro-Lpp), a cell-associated 'mature-like' ScaA protein was detected in their *lgt* mutant. Moreover ScaA with a mature N-terminus was detected in culture supernatants of the mutant, suggesting functional Lsp activity in the absence of lipidation. Henneke *et al.* (2008) also demonstrated that ScaA was further processed by a distinct signal peptidase activity and released into culture supernatants of an *lgt/lsp* double mutant. Similarly, the Eep peptidase has been shown to act on lipoprotein precursors in *Streptococcus uberis* (Denham *et al.*, 2008). In contrast to their results for ScaA, and our results for MtsA and SAK_0932, Henneke *et al.* (2008) did not detect any cell-associated Lmb in their *lgt* mutant but did show that a form of Lmb was released into culture supernatants. Evidently, inactivation of Lpp biosynthesis results in aberrant processing and possibly mislocalization of Lpp precursors, the precise nature of which can vary between different Lpp in the same strain and between the same Lpp in different strains.

Phenotypic characterization of A909ΔLgt

Strain A909ΔLgt exhibited no differences in its general growth characteristics (colony morphology; haemolytic capacity on Columbia blood agar; pigmentation on Granada medium). The fatty acid profile of the whole cells of strain A909ΔLgt was also the same as that of the parent strain (data not shown), suggesting that the failure to turn over membrane lipids into lipoproteins does not significantly affect the cell membrane composition.

Growth curves for A909ΔLgt in unbuffered THB at pH 7 and pH 5 and in media depleted of divalent cations by Chelex treatment were also not significantly different from those of the parent strain (data not shown). These results are in good agreement with the data of Henneke *et al.* (2008), who reported that deletion mutants of *lgt*, *lsp* and *lgt/lsp* in GBS strain NEM316 exhibited normal growth in nutrient-rich media and only minor impairment of growth rates in nutrient-poor or minimal media. In contrast, the growth of an Lgt mutant of *Staphylococcus aureus* was severely impaired in nutrient-poor (iron-depleted) medium (Stoll *et al.*, 2005).

Amino acids are one of the primary groups of substrates transported by putative lipoproteins in their role as solute-

binding proteins in ABC transporter systems (Sutcliffe & Harrington, 2002, 2004). If the ability to transport amino acids was impaired in the A909ΔLgt mutant, then it might be evident by a decrease in the sensitivity of the bacteria to the toxic amino acid analogue aminopterin. However, at doses of 0.5, 5 and 50 nM aminopterin no effect on growth could be seen in either strain (data not shown), suggesting that this amino acid analogue is not imported by either strain.

We assayed sensitivity of the GBS wild-type and A909ΔLgt strains to hydrogen peroxide exposure. The Lgt mutant was significantly more sensitive to oxidative stress in a time- and dose-dependent manner (Fig. 3). Further studies will be needed to establish the basis for this phenotype but it was interesting to note that zymographic analysis revealed no obvious change in the level of the superoxide dismutase activity in strain A909ΔLgt compared with the parent strain (Fig. 3, inset). Since subsequent experiments suggested that the Lgt mutant may have altered levels of encapsulation we also tested the sensitivity of a capsule-deficient mutant (A909ΔCPSE) to hydrogen peroxide exposure. There was no significant difference in the sensitivity of A909ΔCPSE and the parent strain at any of the hydrogen peroxide doses tested (data not shown).

Effect of Lgt mutation on cell envelope properties of GBS

GBS strains display both the teichoic acid-like Lancefield group B carbohydrate (GBC) and type-specific capsular

Fig. 3. Sensitivity of *S. agalactiae* A909 and A909ΔLgt to oxidative stress, as determined by challenge with hydrogen peroxide. The doses shown are the concentrations (mM) of hydrogen peroxide added to cultures. Culture samples were taken immediately post-addition ($t=0$) and after 30 min incubation ($t=30$). Significance was assessed by Student's *t*-test between the parent (P) and ΔLgt (Lgt) strains grown under the same conditions. *, $P=0.02$; **, $P=0.006$; ***, $P=0.046$. Inset: proteins extracted from the parent strain (lane 1) and the A909ΔLgt mutant (lane 2) were separated by native PAGE and superoxide dismutase activity was visualized zymographically. Each lane contains 20 μg total protein.

polysaccharides on their cell surfaces (Sutcliffe *et al.*, 2008; Cieslewicz *et al.* 2005). The principal GBC biosynthesis locus contains a putative Lpp of unknown function (Sutcliffe *et al.*, 2008). We therefore examined if Lgt mutation affected incorporation of the GBC into the cell wall of strain A909 Δ Lgt. The level of surface GBC was estimated by measuring the time taken for visible agglutination to be observed using a commercial diagnostic kit that reacts with GBC. As shown in Fig. 4, there was a statistically significant reduction in the speed at which A909 Δ Lgt cells were agglutinated, suggesting that lower amounts of the GBC are displayed on the cell surface.

To further investigate effects of Lgt mutation on the cell envelope, semiquantitative immunoblotting experiments were carried out with anti-capsular antibody. On the basis of the intensity of spots at different dilutions of bacterial cultures it appeared that there was an approximately twofold reduction in the level of type Ia capsular antigen in the A909 Δ Lgt strain compared with the parent strain (Fig. 5a). Electron microscopic analysis of the strains following immunogold labelling suggested that capsule coverage of the A909 Δ Lgt strain was patchier than in the parent strain (Fig. 5b). These data suggest a perturbation of cell envelope organization that may be an indirect consequence of the effect on GBC noted above.

Effect of Lgt mutation on GBS endothelial cell adherence and invasion *in vitro*

The complete GBS genomes encode at least two putative Lpp that may influence adhesion and cell invasion, i.e. the laminin binding protein Lmb and a paralogue (Spellerberg

Fig. 4. Group B carbohydrate (GBC) production by *S. agalactiae* A909 and A909 Δ Lgt. The ability of the *S. agalactiae* A909 (Parent) and A909 Δ Lgt (Lgt) strains to produce the GBC was evaluated by monitoring the time taken for antibody-coated latex beads to agglutinate in the presence of different dilutions of bacterial cultures. Significance was assessed by Student's *t*-test. * indicates a statistically significant difference in the time taken for agglutination to occur between the parent and Δ Lgt strains ($P < 0.001$). ^ indicates that no agglutination was visible within 15 min of application of latex beads.

Fig. 5. Polysaccharide capsule production by *S. agalactiae* A909 and A909 Δ Lgt. (a) Capsular polysaccharide production by *S. agalactiae* A909, Δ Lgt and Δ CPSE. Aliquots (3 μ l) of serial dilutions of bacterial cultures were spotted onto nitrocellulose and probed with rabbit-anti type Ia CPS antiserum to determine the level of capsule production. Column 1, neat culture (OD₆₀₀ 1.0); column 2, 1 in 2 dilution; column 3, 1 in 4 dilution; column 4, 1 in 8 dilution; column 5, 1 in 16 dilution; column 6, 1 in 32 dilution. (b) Visualization of capsule by immunogold labelling. Preparations of the three *S. agalactiae* strains were probed with rabbit anti-type Ia CPS antiserum, which was then detected using anti-rabbit IgG-conjugated gold particles. Samples were negatively stained to allow visualization by electron microscopy at 75 000 $\times$ magnification. Scale bars represent 0.2 μ m. Arrows indicate regions of the bacterial cells significantly depleted of capsule in the A909 Δ Lgt strain.

et al., 1999; Sutcliffe & Harrington, 2004; Tenenbaum *et al.*, 2007). The loss of Lgt activity in the A909 Δ Lgt strain would thus be predicted to influence adherence and/or invasion. To investigate this, the ability of GBS strains to adhere to and invade HUVEC-C cells in tissue culture was measured. Since umbilical vein endothelial cells are fetally derived they can be considered a relevant model for GBS adherence/invasion in the neonate. Although considerable inter-assay variation was observed (data not shown), the data from five biological replicates revealed that, compared to the parent strain, the A909 Δ Lgt strain exhibited a statistically significant ($P = 0.0079$) reduction in adherence

Fig. 6. Adherence to and invasion of endothelial cells by *S. agalactiae* A909 and A909ΔLgt. Adherence and invasion of HUVEC-C cells by *S. agalactiae* A909 (P) and A909ΔLgt (Lgt) were measured by the method of Rubens *et al.* (1992). The data shown represent combined results from five individual experiments. Significance was assessed by Student's *t*-test. *, $P=0.0079$.

to HUVEC-C cells (Fig. 6) but no significant difference in cell invasion. These data strongly suggest that correctly localized Lpp are required for efficient GBS adherence to endothelial cells. The absence of a significant effect on invasion is surprising given recent data suggesting that Lmb is required for invasion of human brain microvascular endothelial cells (Tenenbaum *et al.*, 2007), whilst several putative lipoproteins have been shown to be upregulated during *in vitro* growth conditions associated with high-level invasion of cervical epithelial cells (Johri *et al.*, 2007).

Concluding comments

This study has demonstrated that a viable GBS mutant in *lgt* can be generated *in vitro* and that the mutant fails to lipidate precursor Lpp (Fig. 1), resulting in their aberrant processing (Fig. 2). These data are in excellent agreement with the recent report by Henneke *et al.* (2008) of an Lgt mutant of GBS strain NEM316. Thus, the phenotype of strain A909ΔLgt would be expected to show pleiotropic changes associated with the loss or reduced efficiency of Lpp function. Surprisingly, many of the phenotypes assayed for A909ΔLgt in this study showed no significant changes compared with the parent strain. Nevertheless there were clear changes in the cell envelope organization (Figs 4 and 5) and these correlated with a statistically significant reduction in the ability of strain A909ΔLgt to adhere to cultured endothelial cells (Fig. 6). Strain A909ΔLgt also showed an increased sensitivity to oxidative stress (Fig. 3), suggesting that it may be less able to resist the reactive oxygen intermediates unleashed by host phagocytes. These data are interesting in the context of the findings of Henneke *et al.* (2008), who observed that an Lgt deletion mutant of GBS strain NEM316 exhibited a 'hypervirulent' phenotype in a subcutaneous mouse infection model of GBS sepsis. It is notable that a *Staphylococcus aureus* Lgt mutant also exhibited a 'hyper-

virulent' phenotype (Bubeck Wardenburg *et al.*, 2006). It would appear that, even though virulence traits such as adhesion and resistance to oxidative stress may be compromised by the loss of specific Lpp, the inability to lipidate Lpp results in a failure to activate immune responses, notably those of the innate immune system (Stoll *et al.*, 2005; Bubeck Wardenburg *et al.*, 2006; Henneke *et al.*, 2008). This immune evasion appears to contribute to a hypervirulence phenotype, at least with some Lgt-deficient strains in some animal models. In contrast, mutations inactivating *lgt* in other Gram-positive pathogens have not resulted in hypervirulent phenotypes and in some cases have led to attenuation (Petit *et al.*, 2001; Hamilton *et al.*, 2006; Baumgärtner *et al.*, 2007; Machata *et al.*, 2008). The data for GBS suggest that it may be prudent for future studies to focus on the roles of individual Lpp as potential virulence factors and vaccine candidates, particularly since the significant effects on adhesion reported here suggest that some Lpp may be important in neonatal colonization. However, for comparison, it would also be of interest to determine the virulence phenotypes of GBS *lsp* and *lgt/lsp* mutants such as those described by Henneke *et al.* (2008).

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