In the absence of Lgt, lipoproteins are shed from *Streptococcus uberis* independently of Lsp

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The role of lipoprotein diacylglyceryl transferase (Lgt) and lipoprotein signal peptidase (Lsp) responsible for processing lipoproteins was investigated in *Streptococcus uberis*, a common cause of bovine mastitis. In the absence of Lgt, three lipoproteins [MtuA (SUB0473), Hap (SUB1625) and an extracellular solute-binding protein (SUB0365)] were detected in extracellular locations. All were shown by Edman degradation analysis to be cleaved on the carboxy side of the LXXC lipobox. Detection of MtuA, a lipoprotein shown previously to be essential for infectivity and virulence, was used as a surrogate lipoprotein marker to locate and assess processing of lipoproteins. The absence of Lgt did not prevent location of MtuA to the cell membrane, its location in the wild-type strain but, in contrast to the situation with wild-type, did result in a widespread location of this protein. In the absence of both Lgt and Lsp, MtuA was similarly released from the bacterial cell. In such strains, however, the cell-associated MtuA represented the full-length gene product, indicating that Lsp was able to cleave non-lipidated (lipo)proteins but was not responsible for their release from this bacterium.

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INTRODUCTION

Lipoproteins are a major class of membrane-bound proteins that were first identified in *Escherichia coli*, but have since been shown in many pathogenic bacteria including *Staphylococcus aureus*, *Mycobacterium tuberculosis* and streptococcal species (Hamilton *et al.*, 2006; Leskela *et al.*, 1999; Petit *et al.*, 2001; Stoll *et al.*, 2005; Sutcliffe & Harrington, 2004; Sutcliffe & Russell, 1995). Lipoproteins perform critical roles within bacteria; these include facilitating nutrient uptake (Janulczyk *et al.*, 2003), mediating antibiotic resistance (Sutcliffe & Russell, 1995), protein folding and the processing of extracytoplasmic proteins emerging from the cell (Kontinen *et al.*, 1991; Wahlstrom *et al.*, 2003). In *Streptococcus uberis*, a common cause of bovine mastitis worldwide, the lipoprotein MtuA was shown to be essential for active transport of Mn²⁺, growth in milk and virulence in the target species (Smith *et al.*, 2003).

Lipoproteins are synthesized with an N-terminal signal sequence that directs the protein into the lipoprotein-processing pathway. The lipobox motif (LXXC) contained within the C-terminal region of the signal peptide (Sutcliffe & Harrington, 2002; von Heijne, 1989) acts as a target for lipoprotein diacylglyceryl transferase (Lgt). This enzyme effects lipid modification and consequent anchoring of the lipoprotein to the cytoplasmic membrane (Hantke &

Braun, 1973; Leskela *et al.*, 1999). In a number of bacteria, it has been demonstrated that lipid modification of the cysteine residue of the lipobox is a prerequisite for cleavage of the signal peptide by the lipoprotein signal peptidase (Lsp) (Hussain *et al.*, 1982; Tjalsma *et al.*, 1999a; Tokunaga & Wu, 1984).

In a study using the signal peptide region of Braun's lipoprotein from *E. coli*, it was revealed that Lgt used phosphatidylglycerol to anchor the protein to the membrane while other, similar, molecules such as phosphatidylethanolamine or cardiolipin were not suitable substrates for this enzyme (Sankaran & Wu, 1994). Lgt transfers the diacylglycerol moiety from phosphatidylglycerol to the thiol group of the cysteine residue with the formation of *N*-acyl-*S*-diacylglyceryl cysteine. Once lipidation has occurred, Lsp recognizes the modified cysteine residue and cleaves the signal peptide sequence, leaving the lipid-modified cysteine residue as the new N terminus of the protein (Hussain *et al.*, 1982).

Lgt is ubiquitous across bacterial species. The biosynthetic anchoring pathway of lipoproteins in Gram-negative bacteria has been elucidated in considerable detail (Braun & Hantke, 1975; Chattopadhyay & Wu, 1977; Dev & Ray, 1984; Hantke & Braun, 1973; Hussain *et al.*, 1982; Innis *et al.*, 1984; Inukai *et al.*, 1984; Lee *et al.*, 1983; Tokunaga & Wu, 1984; Tokunaga *et al.*, 1982, 1985; Yamagata, 1983;

Yamagata *et al.*, 1982; Yamaguchi *et al.*, 1988). In Gram-positive bacteria, Lgt was first reported in *Staph. aureus*, where the gene encoding Lgt was identified by complementation of a temperature-sensitive defect in an *lgt* mutant of *E. coli* (Qi *et al.*, 1995). A high level of similarity was found between the amino acid sequences of Lgt from *E. coli*, *Salmonella typhimurium*, *Haemophilus influenzae* and *Staph. aureus* (Qi *et al.*, 1995).

The functional role of Lgt has also been studied in *Bacillus subtilis* (Antelmann *et al.*, 2001; Leskela *et al.*, 1999), where, unlike the situation in Gram-negative species, a mutant lacking Lgt remained fully viable (Gan *et al.*, 1993; Leskela *et al.*, 1999; Yamagata *et al.*, 1982). A major lipoprotein of *B. subtilis*, PrsA, was shown to be larger in the absence of Lgt activity, suggesting the presence of an intact signal peptide sequence. These findings provided evidence that the pathway for lipoprotein processing in some Gram-positive species of bacteria may be similar to that detected in Gram-negative bacteria, in that lipidation was a prerequisite for removal of the signal peptide by Lsp.

In *B. subtilis*, lipoproteins have been shown to be shaved from the cell surface by cleavage on the carboxy side of the LXXC motif (Antelmann *et al.*, 2001). In *Staph. aureus*, the absence of Lgt resulted in the release of several (lipo)proteins (SitC, PrsA and OppA) (Stoll *et al.*, 2005). In each case, the released proteins were processed to a size similar to that of the mature, fully processed (lipo)protein, whereas cell-associated lipoproteins expressed in the absence of Lgt appeared to retain their signal sequence. In *Listeria monocytogenes*, the absence of Lgt resulted in the release of mature (lipo)proteins from the cell. In this strain, treatment with the Lsp inhibitor globomycin prevented both (lipo)protein cleavage and release, implying that Lsp was able to cleave non-lipidated (lipo)proteins and that such cleavage was a prerequisite for their release.

Lgt homologues are present in all streptococcal species sequenced to date, and lipoproteins represent an abundant group of streptococcal proteins with a wide range of functions (Sutcliffe & Russell, 1995). Interestingly, disruption of Lgt-prelipoprotein processing appears to influence the localization of lipoproteins and consequent pathogenicity of Gram-positive pathogens in a variety of ways (Henneke *et al.*, 2008). A *Streptococcus pneumoniae* *lgt* mutant was shown to be viable, but exhibited reduced virulence (Petit *et al.*, 2001). The pathogenicity of an *lgt* mutant in *Streptococcus equi* was reduced but not eliminated, indicating that it is not an absolute requirement for virulence in a challenge model in the horse (Hamilton *et al.*, 2006). The lipoprotein MtuA from *Strep. uberis* was previously shown to be essential for disease within dairy cattle (Smith *et al.*, 2003) and provided a useful marker with which to help dissect the role of Lgt in the pathogenesis of *Strep. uberis*.

This communication describes the use of isogenic mutants lacking Lgt and, for the first time, Lsp and Lgt to elucidate the processing of lipoprotein. In so doing, it adds to the

understanding of lipoprotein processing in Gram-positive bacteria.

METHODS

Bacterial growth conditions and oligonucleotide primers. *Strep. uberis* was routinely grown on Todd-Hewitt agar or sheep blood aesculin agar (SEA) plates and in Todd-Hewitt broth (THB) (Oxoid) as standing cultures at 37 °C. Uncured pGh9⁺::ISS1 mutants were grown in the same media with the addition of 1 µg erythromycin ml⁻¹ at 37 °C. Globomycin was added to cultures at an OD₆₀₀ of 0.1. Oligonucleotide primers P348 (5'-AGGACGAAATGTTTCAGTTG) and P349 (5'-AGATAACCAGTGCCACAAAG) were used to amplify the *lgt* locus and P432 (5'-GCTCTTCGGATTTTCGGTATC) and P571 (5'-AATATCTTCAGCTTCATAATCC) were used to amplify the *lsp* locus of *Strep. uberis*. Oligonucleotide primers P358 (5'-CATTTCCACGAATAGAAGGACTGTC) and P247 (5'-GCTCTTCGGATTTTCGGTATC) were used to screen pGh9::ISS1 mutant banks, as described below, and a DIG-labelled ISS1 probe was used for Southern blotting as described previously (Ward *et al.*, 2001).

Preparation of chromosomal DNA from *Strep. uberis*. Chromosomal DNA from 3 ml culture was prepared by using a variation of the method of Hill & Leigh (1989) and is described in Denham *et al.* (2008).

Southern blotting to determine location and random distribution of pGh9⁺::ISS1 insertion. Southern blot analysis was performed upon genomic DNA digested with *Eco*RI or *Hind*III. Hybridization of the digoxigenin-dUTP (DIG-dUTP; Roche Diagnostics) labelled probe was carried out overnight at 65 °C. Hybridizing fragments were visualized with the chemiluminescent substrate NBT/BCIP (Roche Diagnostics) as instructed by the manufacturer.

Isolation of mutants from a *Strep. uberis* strain 0140J pGh9⁺::ISS1 mutant bank. The mutants were isolated as described in Denham *et al.* (2008) using the specific oligonucleotide primers described earlier.

Random mutagenesis in *lsp* mutant background of *Strep. uberis*. The procedures described previously by Maguin *et al.* (1996) and Smith *et al.* (2003) were used to generate a bank of random mutants within the *lsp* mutant of *Strep. uberis* using the pGh9::ISS1 vector. Mutants from this bank were isolated in the same manner as described earlier for the isolation of mutants from the *Strep. uberis* strain 0140J pGh9::ISS1 mutant bank.

Detection of MtuA with whole-cell ELISA. Bacteria were grown to mid-exponential phase and the suspensions were adjusted to an OD₅₅₀ of 0.3 with growth medium (THB). Suspensions were mixed with equal volumes of coating buffer (carbonate buffer 0.05 M, pH 9.6) and applied in triplicate to the wells of a 96-well MaxiSorp plate (Nunc). Antigen-negative controls contained only coating buffer in the wells. The plate was incubated overnight in a humid environment at 4 °C to promote binding. Wells were washed three times with ELISA wash buffer [PBS containing 0.1 % (w/v) Tween; PBST] and blocking solution [PBST containing 1 % (w/v) Marvel] was added (200 µl per well) and the plate was then incubated for 90 min at room temperature. Wells were washed again prior to addition of MtuA antisera (diluted 1 in 1000 in blocking solution, 100 µl per well) and incubated for 90 min at room temperature. Wells were washed as described above to remove unbound antibody and then incubated for 1 h with anti-rabbit HRP conjugate antibody (Sigma) (diluted 1 in 1000 in blocking solution). Antibodies were detected

using TMB reagent [0.1 ml TMB dissolved in DMSO and 0.03 % (v/v) H₂O₂ in 10 ml TMB substrate buffer (0.1 M sodium acetate, pH 6.0), 100 µl per well]. After approximately 3 min, 1 M H₂SO₄ was added (100 µl per well) to stop further colour generation. Absorbance readings were taken at 450 nm (Anthos 2001 plate reader; Anthos Labtec Instruments).

Preparation of cleared whole-cell lysates and subcellular fractions. Whole-cell lysates and subcellular fractions (capsule, cell wall, cell membrane and cell contents) were prepared as described previously (Denham *et al.*, 2008).

Preparation of proteins from bacterial growth media by TCA precipitation. Bacteria were grown to an OD₅₅₀ of approximately 0.5. Protease inhibitors were added to a 1 × concentration from a 25 × stock of Complete EDTA-free protease inhibitor (Roche Diagnostics). Bacteria were harvested by centrifugation (10 000 g, 10 min). The culture supernatants (approx. 45 ml) were transferred to a fresh centrifuge vessel before being centrifuged as described previously to remove residual bacteria. The culture supernatants were filtered through a 0.22 µm filter into a fresh centrifuge vessel and placed on ice. Trichloroacetic acid (TCA) was added (10 % v/v from a 100 % w/v stock solution) and mixed thoroughly. Proteins were precipitated on ice for 30 min before being harvested by centrifugation (13 000 g, 30 min, 4 °C). The supernatant was discarded and the precipitate was washed with 5 ml ice-cold acetone. The precipitate was recovered by centrifugation as described previously, before being washed and spun again to remove traces of TCA. The remaining precipitate was air-dried thoroughly before being resuspended in either SDS-PAGE loading buffer (pH 8.0) or Tris/HCl (pH 8.0) and frozen at -20 °C.

Quantification of protein within cell fractions. The protein content of cell fractions was determined using the BCA Protein assay kit (Pierce) as directed by the manufacturer. Protein concentrations were calculated from mean values of triplicate readings for each sample using a standard curve prepared using BSA.

Detection of MtuA within cell fractions prepared from *Strep. uberis*. The proteins present in cell fractions (1 µg) were separated by SDS-PAGE on 12.5 % gels or 10 % Bistris gels (Invitrogen) and detected by Western blotting using MtuA antiserum (Jones *et al.*, 2004) at a concentration of 1:2500 and a secondary goat anti-rabbit-HRP conjugate (Sigma) at a concentration of 1:2500.

RESULTS

The *lgt* locus in the *Strep. uberis* strain 0140J genome

A gene (SUB0578) corresponding to *lgt* was identified within the genome of *Strep. uberis* strain 0140J by similarity searches (BLASTN; Altschul *et al.*, 1990) with functionally characterized *lgt* sequences from *B. subtilis* (GenBank accession no. AAC67287; amino acid sequence identity 46 %), *E. coli* (NP417305; 30 %) and *Strep. pneumoniae* (YP_816710; 65 %). The ORF encoding Lgt in *Strep. uberis* was predicted to generate a product of 259 amino acids and, as in *Staph. aureus* (Stoll *et al.*, 2005), was flanked upstream by an ORF (SUB0577) with homology to *hprK* [an ATP-dependent, metabolite-activated protein kinase/phosphorylase (Reizer *et al.*, 1998)] and downstream by an ORF (SUB0579) encoding an unknown hypothetical protein (data not shown).

Isolation of mutants carrying lesions within the *lgt* locus of *Strep. uberis* strain 0140J

Mutants carrying insertions in *lgt* were isolated using a genotypic selection procedure from a bank of approximately 8800 random insertion mutants (Ward *et al.*, 2001). Three mutants were identified with insertions in the region of the *lgt* locus. Sequence analysis revealed that in two mutants the insertion mapped within SUB0578 (*Strep. uberis lgt::ISSI₁* had an insertion 416 bp from the start codon and *Strep. uberis lgt::ISSI₂* had an insertion 729 bp from the start codon). A further mutant (*lgt::ISSI₃*) carried an insertion that mapped outside the ORF, 13 bp after the stop codon. The insertion in *Strep. uberis lgt::ISSI₁* was predicted to disrupt the Lgt signature sequence (PS01311) described in Prosite (Hulo *et al.*, 2006).

Characterization of the extracellular protein profile of the *lgt* mutants

To determine whether a mutation in the *lgt* gene of *Strep. uberis* had any effect on the extracellular protein profile, capsule fractions were prepared from mid-exponential phase cultures of the wild-type strain and the three mutants (*lgt::ISSI₁*, *lgt::ISSI₂* and *lgt::ISSI₃*). Several proteins that were seen in capsule extracts from *lgt::ISSI₁* were not evident in the wild-type strain, *lgt::ISSI₂* or *lgt::ISSI₃* (Fig. 1). These proteins were immobilized on PVDF membrane and subjected to Edman degradation analysis. Three proteins, including MtuA, were identified (Table 1). These corresponded to predicted lipoproteins described by Denham *et al.* (2008). The N-terminal residue of each protein corresponded to the amino acid carboxyl to the cysteine residue of the lipobox (LXXC) motif, a site distinct from that predicted for the cleavage of lipoproteins by Lsp.

Cell-surface display of MtuA on wild-type and *lgt* mutants

Whole-cell ELISA was able to detect MtuA at similar, low levels on the wild-type, *lgt::ISSI₂* and *lgt::ISSI₃*. Mutant *lgt::ISSI₁* displayed a considerably higher level of this protein on its surface (Fig. 2). This observation, coupled with the different extracytoplasmic protein profile shown for *lgt::ISSI₁* (Fig. 1), suggested that *lgt::ISSI₁*, unlike the wild-type, was unable to anchor lipoproteins conventionally to the membrane. Furthermore, the similarity between the findings for *lgt::ISSI₂*, *lgt::ISSI₃* and the wild-type suggested that the phenotype observed for *lgt::ISSI₁* was not due to polar effects on ORFs located downstream of *lgt*.

The (lipo)protein MtuA is found in all cell fractions from *lgt::ISSI₁*

Immunoblotting of cell fractions with anti-MtuA revealed a single protein of approximately 37 kDa in the membrane

Fig. 1. SDS-PAGE protein profiles of capsule preparations from wild-type and Lgt mutant *Strep. uberis*. Capsule fractions were prepared from mid-exponential phase cultures of wild-type (lane 2) and three *lgt* mutants (*ISS1::lgt₁₋₃*) (lanes 3–5). Lane 1, molecular mass marker proteins with sizes indicated in kDa. Proteins were separated by SDS-PAGE and stained with Simply Blue SafeStain reagent (Invitrogen). At least six additional protein bands were detected in *lgt::ISS1₁* (marked with asterisks 1–6). Three of these were identified by N-terminal sequencing as hyaluronate-associated protein SUB1625 (*2), manganese transporter *uberis* (MtuA) SUB0473 (*4) and an extracellular solute-binding protein SUB0365 (*6).

fraction of the wild-type strain (as shown previously; Jones *et al.*, 2004). In contrast, strain *lgt::ISS1₁* displayed a similar-sized protein in the capsule, cell wall, membrane, cytoplasm and culture supernatant fractions (Fig. 3).

Processing of MtuA is carried out by Lsp in the absence of Lgt, but release of (lipo)proteins occurs independently of Lsp

The three additional (lipo)proteins detected in the extracellular fraction of *lgt::ISS1₁* (Fig. 1) were atypically

Fig. 2. Detection of MtuA on wild-type and *lgt* mutants by whole-cell ELISA. Bacteria were grown to mid-exponential phase and the suspensions were adjusted to OD₅₅₀ 0.3 with THB. Anti-MtuA (Jones *et al.*, 2004) was used as primary antibody to detect the presence of MtuA on the surface of whole cells. MtuA was found on the surface of *lgt::ISS1₁* cells only. Error bars represent SD from assays performed in triplicate.

processed, as cleavage of the signal peptide was shown to have occurred on the carboxyl side of the cysteine residue of the lipobox motif. This suggested the presence of an activity other than Lsp that was able to cleave the signal peptides from (lipo)proteins.

Immunoblotting of whole-cell lysates of *lgt::ISS1₁* prepared from cultures growing exponentially in the presence of globomycin (an inhibitor of Lsp) revealed an additional MtuA protein at a size comparable to the full-length product of *mtuA* (Fig. 4), indicating that Lsp was involved directly or indirectly in cleavage of unlipidated MtuA. In order to substantiate this further, an *lgt/lsp* double mutant with an *ISS1* insertion within *lgt* (546 bp from the start codon) was isolated from a random mutant bank created within a *Strep. uberis* 0140J *lsp::ISS1* background (Denham *et al.*, 2008) and characterized with regard to its lipoprotein-processing properties.

Immunoblotting of cell-associated fractions (cell wall, membrane and cytoplasm) from a mid-exponential phase culture of the *lgt/lsp* double mutant revealed an additional MtuA protein band of a molecular mass comparable to the full-length, uncleaved protein, as shown previously in the presence of this mutation within *lsp* (Denham *et al.*, 2008) (Fig. 5a). However, immunoblotting of extracellular fractions [capsule (Fig. 5a) and culture supernatant (Fig. 5b)] revealed a single protein band comparable in size to

Table 1. Sequence of proteins identified by N-terminal sequencing

Lipobox residues are shown in bold and the N-terminal sequence derived from *lgt::ISS1₁* is underlined.

ORF assignment	Predicted lipoprotein leader sequence
SUB1625. Putative threonine kinase (hyaluronate-associated protein) or OppA1 (Sutcliffe & Harrington, 2002)	MTVAQKSTFKRFLGAVTLASAALL MAC <u>G</u> NKTAAKND
SUB0473. MtuA	MKKKLSLAIMAFLGLL MLGAC <u>S</u> VGNGRKAT
SUB0365. Putative bacterial extracellular solute-binding protein	MMTLKKNLGI SL TLGTLAIL AA C <u>G</u> NKASNNNG

Fig. 3. Immunoblots showing the presence of MtuA within different subcellular fractions prepared from wild-type and the *lgt* mutant strain. (a) Lanes: 1, molecular mass marker (kDa); 2–5, wild-type capsule, cell wall, membrane and cell contents, respectively; 6–9, *lgt* mutant capsule, cell wall, membrane and cell contents, respectively. (b) Immunoblot of proteins prepared from the supernatant of mid-exponential phase *Strep. uberis* wild-type (lane 1) and *lgt* mutant (lane 2). Lane 3 is a membrane fraction prepared from the wild-type. Molecular masses of markers are shown in kDa.

MtuA in all fractions from the single *lgt* mutant, indicating that MtuA was released in the absence of Lsp and that the released protein was cleaved similarly to that released in the presence of Lsp.

DISCUSSION

Lipid modification of the cysteine residue present within the lipobox of lipoproteins by Lgt has been shown to be essential for anchoring such proteins to the membrane of bacteria (Tjalsma *et al.*, 1999a; Tokunaga & Wu, 1984). In Gram-negative bacteria, mutations in *lgt* are lethal, while in Gram-positive bacteria, mutation of *lgt* has resulted in viable bacterial strains displaying variously altered phenotypes (Baumgartner *et al.*, 2007; Hamilton *et al.*, 2006; Petit *et al.*, 2001; Stoll *et al.*, 2005). Lgt in *Strep. uberis* was predicted to have seven transmembrane domains. The insertion in *lgt::ISSI₁* maps immediately after the fourth

transmembrane domain and in the Prosite motif PS01311. The conserved amino acids within this motif are likely to play a role in either the catalysis of the diglyceride bond or the stability of the enzyme (Sankaran & Wu, 1994). Therefore, any truncated Lgt from *lgt::ISSI₁* would not be predicted to be functional. The mutations in *lgt::ISSI₂* and *lgt::ISSI₃* mapped 729 bp from the start codon and 13 bp after the stop codon, respectively. No difference was detected in the (lipo)protein distribution between *lgt::ISSI₂* and the wild-type strain, indicating that disruption of *lgt* at this point has no major effect on the activity of Lgt. The last transmembrane domain is predicted to terminate at a sequence corresponding to 741 bp from the start codon. A mutant strain of *B. subtilis* selected for defects in the KinB-dependent sporulation pathway (Dartois *et al.*, 1997) was shown to carry a mutation in *lgt* that was located at a position upstream of the lesion present in *lgt::ISSI₂*, suggesting a discrepancy between the essential regions of Lgt in these two bacteria.

Fig. 4. Immunoblot of whole-cell lysates from mid-exponential phase cells. Samples were prepared from whole washed cells grown with/without globomycin, separated by SDS-PAGE and immunoblotted using anti-MtuA. Lanes: 1, wild-type; 2, wild-type plus globomycin; 3, *lgt* mutant; 4, *lgt* mutant plus globomycin. Molecular masses of markers are shown in kDa.

Analysis of the proteins found within the capsule fractions from the three *Strep. uberis* *lgt::ISSI* mutants revealed at least six additional proteins in *lgt::ISSI₁* that were not present either in the wild-type or in the other *lgt* mutants. Three of these were identified as putative lipoproteins and, in each case, the N terminus was shown to be the amino acid following the conserved cysteine of the lipobox. Lipid modification of cysteine within the lipobox motif by Lgt has been shown to be a prerequisite for the cleavage of the signal peptide from lipoproteins by Lsp in *E. coli* (Tokunaga & Wu, 1984) and has been implied in *Staph. aureus* (Sibbald *et al.*, 2006). However, cleavage of non-lipidated (lipo)proteins has been implied through the use of the selective inhibitor globomycin in *L. monocytogenes* (Baumgartner *et al.*, 2007). Interestingly, in the extracellular proteome of wild-type *B. subtilis*, seven lipoproteins were shed into the extracellular medium and six of these were cleaved after LXXC (Antelmann *et al.*, 2001) in a manner similar to those identified from *lgt::ISSI₁* in the present study. The data presented here suggest the presence of an enzyme capable of shaving lipoproteins from the

Fig. 5. Immunoblots of subcellular fractions of wild-type and the *lgt/lsp* double mutant using anti-MtuA. Samples were prepared using the subcellular fractionation procedure and immunoblotted using anti-MtuA to visualize differently processed forms of MtuA protein. (a) Lanes: 1–4, wild-type capsule, cell wall, membrane and cell contents, respectively; 5–8, *lgt/lsp* mutant capsule, cell wall, membrane and cell contents, respectively; 9, *lgt* capsule. Arrow 1 indicates the size of MtuA processed in the wild-type, *lgt* mutant and *lgt/lsp* mutants and arrow 2 indicates unprocessed MtuA detected in the *lsp* and *lgt/lsp* mutants. (b) Proteins from the supernatant prepared from mid-exponential-phase *Strep. uberis* wild-type (lane 1), *lgt* mutant (lane 2) and *lgt/lsp* mutant (lane 3). Membrane from the wild-type strain was included as a control (lane 4). MtuA was found in the supernatant of the two lipoprotein-processing mutants. Molecular masses of markers are shown (kDa).

surface of Gram-positive bacteria, the activity of which is only apparent in *Strep. uberis* in the absence of Lgt.

In the absence of Lgt, MtuA was mislocated, and it could be envisaged that the non-lipidated protein might be positioned incorrectly with respect to accessory proteins (MtuB/C), thereby precluding active transport of Mn^{2+} . Mutation within *mtuA/B/C* resulted in a strain that was unable to grow in bovine milk, a medium in which Mn^{2+} is restricted (C. L. Jones and J. A. Leigh, unpublished). However, in the absence of Lgt, *Strep. uberis* was able to grow in milk at a rate and final cell yield similar to that of the wild-type, indicating that sufficient functional MtuA was present to acquire Mn^{2+} at a rate not limiting to growth. Mutation of the *Strep. pneumoniae lgt* gene produced bacteria that were 5-fold reduced in their ability to cause infection (Petit *et al.*, 2001). An *lgt* mutant and a maturase lipoprotein mutant have both been studied in *Strep. equi*, where it was found that the maturase mutant was less virulent than both the wild-type and *lgt* mutant (Hamilton *et al.*, 2006). These data indicate that, while elimination of Lgt can constrain the pathogenesis of some Gram-positive pathogens, mutation of essential lipoproteins processed by Lgt can have a greater effect on the virulence of the pathogen. However, *lgt* mutants of *Staph. aureus* (Bubeck Wardenburg *et al.*, 2006) and *Streptococcus agalactiae* (Henneke *et al.*, 2008) were both hypervirulent within animal infection models through escaping detection by the host's innate immune system. Lipoproteins are therefore of high relevance to the host in mounting an effective immune responses to these two pathogens (Bubeck Wardenburg *et al.*, 2006; Henneke *et al.*, 2008). Contrastingly, while the lipoproteins of *L. monocytogenes* constitute immunological targets, an *lgt* mutant was found

to be avirulent (Machata *et al.*, 2008). A reduced Lgt mutant bacterial load within the spleen and liver was identified under these circumstances, suggesting the essential nature of Lgt-processed proteins in enabling the establishment of *L. monocytogenes* infections (Machata *et al.*, 2008).

In contrast to the situation in the wild-type, in *lgt::ISS1*, MtuA was detected in all fractions of the bacterium (cell contents, cell wall, cell membrane, capsule and culture supernatant). Full-length MtuA was not detected, demonstrating that MtuA [and probably other (lipo)proteins] was cleaved despite the absence of a lipid anchor. MtuA was detected in the cell contents of the *lgt* mutant in a processed form. It is important to note that the cell-content fractions were prepared from disrupted protoplasts, i.e. a mixture of membrane and cytosolic contents. The lipoproteins in the Lgt mutants are not anchored and are therefore free to move into the cytoplasmic fraction (Figs 3 and 5a). The cytoplasmic cell fractions are likely to be enriched for proteins from that specific location and those loosely associated with the membrane, but it is highly unlikely that they will be contaminated by proteins from other locations, the fractions for which were removed prior to disruption of the protoplasts. In an *lsp* mutant of *Strep. uberis*, alternative processing of MtuA was shown to be carried out by Eep (Denham *et al.*, 2008). Eep activity was not detectable in the *lgt* mutant background reported in this study.

The localization of lipoproteins against backgrounds of *lgt* or *lsp* mutations has been shown to be altered in other bacterial species including *B. subtilis* (Antelmann *et al.*, 2001; Tjalsma *et al.*, 1999a, b), *Strep. agalactiae* (Henneke

et al., 2008), *L. monocytogenes* (Baumgartner *et al.*, 2007) and *Staph. aureus* (Bubeck Wardenburg *et al.*, 2006; Stoll *et al.*, 2005). Lipoproteins in wild-type bacteria are in general anchored to the membrane. In *B. subtilis* and *Staph. aureus*, several lipoproteins have been found in the extracellular space as a result of alternative processing of the signal peptide (Antelmann *et al.*, 2001; Sibbald *et al.*, 2006). Inactivation of *lsp* has not been shown to affect the location of lipoproteins. Furthermore, immunoblotting of cell fractions has shown that lipoproteins remain localized within the membrane under such circumstances, but alternatively processed forms of lipoproteins have been identified (Denham *et al.*, 2008; Tjalsma *et al.*, 1999a). These alternative forms are likely to contain varying amounts of the signal peptide. In *Strep. uberis*, full-length lipoprotein was detected along with an alternatively processed form, where Eep was shown to be responsible for the cleavage event at a site approximately 8 amino acids away from the Lsp cleavage site within the signal peptide (Denham *et al.*, 2008). When *lgt* is inactivated, the location of at least some lipoproteins is altered. This alteration of location can vary for different lipoproteins in an *Lgt*[−] background; for example, in *Strep. agalactiae*, the location of Lmb is the supernatant, whereas ScaA is released in part to the supernatant but also appears retained within the bacteria. This is also the case in an *lgt/lsp* double mutant (Henneke *et al.*, 2008).

Evidence collected during analysis of extracts from *lgt*: :ISS1₁ grown in the presence of globomycin and from a strain of *Strep. uberis* 0140J lacking both Lsp and Lgt indicated that Lsp was able to remove the signal peptide from cell-associated (lipo)proteins in the absence of Lgt. The N-terminal sequence data from the *lgt* mutant suggested that the lipoproteins were processed on the C-terminal side of the cysteine residue of the lipobox. While it is conceivable that conventionally Lsp-cleaved forms of the proteins were also present in the capsule fraction, the relative abundance of the alternatively processed form was reflected in N-terminal sequence data. Cleavage of non-lipidated (lipo)proteins by Lsp of *L. monocytogenes* was implied by inhibition by globomycin (Baumgartner *et al.*, 2007). However, in contrast to the situation in the present study with *Strep. uberis*, addition of globomycin to cultures of the *lgt* mutant of *L. monocytogenes* also prevented release of (lipo)proteins into the supernatant fraction. Consequently, it was hypothesized that the presence of a hydrophobic signal peptide (removed by Lsp and retained in the presence of globomycin) prevented the release of (lipo)proteins into the supernatant. This appeared not to be the case in our study of *Strep. uberis*, where the *lgt/lsp* double mutant was capable of (lipo)protein release (Fig. 5b).

The data presented here show clearly that, in *Strep. uberis*, similar to other bacteria, Lgt and Lsp act to process lipoproteins; like the situation in *L. monocytogenes*, Lsp can cleave non-lipidated substrates, but, in contrast, Lsp from *Strep. uberis* is not required for release or shaving of such proteins from the bacterium. The observations on

lipoprotein processing have indicated the pitfalls of extrapolating conclusions from one species to bacteria in general. The demonstration in *E. coli* that lipidation was a prerequisite for signal peptide cleavage has not proved to be universal. Similarly, the observation that Lsp acts as the release or shaving activity in *L. monocytogenes* does not appear to be true for *Strep. uberis*, where the shaving factor remains unidentified.

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