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

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The role of lipoprotein diacylglycerol 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 (lip)proteins but was not responsible for their release from this bacterium.

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 diacylglycerol 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 and 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-diacylglycerol 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).

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., 2001). 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).

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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 ascuclin 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′-AGGACGAATTGTTCCAGTGTG) and P349 (5′-AGATAACCAGTGCCACAAAG) were used to amplify the lgt locus and P432 (5′-GCTCTTGGATTTTCGATTC) and P571 (5′-AATATCTTACGTCCTATAATCC) were used to amplify the lsp locus of Strep. uberis. Oligonucleotide primers P358 (5′-CATTTCACGAATTAGGACTGTC) and P247 (5′-GCTCTCGGATTTCGATTC) 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 EcoRI or HindIII. 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 Maquin 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 OD600 of 0.3 with growth medium (THB). Suspensions were mixed at room temperature. Wells were washed again prior to addition of anti-MtuA antisera (diluted 1 in 1000 in blocking solution, 100 µl per well) and the plate was then 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) H$_2$O$_2$ in 10 ml TMB substrate buffer (0.1 M sodium acetate, pH 6.0), 100 μl per well]. After approximately 3 min, 1 M H$_2$SO$_4$ 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 OD$_550$ 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 (Perbio) 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::ISS1 had an insertion 416 bp from the start codon and *Strep. uberis* lgt::ISS2 had an insertion 729 bp from the start codon). A further mutant (lgt::ISS3) carried an insertion that mapped outside the ORF, 13 bp after the stop codon. The insertion in *Strep. uberis* lgt::ISS1 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::ISS1, lgt::ISS2* and *lgt::ISS3*). Several proteins that were seen in capsule extracts from *lgt::ISS1* were not evident in the wild-type strain, *lgt::ISS2* or *lgt::ISS3* (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::ISS1* and *lgt::ISS3*. Mutant *lgt::ISS1* 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::ISS1* (Fig. 1), suggested that *lgt::ISS1*, unlike the wild-type, was unable to anchor lipoproteins conventionally to the membrane. Furthermore, the similarity between the findings for *lgt::ISS2*, *lgt::ISS3* and the wild-type suggested that the phenotype observed for *lgt::ISS1* was not due to polar effects on ORFs located downstream of lgt.

**The (lipo)protein MtuA is found in all cell fractions from lgt::ISS1**

Immunoblotting of cell fractions with anti-MtuA revealed a single protein of approximately 37 kDa in the membrane
fraction of the wild-type strain (as shown previously; Jones et al., 2004). In contrast, strain \textit{lgt}::\textit{IS}$_{S1}$ 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 \textit{lgt}::\textit{ISS}$_1$ (Fig. 1) were atypically 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 \textit{lgt}::\textit{ISS}$_1$ 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 \textit{mtuA} (Fig. 4), indicating that Lsp was involved directly or indirectly in cleavage of unlipidated MtuA. In order to substantiate this further, an \textit{lgt}/\textit{lsp} double mutant with an \textit{IS}$_{S1}$ insertion within \textit{lgt} (546 bp from the start codon) was isolated from a random mutant bank created within a \textit{Strep. uberis} \textit{lsp}::\textit{ISS}$_1$ 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 \textit{lgt}/\textit{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 \textit{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

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

Lipobox residues are shown in bold and the N-terminal sequence derived from \textit{lgt}::\textit{ISS}$_1$ is underlined.

<table>
<thead>
<tr>
<th>ORF assignment</th>
<th>Predicted lipoprotein leader sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUB1625. Putative threonine kinase (hyaluronate-associated protein) or OppA1 (Sutcliffe &amp; Harrington, 2002)</td>
<td>MTVAQKSTFKRFLGAVT ASDALLMAC GNKTA AKND</td>
</tr>
<tr>
<td>SUB0473. MtuA</td>
<td>MKKKSLAIALLGMLGAC SVGNRKAT</td>
</tr>
<tr>
<td>SUB0365. Putative bacterial extracellular solute-binding protein</td>
<td>MTMLKKNLGSLTGTALAC GNKASNNNG</td>
</tr>
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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 (lipoprotein) 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.

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 (lipoproteins 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

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**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.

**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.
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 Mn²⁺. Mutation within mtuA/B/C resulted in a strain that was unable to grow in bovine milk, a medium in which Mn²⁺ 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 Mn²⁺ 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 ISP 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 ISP 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

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**Fig. 5.** Immunoblots of subcellular fractions of wild-type and the lgt/ISP 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/ISP 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/ISP mutants and arrow 2 indicates unprocessed MtuA detected in the ISP and lgt/ISP mutants. (b) Proteins from the supernatant prepared from mid-exponential-phase Strep. uberis wild-type (lane 1), lgt mutant (lane 2) and lgt/ISP 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).
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::ISS1, 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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REFERENCES


Dartois, V., Djavakhishvili, T. & Hoch, J. A. (1997). KapB is a lipoprotein required for KinB signal transduction and activation of the phosphor- 

day to sporulation in Bacillus subtilis. Mol Microbiol 26, 1097–1108.


Hanke, K. & Braun, V. (1973). Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the


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