INTRODUCTION

Enterococcus faecalis is a natural commensal of the gastrointestinal tract of humans and animals. It is also readily isolated from foods, plants, water and soils because of the dissemination of faecal sources and its capacity to adapt to environmental changes. However, E. faecalis can be pathogenic for humans, causing urinary tract and surgical wound infections, bacteraemia and endocarditis (Murray, 1990), and it represents one of the main causes of nosocomial infection in the USA and Europe (Wisplinghoff et al., 2004). The intrinsic and acquired resistance of E. faecalis to commonly used antibiotics and the range of virulence determinants, which include cytolysin, gelatinase, extracellular superoxide, surface-exposed proteins (Ace, EfaA, Esp, AS) and surface carbohydrates (for reviews, see Hutchings et al., 2010; Tendolkar et al., 2003), are likely to be factors that contribute at least in part to survival in the hospital environment and the incidence of infections.

The location of lipoproteins in Gram-positive bacteria, anchored in the outer leaflet of the membrane, suggests a role in interaction with the environment. In consequence, they are thought to play an important part in host–bacteria interactions, including adaptive responses to environmental changes, adherence, internalization, colonization, toxin synthesis and escape from the immune system (for reviews, see Hutchings et al., 2009; Kovacs-Simon et al., 2011). Moreover, lipoproteins have been reported to play a role in cell envelope stability (Sutcliffe & Harrington, 2004; Nguyen et al., 2010), in post-translocational folding of exported proteins (Kontinen & Sarvas, 1993; Hermans et al., 2006) and in pheromone production (Clewell et al., 2000). Finally, lipoproteins can have diverse enzymic activities and can initiate proinflammatory responses, activating many types of host cells such as monocytes, macrophages, neutrophils and B cells (Hutchings et al., 2009; Kovacs-Simon et al., 2011). Toll-like receptor 2 is activated by lipoproteins, and in consequence induces cellular and organ inflammation, which contributes to the pathogenesis of sepsis (Henneke et al., 2009; Kurokawa et al., 2009; Shin et al., 2011; Schmaler et al., 2009). As recently reviewed (Kovacs-Simon et al., 2011), lipoproteins also participate in bacterial adhesion and translocation.

Lipoprotein synthesis in Gram-positive bacteria starts with the production of pre-lipoproteins containing a peptide signal sequence characterized by a lipobox motif, which consists of four amino acids and corresponds to the consensus sequence L-x-[A/S/T]-x-[G/A]-x-C+1 (Sutcliffe & Harrington, 2002). The cysteine in position +1 (the first amino acid of the mature lipoprotein) is always conserved...
and corresponds to the anchoring site of a diacylglycerol moiety (Sutcliffe & Harrington, 2002). Initially, lipoproteins are translocated across the cytoplasmic membrane by the Sec or Tat pathway. After translocation, lipoprotein biogenesis in Gram-positive bacteria requires two steps. The first depends on Lgt, a prolipoprotein diacylglycerol transferase which catalyses the transfer of a diacylglycerol moiety from a glycerophospholipid onto the thio group of the conserved cysteine via a thioether linkage. In the second step, the signal peptide is cleaved by Lsp, a type II signal peptidase, at the conserved cleavage site of the lipobox, leaving the lipid-modified cysteine at the N terminus of the mature lipoprotein anchored to the membrane with the protein moiety exposed at the surface (Hutchings et al., 2009).

While lipoprotein biosynthesis has been shown to be essential in Gram-negative bacteria, this is not so in almost all Gram-positive bacteria tested (except Streptomyces coelicolor; Thompson et al., 2010). Therefore, the involvement of Lgt and Lsp in the virulence of Gram-positive bacteria has been tested in several species. Loss of Lgt or Lsp leads to attenuation of virulence in Streptococcus pneumoniae (Petit et al., 2001), to a decrease in immune activation for group B Streptococcus, Staphylococcus aureus and Listeria monocytogenes (Henneke et al., 2008; Stoll et al., 2005; Machata et al., 2008), and to a reduction in adherence of Streptococcus agalactiae (Bray et al., 2009) and in intracellular growth of L. monocytogenes (Baumgartner et al., 2007). While S. agalactiae and Staphylococcus aureus lgt mutants show increased sensitivity to oxidative stress (Bray et al., 2009) and growth attenuation in whole human blood (Stoll et al., 2005), respectively, both display a hypervirulent phenotype (Henneke et al., 2008; Bubeck Wardenburg et al., 2006).

In *E. faecalis*, transport of lipoproteins across the membrane uses the Sec system, as no Tat mechanism is present and none of the 90 lipoproteins predicted in *E. faecalis* V583 harbours a Tat signal peptide (Reffuveille et al., 2011). Analysis *in silico* has revealed that prolipoprotein diacylglycerol transferase (Lgt) and the signal peptidase II (Lsp) would be encoded by genes *ef1748* and *ef723*, respectively (Paulsen et al., 2003; Reffuveille et al., 2011). However, no description of the involvement of these genes in the processing of lipoproteins has been reported. In this study, we constructed and characterized an *lgt* mutant in order to evaluate the role of *E. faecalis* lipoproteins in the stress response and virulence.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains used in this study are listed in Table 1. The parental *E. faecalis* strain used was V19, a plasmid-cured derivative of the vancomycin-resistant clinical isolate V583 (Zhao et al., 2010). *Escherichia coli* XLI Blue was used as the recipient for cloning (Table 1). *E. faecalis* V19 and its derivatives were grown at 37 °C in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% glucose (GM17). When required, erythromycin (150 μg ml⁻¹) was added to *E. faecalis* cultures. Cultures of *E. faecalis* strains were also performed on CCM17 MOPS medium with glycerol (0.5%, v/v) as the sole carbohydrate (CCM17-gly), as described by La Carbona et al. (2007). Different pH conditions (4.0–9.0) were tested using microplates with CCM17-gly medium. *Escherichia coli* strains were cultured with shaking at 37 °C in Luria–Bertani medium (Sambrook et al., 1989), with ampicillin (100 μg ml⁻¹) or erythromycin (150 μg ml⁻¹) when required. Plasmid vectors used in this study are listed in Table 1. For some experiments, a chemically defined medium, MCDE Caa Trp medium, was used (MCDE Caa Trp medium contains KH₂PO₄, K₂HPO₄, ammonium citrate and sodium acetate at 20, 60, 2.47 and 7.35 mM respectively; pyridoxal, nicotinic acid, thiamine hydrochloride, riboflavin, DL-pantothenic acid salt, 4-aminobenzoic acid,

<table>
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<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. faecalis</em></td>
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<td>V19</td>
<td>Strain V583 (clinical isolate), without plasmid</td>
<td>Zhao et al. (2010)</td>
</tr>
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<td>Slgt</td>
<td>V19 isogenic derivative lgt mutant with a stop codon insertion</td>
<td>This study</td>
</tr>
<tr>
<td>SlgtC</td>
<td>Strain Slgt mutant derivative, complemented</td>
<td>This study</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<td>XL1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacZΔM15] Tn10 (TetR)</td>
<td>Stratagene</td>
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<td>Qiagen</td>
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<td>M15/pQE6085</td>
<td>Strain harbouring pQE6085</td>
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<tr>
<td>pGEMt</td>
<td>System for cloning PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pGHost9</td>
<td>Plasmid with thermosensitive replication</td>
<td>Maguin et al. (1992)</td>
</tr>
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<td>pGEMt derivative carrying a 3.7 kb DNA fragment from <em>E. faecalis</em> V19 containing lgt</td>
<td>This study</td>
</tr>
<tr>
<td>pGEMtSlgt</td>
<td>pGEMt derivative carrying a 3.7 kb lgt DNA fragment with two stop codons</td>
<td>This study</td>
</tr>
<tr>
<td>pGHost9wtlgt</td>
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<td>This study</td>
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<td>pGHost9 derivative carrying a 3.7 kb DNA fragment with two stop codons</td>
<td>This study</td>
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<tr>
<td>pQE30</td>
<td>Plasmid for overexpression of proteins</td>
<td>Qiagen</td>
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bion, folic acid, vitamin B12, orotic acid, thymidine, inosine, DL-thioctic acid and pyridoxamine at 9.8, 8.1, 3.0, 2.7, 4.2, 72.9, 4.1, 2.1, 0.7, 32, 20.6, 18.6, 12.2 and 20.7 μM, respectively, pH adjusted to 7.0; MgCl2, CaCl2, FeCl2, ZnSO4, CoCl2, CuSO4 and MnSO4 at 984, 340, 25, 17.4, 10.5, 0.4 and 165 μM, respectively; adenine, uracil, xanthine and guanine at 74, 89.2, 65.7 and 66.2 mM, respectively; 1.5% Casamino acids, 0.49 mM tryptophan and 0.5% glucose). When required, a higher MnSO4 concentration of 1.76 mM was used. The pH was adjusted to 7.25 and the medium was sterilized by filtration through a 0.22 μm pore-size filter.

Survival experiments. Cultures of *E. faecalis* wild-type, *lgt* mutant and complemented strains at OD600 0.5 were harvested and the pellet was resuspended in normal saline (0.9% NaCl in distilled water) containing sodium chloride (28.5%), bile salts (0.3%) or ethanol (22%), or in urine from humans (healthy volunteers) or in GM17 medium at a temperature of 62°C. After different exposure times (24 and 48 h for NaCl, 15 and 30 min for bile salts and heat, 30 and 60 min for urine and ethanol, respectively), serial dilutions were plated on GM17 to evaluate survival. Antibiotic exposure was performed using Mueller–Hinton agar with antibiotic discs: β-lactams [penicillin (6 μg) and ampicillin (10 μg)], aminoglycosides [streptomycin (500 μg), kanamycin (1 μg), gentamicin (500 μg), tobramycin (10 μg) and netilmicin (30 μg)], cyclins [tetracycline (30 μg), minocycline (30 UI) and doxycycline (30 μg)], quinolones [ofloxacin (5 μg), norfloxacin (5 μg), ciprofloxacin (5 μg), levofloxacin (5 μg) and nalidixic acid (30 μg)] and glycopeptides [vancomycin (30 μg) and teicoplanin (30 μg)].

**General molecular methods.** PCR was performed with GoTaq Flexi polymerase (Promega). PCR primers are listed in Table 2. PCR products and plasmids were purified using the NucleoSpin Extract II kit (Macherey-Nagel), respectively. Restriction endonucleases and T4 DNA ligase were purchased from Promega and used according to the manufacturer’s instructions. Genomic DNA extraction and other standard techniques were carried out as described by Sambrook *et al.* (1989).

**Genetic construction of an *lgt* mutant and complementation.** A mutant with an insertion of two stop codons in *lgt* (*Slgt*) was constructed by allelic replacement as follows. Briefly, a 3.7 kb DNA fragment (obtained using chromosomal DNA of *E. faecalis* V19 as template) containing the *lgt* gene flanked by 1384 and 1452 bp upstream and downstream, respectively, was amplified using primers Ef1748m1 and Ef1748m6 (Table 2) and cloned into plasmid pGemT (Table 1) to produce pGEMwtlgt. The reverse PCR product (using primers Ef1748stop2 and Ef1748stop1; Table 2) was digested with SacI and ligated to obtain plasmid pGEMSlgt, harbouring two stop codons within *lgt*. The SacII restriction site common to plasmids pGEMT and pGHost9 (Table 1) was used to add pGHost9 to the above constructs. Next, the restriction endonuclease NotI was used to remove the pGEMt vector and obtain plasmids composed of pGHost9 with the fragment carrying the stop codons (pGHost9Slgt) or the original fragment (pGHost9wtlgt). The recombinant plasmid pGHost9Slgt was then introduced into *E. faecalis* V19. Transformants (erythromycin-resistant colonies) obtained at 30°C were used for temperature shifts in order to select clones with the plasmid integrated within the chromosome (first crossing over) and subsequently clones resulting from a double crossing over. These were analysed for the presence of a mutated *lgt* gene, and the corresponding region was verified by sequencing.

For complementation studies, the pGHost9wtlgt plasmid was introduced into the *Slgt* mutant, and double-crossover events were used to allow the construction of the complemented strain *Slgt*C.

**RNA isolation and quantitative reverse transcriptase PCR (RT-qPCR).** In order to assess comparative transcriptional gene expression, we used the V19 wild-type strain and its *lgt* mutant derivative cultured on GM17 medium, with or without shaking, and

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**Table 2.** Primers used in this study

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5’–3’)*</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ef1748m0/m7</td>
<td>TGAATCACTGGCATTCTAGG</td>
<td>Cloning verification</td>
</tr>
<tr>
<td>Ef1748m1/m6</td>
<td>TCCGGCAACAAATTTCAATCCT</td>
<td>Cloning in pGEMt</td>
</tr>
<tr>
<td>Ef1748stop2/stop1</td>
<td>TATAGAGCCTCCTTTTGG</td>
<td>Reverse PCR for mutant with insertion of stop codons</td>
</tr>
<tr>
<td>Ef0685s1/Ef0685s2</td>
<td>AGGGACGATCGGGTCAA</td>
<td>Overproduction of EF0685</td>
</tr>
<tr>
<td>PU/PR</td>
<td>TGTAAAACGACGGCCAG</td>
<td>Cloning verification</td>
</tr>
<tr>
<td>EF0636R/EF0636L</td>
<td>GGCATTAAAAAGGCAATCCAAA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>EF0273R/EF0273L</td>
<td>GGCCTTACGAAAGACTATGCT</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>EF0577R/EF0577L</td>
<td>TCCCAACAAATGACTACCG</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>EF0685R/EF0685L</td>
<td>GGCAGAATGGCAGAAACCA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>EF1234R/EF1234L</td>
<td>TTTATTTCCCGAGATTTAC</td>
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<td>EF1362R/EF1362L</td>
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<td>EF3082R/EF3082L</td>
<td>CCGTTACGGGAAAGGCTCT</td>
<td>RT-qPCR</td>
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*Underlined sequences correspond to the recognition sites of the restriction endonucleases shown in parentheses.*
supplemented with 2 mM H₂O₂ when required. Using the RNasey Midi kit (Qiagen), two or three independent samples of total RNA were isolated for each condition. For RT-qPCR, specific primers were designed using the E. faecalis V583 genome sequence and Primer3 software (http://frodo.wi.mit.edu/primer3/) to produce amplions of equivalent length (100 bp; Table 2). Two micromolars of total RNA were reverse-transcribed with random hexamer primers and the QuantiTect enzyme (Qiagen). Quantification of 23S rRNA and gyrA (encoding the A subunit of DNA gyrase) mRNA provided internal controls. Amplification (using 5 μl of a 1:100 cDNA dilution), detection (with automatic calculation of the threshold value), and real-time analysis were performed twice for each cDNA sample using the iCycler IQ Detection System (Bio-Rad Laboratories). Relative mRNA levels for each gene in each sample were calculated using comparative cycle time, as described elsewhere (Meijerink et al., 2001).

Protein extraction. Cells from E. faecalis strains were harvested from the exponential growth phase in GM17 medium (OD₆₀₀ 1) for the extraction of surface-exposed or total proteins, and from the exponential growth phase in MCDE Caa Trp medium (OD₆₀₀ 0.5) for secreted proteins.

Surface-exposed proteins were extracted by exposure of cells to high pH using a protocol based on that described by Morschzek et al. (2008). Briefly, a cell pellet from a 200 ml culture was washed with a PBS sucrose solution (100 mM NaCl, 60 mM sucrose, 55 mM sodium phosphate, pH 7.2) and shaken gently for 1 h at room temperature with 2 ml NaOH glycine sucrose (50 mM glycine, 60 mM sucrose, pH 12.4). After centrifugation (30 min, 10,000 g), 108 μl 1 M HCl and 100 μl 1 M Tris/HCl (pH 7.0) were added to 1 ml of supernatant. Proteins were precipitated by the addition of 8 ml cold acetone with 20 min incubation at 4 °C. The protein pellet obtained after further centrifugation (10 min at 10,000 g) was resuspended in 200 μl Tris/HCl, pH 7.5.

For the extraction of secreted proteins, culture supernatants were sterilized by filtration through a 0.22 μm pore-size membrane. Proteins were precipitated by the addition of TCA (final concentration 10 %) and incubation on ice for 30 min. After centrifugation, the pellet was washed twice in acetone, and the proteins were resuspended in 20 μl 1 M Tris/HCl (pH 8.8). The concentration of secreted proteins in culture supernatants (E. faecalis wild-type, Sglt mutant or complemented strain in MCDE Caa Trp medium) was measured using the method of Bradford (1976).

For the extraction of total proteins, cells were washed with a solution containing 50 mM Tris buffer (pH 7.5), 50 mM Na₂SO₄ and 15 % (v/v) glycerol, resuspended in the same solution and broken open using two 30 s treatments in a FastPrep instrument (MP Biomedical). Unbroken cells were removed by centrifugation (10 000 r.p.m., 10 min, 4 °C).

For electrophoresis, proteins were mixed with Laemmli buffer (Laemmli, 1970), heated for 5 min at 95 °C, and subjected to SDS-PAGE.

Construction of EF0685-overproducing strain Escherichia coli M15/pQEsE‑ef0685. In order to produce antibodies against E. faecalis lipoprotein, protein EF0685 (from the rotamase family; Paulsen et al., 2003) was overproduced. The corresponding gene (excluding the nucleotides encoding the signal peptide) was amplified using primers EF0685s1/EF0685s2 (Table 2) and inserted downstream of the IPTG-inducible promoter in the pQEtet expression vector (Qiagen) to obtain an N-terminal His tag. The resulting construct was electroporated into Escherichia coli M15pRep4, creating M15/pQEsE‑ef0685 (Table 1). Recombinant EF0685 protein was over-produced and purified as described by Muller et al. (2006).

Production of anti-EF0685 antibodies. Purified recombinant EF0685 protein was injected with Freund’s complete adjuvant into a New Zealand white rabbit. After three injections (one every 2 weeks), serum was collected and tested for anti-EF0685 antibodies by Western blotting with purified recombinant EF0685, and total proteins extracted from E. faecalis wild-type and ef0685 mutant strains (F. Reffuveille and others, unpublished results).

Western blotting. After electrophoresis, proteins were transferred onto a PVDF membrane, which was then stained with Coomassie blue in order to verify that equal amounts of protein were present in all lanes. Blocking, incubation with antisera against His-tagged EF0685, and enhanced-chemiluminescence detection (ECL detection kit, GE Healthcare) were carried out as described previously (Riboulet-Bisson et al., 2008).

Protein identification by MS. Following SDS-PAGE and Coomassie blue staining, the protein-containing regions (bands) were excised, and washed twice in ultrapure water and once in acetonitrile/50 mmol ammonium bicarbonate 1⁻¹ (1 : 1, v/v). Samples were stirred for 15 min and vacuum-dried for 30 min. In-gel digestion of the excised protein bands was carried out using 0.5 μg trypsin (Promega), incubated overnight at 37 °C. MS experiments were carried out on an AB SCIEX 5800 proteomics analyser equipped with TOF ion optics and OptiBeam on-axis laser irradiation with a 1000 Hz repetition rate. The system was calibrated immediately before analysis using a mixture of des-arg-bradykinin, angiotensin I, glu-fibrinopeptide B, ACTH (18–39) and ACTH (7–38), with a mass precision better than 5 p.p.m. A 0.8 μl volume of the peptide solution was mixed with 1.6 μl volumes of a solution of α-cyano-4-hydroxycinnamic acid matrix prepared in a solution of 50 % acetonitrile with 0.1 % trifluoroacetic acid. The mixture was spotted onto stainless steel Opti-TOF 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. All acquisitions were taken in automatic mode. A laser intensity of 3000 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (10 × 200) in the mass range 700–4000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 3900. For the tandem MS experiments, the acceleration voltage applied was 1 kV using air as the collision gas. A gas pressure of medium was used. The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the MASCOT 2.3.02 program (Matrix Science). A database corresponding to an updated compilation download from the NCBI database was used with E. faecalis as the selected species (including 282 253 entries). The variable modifications allowed were as follows: N-terminal acetylation, methionine oxidation, and dioxidation. Trypsin was selected as the enzyme, with three miscleavages also allowed. Mass accuracy was set to 100 p.p.m. and 0.6 Da for MS and MS/MS modes, respectively.

Detection of superoxide dismutase (SOD) activity. Non-denaturing PAGE was carried out according to the protocol of Laemmli (1970), omitting SDS and mercaptoethanol. Equal amounts of protein (30 μg) were loaded in each lane, and the gel was used to detect SOD activity according to the protocol of Beauchamp & Fridovich (1971).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For electron microscopy, E. faecalis cells were washed in PBS buffer and fixed with 2.5 % glutaraldehyde in PBS buffer X (0.1 M cacodylate buffer (pH 7.0) in the presence of 0.4 mg ruthenium red 0.1 mol⁻¹) overnight at 4 °C. For TEM, cells were rinsed in buffer X three times, post-fixed for 2 h with buffer X supplemented with 1 % osmium tetroxide, and washed three times with buffer X alone. Cells were pelleted in 1.5 % low-melting-point agar at 40 °C,
dehydrated in progressive baths of ethanol (70–100%), and embedded in Epon resin. Ultrathin sections were cut and contrasted with uranyl acetate (2.5% in ethanol) and lead citrate. Cells were observed using a JEOL 1011 transmission electron microscope, with images taken using a MegaView 3 camera and Analysis VIVE software (SIS). For SEM, cells were rinsed in PBS buffer and fixed with 2.5% glutaraldehyde in PBS buffer X overnight at 4 °C. Then, cells were sedimented on a Thermanox coverslip coated with poly-l-lysine. The cells were rinsed in buffer X three times and post-fixed for 2 h with buffer X supplemented with 1% osmium tetroxide. The cells were rinsed in buffer X three times, dehydrated in progressive baths of ethanol (70–100%), critical point-dried (CPD 020 Baltec), sputtered with platinum and observed with a JEOL 6400F scanning electron microscope.

Infection and survival experiments. Infection of Galleria mellonella larvae with E. faecalis was accomplished as previously described by Lebreton et al. (2009). Briefly, using a syringe pump (KD Scientific), larvae (about 0.3 g and 3 cm in length) were infected subcutaneously with washed E. faecalis cells from an overnight culture in GM17, with 1.5 x 10^6 ± 0.15 x 10^6 or 3 x 10^6 ± 0.3 x 10^6 c.f.u. per larva administered in 10 μl sterile saline buffer. In each test, 15 insects were infected and the experiments were repeated at least three times. Larval killing was monitored each hour from 18 to 24 h post-infection.

RESULTS AND DISCUSSION

Lgt and construction of an lgt-deficient strain of E. faecalis

In order to evaluate the role of E. faecalis lipoproteins in stress resistance and virulence, an lgt mutant was constructed. ORF ef1748 in the genome of the E. faecalis V583 wild-type strain has been annotated as a prolipoprotein diacylglyceryl transferase gene (lgt). ef1748 encodes a protein consisting of 278 aa with a calculated molecular mass of 32.0 kDa. Bioinformatic analysis identified a single candidate for prolipoprotein diacylglyceryl transferase in the genome of strain V583 (ef1748) that exhibits 134/273 (49%) amino acid sequence identity and 189/273 (69%) amino acid sequence similarity to the characterized Lgt of L. monocytogenes (Baumgärtnner et al., 2007; Stoll et al., 2005). In the genome of E. faecalis strain V583, lgt (ef1748) is flanked upstream by hprK (HPr serine kinase/phosphatase) and downstream by two genes, gpsA and galU, encoding a glycerol-3-phosphate dehydrogenase and a UTP glucose-1-phosphate uridylyltransferase, respectively (Fig. 1). The short distance observed between hprK and lgt (14 nt) strongly suggests that they could be co-transcribed and that hprK could be involved in lipoprotein maturation. Indeed, the presence of hprK immediately upstream of lgt has been found in numerous genomes from Gram-positive bacteria, including Listeria, Pediococcus, Lactobacillus, Streptococcus, Bacillus, Lactococcus and Staphylococcus. Stoll et al. (2005) have suggested that HPr kinase could carry out dephosphorylation of phosphatidylglycerol to yield the diacylglycerol group that is then transferred to the thiol group of the cysteine residue by Lgt.

In order to study the role of lgt in the physiology and virulence of E. faecalis, we constructed a mutant by the insertion of two stop codons into lgt (Slgt). As the Slgt mutation was successful in E. faecalis, we can conclude that the prolipoprotein diacylglyceryl transferase is not essential in this bacterium. The generation of a viable lgt-deficient mutant is consistent with other reports indicating that loss of lgt generally does not significantly affect the in vitro viability of Gram-positive bacteria (Leskelä et al., 1999; Petit et al., 2001; Baumgärtnner et al., 2007; Stoll et al., 2005; Hamilton et al., 2006).

Effect of Lgt inactivation on lipoprotein processing

Lgt is an enzyme thought to be responsible for the transfer of the diacylglyceryl group from phosphatidylglycerol to the thiol group of the invariant cysteine of lipoproteins. Consequently, its absence would be expected to modify the abundance and/or electrophoretic mobility of secreted and surface-exposed proteins. Indeed, the absence of properly modified lipoprotein will disturb the pattern not only of surface-exposed proteins but also of secreted proteins, if Lsp cleaves non-lipidated pre-prolipoproteins. We evaluated the effect of lgt mutation on the level of secreted proteins by measuring their concentrations in the exponential growth phase in MCDE Caa Trp medium. Secreted protein concentrations were estimated to be 0.313 (±0.030), 0.179 (±0.017) and 0.193 (±0.028) mg ml⁻¹ for the Slgt mutant, the wild-type and the complemented strain, respectively. Thus, the absence of Lgt clearly led to increases in the concentrations of secreted proteins, as observed by Stoll et al. (2005) for Staphylococcus aureus, which suggests that unmodified lipoproteins are released into the culture supernatant of the Slgt mutant. It is also possible that some other secreted proteins could be more abundant due to perturbations of cell wall integrity in the

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**Fig. 1.** Genetic organization of the lgt (ef1748) chromosomal region of E. faecalis. Large arrows represent the ORFs and their orientation shows the transcriptional direction.
Sltg mutant. This increase in secretion in the mutant strongly suggests that the signal peptidase(s), responsible for the signal peptide cleavage of lipoproteins, are active even on non-lipidated lipoproteins, as observed by Henneke et al. (2008) and Stoll et al. (2005) for group B Streptococcus and Staphylococcus aureus, respectively.

The analysis of surface-exposed proteins by SDS-PAGE also revealed differences between the wild-type and Sltg mutant strains (Fig. 2). MS experiments allowed the identification of three lipoproteins corresponding to two putative units of ABC transporters (EF0907 and EF0177), and EF2556 (Fig. 2), among the proteins which were more abundant in the Sltg mutant extracts. This may be due to either a weaker attachment of the lipoproteins to the membrane or a differential shaving efficiency linked to conformational changes of the surface-exposed proteins. Even if the three proteins identified here are members of the E. faecalis proteins previously predicted to be lipoproteins (Reffuveille et al., 2011), the conformation of other surface proteins also could be affected. The alkaline treatment used for extraction of surface-exposed proteins released lipoproteins from the Sltg mutant. Consequently, it is likely that at least some of the lipoproteins lacking a diacylglycerol moiety are still linked to the membrane (probably temporarily attached by their signal peptide).

Using a polyclonal antibody, we tested the effect of the lgt mutation on the EF0685 lipoprotein by Western blotting after total protein extraction. In the wild-type strain, a cross-reactive band of about 43 kDa was seen (Fig. 3). The failure to lipidate EF0685 in the Sltg mutant was shown by the presence of only a light band at the corresponding position. However, an additional cross-reactive band of 100 kDa was observed in the Sltg mutant. Bray et al. (2009) observed the same phenomenon and suggested that the band corresponding to the higher molecular mass represented aggregated forms of the prolipoproteins.

The higher concentrations of secreted proteins and of lipoproteins in the surface-exposed protein extracts in the Sltg mutant, and the effect of lgt mutation in EF0685, clearly confirm the involvement of EF1748 in lipoprotein maturation.

The expression of nine genes encoding particular lipoproteins [ef0063, ef0273, ef0577, ef0685, ef1234, ef1362, ef1534, ef2076 (efaA) and ef3082 (fatB)] was evaluated in the Sltg mutant and the wild-type strains grown in GM17 medium. No significant difference was observed (data not shown), so we can conclude that the lgt mutation has no effect on the transcriptional regulation of these lipoproteins, and suggest that it causes disturbances only in the processing of lipoproteins.

**Phenotypic characterization of the Sltg mutant**

No major change was observed between the mutant and the wild-type strain by either TEM or SEM (data not shown).

Growth of the Sltg mutant in GM17 medium was compared with that of the wild-type strain V19 with and without shaking. Without shaking, there was no significant difference in growth between the strains (data not shown), and the generation times were 43.8 min (±1.5 min) and 43.4 min (±1.3 min) for the wild-type and the mutant, respectively. This suggests that the lack of lipid modification of the 90 putative E. faecalis lipoproteins does not

![Fig. 2. SDS-PAGE of surface-exposed proteins. Coomassie blue-stained monodimensional gel electrophoresis of surface proteins from exponentially growing cells of E. faecalis wild-type (Wt) and the Sltg mutant. The arrows indicate the protein bands excised from the gel, digested with trypsin and subjected to MS identification. Four peptides corresponding to 21.3% coverage were identified by MS for EF0177, while three (10.1% coverage) and five (17.6% coverage) peptides were identified for EF0907 and EF2556, respectively. The molecular masses of ladder polypeptides are shown on the left.](image)

![Fig. 3. Effect of lgt mutation on the EF0685 lipoprotein determined by Western blotting. Total protein extracts of the wild-type (Wt), Sltg mutant and complemented (SltgC) E. faecalis strains were probed with anti-EF0685 antibodies. Arrows indicate the molecular masses of the cross-reactive bands. Other observed bands are the result of non-specific reactions, as they were also present in an EF0685-deficient strain (data not shown).](image)
affect growth under these conditions. We conclude that either lipoproteins are not involved in growth without shaking in GM17, or that lipoproteins lacking the lipid moiety are still attached to the membrane (probably by their signal peptide), and so provide the functions normally performed by the corresponding mature lipoproteins. In contrast, when cultures were shaken, a slight difference in generation time between the mutant and the wild-type strain was noted, with generation times of 42.0 min (±2.0 min) for \( \text{S} \) and 49.2 min (±1.1 min) for the wild-type strain.

The sensitivity of the wild-type strain and the \( \text{S} \) mutant to sodium chloride, bile salts, urine and ethanol, and to exposure to antibiotics, different pH conditions (4.0–9.0) and heat shock, was tested as described above. No difference in survival was observed between the wild-type and mutant strains (data not shown).

Since differences in the behaviour of the mutant were noted only when incubated with shaking, we were interested in the growth of the mutant under oxidative stress conditions. We first studied growth of the \( \text{S} \) mutant in GM17 medium supplemented with 1.5 mM \( \text{H}_2\text{O}_2 \). The strain lacking Lgt clearly grew to higher densities in the presence of \( \text{H}_2\text{O}_2 \) than the wild-type and the complemented strain, which both showed very little growth (Fig. 4a). In \( \text{E. faecalis} \), SOD has been shown to be important in protecting cells from damage under oxidative stress (Verneuil et al., 2006). However, zymographic analysis revealed no obvious change in the level of SOD activity in the \( \text{S} \) mutant compared with the wild-type strain (data not shown). Growth of the mutant was also measured in a stable pH medium in the presence of glycerol (CCM17-gly), which allows \( \text{H}_2\text{O}_2 \) production within the cytoplasm (Bizzini et al., 2010). Again, under these conditions, the growth of the mutant exceeded that of the wild-type strain (Fig. 4b), a phenotype similar to that observed in GM17 supplemented with \( \text{H}_2\text{O}_2 \).

Of the stress conditions tested here (NaCl, bile salts, urine, ethanol, \( \text{H}_2\text{O}_2 \), heat, pH and antibiotic resistance), a difference was observed between the wild-type and mutant strains only under oxidative stress. Indeed, the growth of the \( \text{S} \) mutant was less affected by \( \text{H}_2\text{O}_2 \) than that of the wild-type strain. Involvement of Lgt in oxidative stress has been reported for \( \text{S. agalactiae} \) (Bray et al., 2009), although in that study, the mutant appeared more sensitive to oxidative stress. Using RT-qPCR, we determined the expression of genes encoding particular lipoproteins [\( \text{ef}0063, \text{ef}0273, \text{ef}0577, \text{ef}0685, \text{ef}1234, \text{ef}1362, \text{ef}1534, \text{ef}2076 \) (\( \text{efaA} \)) and \( \text{ef}3082 \) (\( \text{fatB} \))] during growth in GM17 supplemented with \( \text{H}_2\text{O}_2 \). However, the ratios of the expression of these genes in the oxidative stress versus the control (no \( \text{H}_2\text{O}_2 \)) condition were similar in the \( \text{S} \) mutant and the wild-type strain. Based on the RT-qPCR data, we conclude that the more extensive growth of the \( \text{S} \) mutant in the presence of \( \text{H}_2\text{O}_2 \) is probably due to two factors: the defective conformation of lipoproteins in the

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**Fig. 4.** Effect of lgt inactivation on growth. Growth of \( \text{E. faecalis} \) wild-type strain (○), \( \text{S} \) mutant (●) and complemented \( \text{S} \) strain (●) determined by \( \text{OD}_{600} \). (a) Growth with shaking in GM17 medium supplemented with 1.5 mM \( \text{H}_2\text{O}_2 \). (b) Growth with shaking in CCM17 MOPS medium supplemented with 0.5 % (v/v) glycerol. (c) Growth in MCDE Caa Trp medium with 1.76 mM manganese as \( \text{Mn}^{2+} \). The dotted line represents the growth of the wild-type strain in MCDE Caa Trp medium without manganese.
absence of Lgt, and the secretion of the corresponding unlipidated proteins, and is not due to transcriptional regulation. The lgt mutation could have an effect on the levels of many transporters (or other important proteins) and their activity. Factors such as divalent cations (Prousek, 2007) are known to be able to enhance the effect of oxidative stress. The phenotype observed here could (at least in part) be related to a reduction of the ability of the Slgt mutant to transport these compounds.

Divalent transition metal ions such as Mn$^{2+}$ are essential for both the growth and the survival of micro-organisms; moreover, Mn$^{2+}$ is important as a cofactor for many enzymes and as a regulator of oxidative stress regulons. However, Mn$^{2+}$ is potentially toxic at high concentrations (Low et al., 2003). As many lipoproteins could be involved in the transport of these components, we tested the effect of a high manganese concentration on the growth of the wild-type strain and the Slgt mutant in a chemically defined medium (Fig. 4c). We observed a clear growth advantage for the Slgt mutant in the presence of a high concentration of Mn$^{2+}$ compared with the wild-type strain. Indeed, final OD$_{600}$ readings were 2.32 ($\pm$ 0.25) for Slgt versus 0.76 ($\pm$ 0.12) for the wild-type strain, showing that although a high Mn$^{2+}$ concentration reduced *E. faecalis* V19 growth, it had less effect on the Slgt mutant. The phenotype observed in this high-manganese-concentration medium suggests that the reduction of the amount of lipoproteins on the cell surface, or their defective conformation, protects the cell when the Mn$^{2+}$ concentration is high enough to limit *E. faecalis* growth. As lipoproteins showing high homologies with subunits of Mn$^{2+}$ ABC transporters have been identified in *E. faecalis* [EF0055, EF0577, EF2076 (EfaA) and EF3206] (Reffuveille et al., 2011), the phenotype observed here could be explained by a reduction in the internalization of Mn$^{2+}$ and consequently reduced toxic effects on intracellular targets.

**Involvement of Lgt in *E. faecalis* virulence**

In order to study the involvement of Lgt in *E. faecalis* virulence, *G. mellonella* caterpillars were infected with the wild-type or the Slgt mutant strain at two different bacterial concentrations. More larvae infected with the Slgt mutant survived bacterial infection, as demonstrated by the survival rate monitored from 18 to 24 h post-infection (Fig. 5). The same increased survival was observed at both concentrations, and the killing effect was dose-dependent (Fig. 5a, b). According to Machata et al. (2008), *L. monocytogenes* entry into and survival inside epithelial cells or macrophages can be affected in the absence of Lgt, and there is certainly a balance between adhesion, cytotoxicity, activation of the immune system and lipidation of lipoproteins. Of the 90 predicted lipoproteins in *E. faecalis*, two [EF1818 (Ge) and EF2076 (EfaA)] are involved in virulence (Qin et al., 2000; Singh et al., 1998), one (EF2488) is encoded by a gene which is a part of an operon responsible for the synthesis of a capsular carbohydrate also involved in virulence (Hancock & Gilmore, 2002), and 39 correspond to putative components of ABC transporters, among which 24 have been predicted to be important for virulence (Reffuveille et al., 2011). Indeed, metal transport is a prerequisite for growth during infection and virulence, and amino acid, peptide and amine ABC transporters, as well as pheromone-binding proteins, could have direct or indirect effects on bacterial fitness.

**Fig. 5.** Effect of lgt inactivation on virulence. Kaplan–Meier survival analysis of *G. mellonella* from 18 to 24 h post-infection with *E. faecalis* wild-type strain (●), Slgt mutant (○) and complemented SlgtC strain (▲). We used 1.5×10$^6$ c.f.u. (a) or 3×10$^6$ c.f.u. (b), counted on agar plates, per injection. Data are representative of three separate survival experiments with 15 insects inoculated for each experiment. *G. mellonella* survival was significantly ($P<0.05$) greater following infection with the Slgt mutant than it was with the wild-type strain or the complemented SlgtC strain.
roles in virulence (Reffuveille et al., 2011). Also, two lipoproteins of *E. faecalis* V583 show high homology with the peptidyl-prolyl *cis*-trans isomerases of *S. pneumoniae*, which are involved in colonization (Hermans et al., 2006; Cron et al., 2009).

**Conclusion**

This study has demonstrated the involvement of the EF1748 protein, as the enzyme prolipoprotein diacylglycerol transferase (Lgt), in the processing of lipoproteins. Its involvement in virulence was clearly demonstrated, and the results also highlight a role for Lgt in the sensitivity of *E. faecalis* to oxidative stress. These observations show that the study of the specific roles of particular lipoproteins in virulence can be of significant interest. Another important question is why the Sltg mutant was less virulent, while still having an advantage under oxidative stress.

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