Enterococcus faecalis Glycolipids Modulate Lipoprotein-Content of the Bacterial Cell Membrane and Host Immune Response

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Abstract

In this study, we investigated the impact of the cell membrane composition of E. faecalis on its recognition by the host immune system. To this end, we employed an E. faecalis deletion mutant (ΔbgsA) that does not synthesize the major cell membrane glycolipid diglycosyl-diaclylglycerol (DGlcDAG). Proteomic analysis revealed that 13 of a total of 21 upregulated surface-associated proteins of E. faecalis ΔbgsA were lipoproteins. This led to a total lipoprotein content in the cell membrane of 35.8% in ΔbgsA compared to only 9.4% in wild-type bacteria. Increased lipoprotein content strongly affected the recognition of ΔbgsA by mouse macrophages in vitro with an increased stimulation of TNF-α production by heat-fixed bacteria and secreted antigens. Inactivation of the prolipoprotein diacylglycerol transferase (lgt) in ΔbgsA abrogated TNF-α induction by a ΔbgsA_lgt double mutant indicating that lipoproteins mediate increased activation of mouse macrophages by ΔbgsA. Heat-fixed ΔbgsA bacteria, culture supernatant, or cell membrane lipid extract activated transfected HEK cells in a TLR2-dependent fashion; the same was not true of wild-type bacteria. In mice infected intraperitoneally with a sublethal dose of E. faecalis we observed a 70% greater mortality in mice infected with ΔbgsA compared with wild-type-infected mice. Increased mortality due to ΔbgsA infection was associated with elevated plasma levels of the inflammatory cytokines TNF-α, IL-6 and MIP-2. In summary, our results provide evidence that an E. faecalis mutant lacking its major bilayer forming glycolipid DGlcDAG upregulates lipoprotein expression leading to increased activation of the host innate immune system and virulence in vivo.

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Introduction

In invasive bacterial infections, host inflammation may vary from low-grade to a strong systemic response associated with multi-organ failure and severe sepsis. The differences in the host response are thought to result mainly from activation of the innate immune system by pathogen- and danger-associated molecular patterns. In Gram-positive sepsis, a variety of microbial compounds such as peptidoglycan and its derivatives, bacterial DNA, lipoteichoic acid, and lipoproteins are believed to activate the host immune system [1]. Numerous studies in mice have underlined the role of Toll-like receptor 2 (TLR2) as a major sensor of Gram-positive bacteria, yet its role in vivo is strongly dependent on the specific infectious microorganism [2–6]. In contrast, no clear association has been established between TLR2 variants and susceptibility to Gram-positive infection in humans [7,8].

Several TLR2 ligands have been identified in Gram-positive bacteria, including peptidoglycan, LTA, and lipoproteins/lipopeptides [9]. Studies with mutants of the lipoprotein-acyl transferase (lgt) gene and considerations regarding the structure-function relationship suggest that lipopeptides/lipoproteins are the predominant agonists of the TLR2/TLR6 dimer [10,11]. Lipoproteins/lipopeptides are important amphiphiles of the cell membrane in Gram-positive bacteria. They are found both in the cell envelope and culture supernatant [12]. In Mycobacterium tuberculosis, membrane-derived vesicles have been described as a vehicle to release lipoproteins into the environment and similar vesicles have also been described for S. aureus [13,14]. Together with phospholipids, glycolipids, and lipoteichoic acid they constitute the lipid bilayer of the cell membrane.

We have previously studied the impact of cell membrane composition on the virulence of E. faecalis using mutants deficient in glycolipid biosynthesis. For this purpose we constructed two deletion mutants in E. faecalis strain 12030 (ΔbgsA and ΔbgsB) that are defective in the glycosylation of glycolipids [15,16]. Inactivation of bgsA leads to a complete loss of DGlcDAG from the cell membrane and accumulation of high concentrations of its precursor molecule mono-glycosyl-diacylglycerol (MGlcDAG) [15]. Inactivation of ΔbgsB, on the other hand, results in a cell membrane devoid of glycolipids [16]. Both ΔbgsA and ΔbgsB elaborate a longer poly-glycero-phosphate polymer of LTA than wild-type bacteria and show impaired biofilm formation and attachment to colonic epithelial cells. In a mouse bacteremia model, both mutants were cleared more rapidly from the bloodstream [15,16]. Interestingly, defects in glycolipid biosynthesis in ΔbgsA and ΔbgsB were not associated with changes in the bacterial cell shape or ultrastructure, in the growth rate, or in sensitivity to osmotic stress. This finding was surprising, since the ratio of the bilayer-forming DGlcDAG and the nonbilayer-prone MGlcDAG was shown to be critical for cell membrane architecture and curvature stress in studies using Acholeplasma laidlawii [17,18].

Here we examined the consequences of the altered glycolipid composition in ΔbgsA on the cell-surface proteome of the bacteria and studied the impact of these changes on the interaction between bacteria and the host immune system. For the investigation of the virulence of glycolipid-deficient E. faecalis strains we used a mouse peritonitis model that has been validated in several previous studies [19–22]. Our results show that in the absence of DGlcDAG, lipoprotein expression is upregulated in E. faecalis, which substantially increases the activation of TLR2 and virulence in vivo.

Materials and Methods

Bacterial strains, growth conditions, and medium

The bacterial strains and plasmids used in this study are listed in Table 1. Enterococci were cultured in tryptic soy broth (TSB, Merck), M17 broth (Difco Laboratories), Caso Bouillon (Carl
Roth), or TSB plus 1% glucose (TSG) as indicated. In addition, tryptic soy agar or M17 agar plates were used. *Escherichia coli* DH5α and TOP10 (Invitrogen) were cultivated aerobically in LB-broth. For cell culture stimulation studies, bacteria were grown in chemically defined medium (CDM) prepared from endotoxin-free water [23].

### Construction of deletion mutant \( \Delta lgt \)

A non-polar deletion of a portion of gene \( lgt \) (EF1748 in *E. faecalis* V583, GenBank ID accession number NP_815451) was created using the method described by Cieslewicz et al., [24] with the following modifications: primers 1 and 2 (Table 2) were used to amplify a 503-bp fragment from the region upstream of gene \( lgt \), and also the end part of EF1747. Primers 3 and 4 were used to amplify a 546-bp fragment downstream of the \( lgt \) gene and the beginning of EF1749. Primers 2 and 3 contain a 21-bp complementary sequence (underlined in Table 2). Overlap extension PCR was used to create a PCR product lacking a portion of gene EF1748. The resulting fragment was cloned into Gram-negative cloning vector pCRII-TOPO (Invitrogen) and cut with the restriction enzyme EcoRI (Invitrogen); the resulting fragment was then inserted into shuttle vector pCASPER containing a temperature-sensitive origin of replication. The resulting plasmid, pCASPER/\( \Delta lgt \), was transformed into *E. faecalis* 12030 by electroporation, and integrants were selected at a non-permissive temperature (42°C) on TSA plates with kanamycin (1 mg/ml). A single colony was picked, and insertion of plasmid into the chromosome was confirmed by PCR. The integrant was passaged 10 times in liquid culture without antibiotic at the permissive temperature (30°C), and colonies were replica-plated to screen for loss of kanamycin resistance. The excision of the plasmid either creates a reconstituted wild-type strain or leads to

### Table 1. Bacterial strains used and plasmids used in this study.

| strain or plasmid         | characterization                      | reference |
|---------------------------|---------------------------------------|-----------|
| *E. faecalis* 12030       | Clinical isolate, strong biofilm producer | [54]      |
| *E. faecalis* 12030ΔbgsA  | (EF2891) bgsA deletion mutant          | [15]      |
| *E. faecalis* 12030ΔbgsB  | (EF2890) bgsB deletion mutant          | [25]      |
| *E. faecalis* 12030Δlgt   | (EF 1748) lgt deletion mutant          | this study|
| *E. faecalis* 12030ΔbgsA_\( \Delta lgt \) | double bgsA-lgt deletion mutant      | this study|
| *E. faecalis* 12030ΔbgsB_\( \Delta lgt \) | double bgsB-lgt deletion mutant      | this study|
| *Escherichia coli* DH5α   | Gram-negative cloning host             | Invitrogen|
| *Escherichia coli* TOP10  | Gram-negative cloning host             | Invitrogen|

| plasmids                  |                                          |           |
|---------------------------|------------------------------------------|-----------|
| pCASPER                   | Gram-positive, temperature-sensitive mutagenesis vector | [55]      |
| pCRII-TOPO                | Gram-negative cloning vector             | Invitrogen|
| pCASPER/\( \Delta lgt \)  | pCASPER carrying a \( lgt \) deletion    | this study|

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|---------------------------------------|

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

| name               | sequence (5'-3')<sup>a</sup> |
|--------------------|-------------------------------|
| 1 pEF1748delF      | CCTTGTTTCGAGCCCCCTTACTT       |
| 2 pEF1748OEF       | ACTAGCGCGGCCGCTGACTACCTT      |
| 3 pEF1748delR      | ACGTACAGATACCTTTGGAG          |
| 4 pEF1748OER       | GAGACGACGCGCCGCTGTAATCGATTTCC |

<sup>a</sup>Linkers are underlined.

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an allelic replacement with the deleted sequence in the chromosome. The deletion mutant created was designated *E. faecalis* 12030Δlgt, containing a 507-bp (169 amino acids) in-frame deletion. The genotype was confirmed by PCR and automated sequencing.

**Construction of a ΔbgsA_lgt and ΔbgsB_lgt double mutant**

For construction of the double mutants bgsA_lgt and bgsB_lgt the plasmid pCASPER/Δlgt was transformed into prepared electroporation-competent cells of *E. faecalis* 12030ΔbgsA or ΔbgsB following the procedure described above for the construction of the single Δlgt deletion mutant.

**Preparation of *E. faecalis* antigens for stimulation experiments**

*E. faecalis* strains were grown for 16 h in CDM to stationary phase, collected by centrifugation and washed twice in phosphate buffered saline (PBS). The multiplicity of infection (MOI) for the cell culture experiments was calculated by quantification of colony-forming units (CFU) of serially diluted live bacteria of the respective strain on agar plates, with subsequent adjustment of the suspension of heat-fixed cells to the desired MOI. Cell culture supernatant was filter-sterilized with a 0.2 μm membrane, dialyzed for 24 h against endotoxin-free water, and lyophilized. Bacteria were fixed at 65°C for 60 min. Extraction according to the method of Bligh and Dyer was used to obtain total cell membrane lipids as described previously [15,25]. The concentration of the supernatant and total lipid extracts in the stimulation experiments is expressed as weight per volume.

Lipoproteins were enriched from cell membrane fractions by phase-partitioning with Triton X-114 (TX114) [11,26,27]. To this end, bacterial cells were grown in CDM medium as described above and quantified by serial dilutions and viable counts after culture on agar plates. Bacterial cells were disrupted by vibration with glass beads as described previously [15]. Cell debris and glass beads were pelleted by centrifugation and the supernatant was diluted in TN-buffer (20 mM Tris, 100 mM NaCl, pH 8.0) and passed through a 0.2 μm membrane. For separation of the cell membrane fraction, the filtered supernatant was ultracentrifuged at 50,000 rpm at 4°C for 1 h. The supernatant was discarded and the cell membrane fraction redissolved in TN-buffer plus 2% TX114 (v/v) and incubated at 4°C for 2 h, followed by a second incubation step at 37°C for 30 min. The solution was centrifuged (6,000 rpm, 10 min, 37°C) for phase separation. The lower detergent-soluble phase was carefully collected. The detergent-soluble phase was further purified by adjusting the TX114 concentration to 2% and repeating the phase partitioning, as described above. For removal of TX114, the detergent phase was mixed with 90% ethanol, incubated at -20°C for 18 h and precipitated proteins were collected by centrifugation. After resuspension in TN-buffer the protein concentration of the lipoprotein extracts was determined photometrically and normalized to bacterial cfu.

**Metabolic labeling, isolation of surface-associated proteins**

Surface-associated proteins were isolated by biotin labeling and affinity chromatography. Proteins were quantified with the spike-in of a stable isotope-labeled standard. For metabolic labeling the method of Becher *et al.* was used with modifications [28]. *E. faecalis* 12030 wild-type and ΔbgsA were each grown separately in both labeled and unlabeled rich growth media for *E. coli*, (*E. coli*-ODS, Silantes) supplemented with 2% glucose, vitamins (p-amino benzoic acid, biotin, folic acid, niacinamide, pantothenate, riboflavin, thiamine), and nucleotides. Cultures were grown at 37°C for 18 h while shaking at 140 rpm. As an internal standard, equal volumes of wild-type and ΔbgsA grown to the same OD600nm in 15N-labeled medium were mixed. Subsequently, equivalent OD units of wild-type and ΔbgsA cell culture grown in unlabeled medium were each mixed with the internal standard and centrifuged.
Cell pellets of wild-type and ΔbgsA were each washed once with PBS (pH 8) plus PMSF (1 mM). Cells (0.15 g) were resuspended in 1 ml PBS/PMSF, and Sulfo-N-hydroxysulfosuccinimide-disulfide-Biotin (Sulfo-NHS-SS-Biotin; Thermo Scientific) was added to produce a final concentration of 1 mM. The cell suspension was shaken carefully on ice for 1 h. After centrifugation, biotinylation was stopped by washing cells three times in 1 ml PBS plus 500 mM glycine. Next, cells were resuspended in 1 ml PBS and disrupted with glass beads (0.1 mm diameter) in two cycles at 6,800 rpm for 30 s using a ribolyser (Roche). To obtain cell membrane proteins, cell debris was washed six times with PBS and centrifuged at 45,000 rpm for 1 h at 4°C. Cell debris was resuspended in 0.4 ml PBS/I2-Iodoacetamide (5%) and homogenized with glass beads (0.1 mm diameter) using a ribolyser (6,800 rpm; 2x 30 s). To each sample 100 μl PBS plus 20% CHAPS and 20% Amidosulfobetaine-14 was added, homogenized in a ribolyser (6,800 rpm; 2 x 30 s) and shaken gently for 1 h at 20°C. Cell debris was removed again by centrifugation (14,000 rpm; 15 min; 4°C). Biotinylated proteins were recovered by incubation of the protein lysate with NeutrAvidin-agarose-beads equilibrated in PBS/Nonidet P40 (1%) for 1 h on ice while shaking. Unbound proteins were removed by washing beads four times with PBS/NP-40 plus 6% CHAPS and two times with 1 ml PBS/NP-40 plus 2% SDS. For elution of bound proteins, the disulfide bond of Sulfo-NHS-SS-Biotin was cleaved by incubation with 5% β-mercaptoethanol in deionized water for 1 h at 20°C. NeutrAvidin-Agarose-Beads were removed by centrifugation and the supernatant was transferred to 8 ml acetone (-20°C). Elution buffer was once again added to each sample, centrifuged and the resulting supernatant was also added to the acetone-elution-buffer-mix. Proteins were precipitated with acetone overnight at -20°C. Precipitated proteins were centrifuged (8,500 rpm; 30 min; 4°C) and washed with ethanol (98%; 4°C). Finally, the pellet was dried in 6 M urea/2 M thiourea under vacuum (SpeedVac; Bachofer).

### Protein identification and quantification by ESI-LC-MS/MS

Subsequent to preparation of the mixed and labeled protein extracts, samples were subjected to SDS-gel electrophoresis. Resulting gel lanes were cut into equidistant pieces, followed by tryptic in-gel digestion as described elsewhere [29]. The resulting proteolytic digests were subjected to LC-MS/MS analyses as described elsewhere [30]. In brief, peptides were applied to reversed-phase C18 chromatography on an EASYnLC system (Thermo Fisher) online coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher). For LC-MS analyses a full survey scan in the Orbitrap (m/z 300–2000) with a resolution of 30,000 was followed by MS/MS experiments of the five most abundant precursor ions acquired in the linear trap quadrupole (LTQ) via collision-induced dissociation (CID).

Database searching for light and heavy extracts and subsequent quantitation were done as described in Otto et al. [30]. For unambiguous identification data, database searching by Sorcerer- Sequest (version 3.5) relied on an *E. faecalis* target-decoy protein sequence database (*E. faecalis* V583 (NC_004668.1) including a set of common contaminants). The following parameters were set for database searching: enzyme type, trypsin maximum of two missed trypsin cleavage sites; peptide tolerance, 10 ppm; tolerance for fragment ions, 1amu.; b- and y-ion series; variable modification, oxidation of methionine (15.99 Da). For database searches for 15N-labeled peptides, the mass shift of all amino acids completely labeled with 15N-nitrogen was taken into account. Relative quantification was carried out as described previously [31]. Protein identifications were considered significant for the biological system if the protein was identified in at least two out of three samples in wild type or the mutant. The crude search results served as the base for the further analysis using Census software to obtain quantitative data of 14N peaks (sample) and 15N peaks (standard) [32].
Relative quantification of surface-associated proteins by spectral counting

For estimation of relative proportions of proteins within the surface proteome of *E. faecalis*, the normalized spectral abundance factor (NSAF) for the light mass traces (\(^{14}\text{N}\)) were calculated as described elsewhere [33]. For calculation of NSAF, spectra from raw data were extracted and searched as described above taking into account only light masses. Scaffold (version Scaffold_4.3.2, Proteome Software Inc.) was then used to validate MS/MS based peptide and protein identifications. Peptide identifications were only accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 2.2, 3.3, and 3.8 for doubly, triply, and quadruply charged peptides, respectively. Protein identifications were accepted if they contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Calculation of NSAF was carried out as described elsewhere [34].

RAW 264.7 mouse macrophage stimulation

RAW 264.7 macrophages were seeded at a density of 1 \(\times\) 10⁵ cells/ml in 24-well dishes in endotoxin-free DMEM containing 10% fetal calf serum. Cultures were stimulated with either heat-killed bacteria, lyophilized supernatant or lipoprotein TX114-extracts from bacterial culture for 16 h at 37°C in a 5% humidified CO₂ environment. After the incubation period, cell culture supernatant was collected and TNF-α production measured by commercial ELISA (R&D Systems) according to the manufacturer’s protocols. A commercial LPS preparation from *E. coli* 0111:B4 (Sigma Chemicals) was used as positive control.

Reporter gene analysis

HEK293 cells (Sigma Aldrich) and the stable cell line HEK-TLR2YFP were used in reporter gene studies as described [2]. In brief, cells were seeded into 96-well tissue-culture plates at a density of 5 \(\times\) 10⁵ cells/well. Cells were transiently transfected 16 hours later with an ELAM.luc reporter construct with TransIT-LT1 transfection reagent (Mirus Bio). Plasmid pcDNA was used to assure equal amounts of transfected DNA. The following day cells were incubated for 6 h with bacterial cells or cell wall extracts as indicated. After incubation, cultured cells were lysed in passive lysis buffer (Promega) and reporter gene activity was measured using a plate reader luminometer (MicroLumat Plus; Berthold).

Mouse peritonitis model

The virulence of *E. faecalis* strains was evaluated in a mouse peritonitis model. The mice were housed in groups of 5 per cage. All procedures were carried out between 8 a.m. and 18 p.m. in the animals home cage. Fourteen female BALB/C mice (Charles River Laboratories) 6–8 weeks old per group were assigned randomly to infection by intra-peritoneal injection of *E. faecalis* strains as indicated. The inoculum for the infection model was grown in TSB for 16 h to stationary phase, washed in PBS, adjusted to the desired concentration and injected intraperitoneally (i.p.) in 200 \(\mu\)l of PBS. The bacterial inoculum was confirmed by plating serial dilutions on agar plates. Mice were monitored twice daily for mortality or signs of illness. If mice had reached an unconscious or moribund state they were euthanized by carbon dioxide inhalation and counted as dead. A moribund condition was defined as impaired mobility, the inability to reach food and water or to keep an upright position, labored breathing or cyanosis, or a hunched position for more than 48 h. Moribund mice were placed in a chamber and CO₂ was...
introduced at a displacement rate approximately 20% of the chamber volume per min. The CO₂ flow was maintained for at least 1 minute after respiratory arrest of the animal. For quantification of bacterial counts and measurement of cytokines, mice (six per group) were sacrificed as described above. Peritoneal lavage fluid (PLF) was obtained by injecting 2 ml of sterile PBS with a 18-gauge needle. Blood was drawn under sterile precautions by cardiac puncture and transferred to heparin tubes. Next, the abdomen was opened, and the right kidney was harvested. All samples were directly placed on ice and processed immediately. The number of E. faecalis CFU in the PLF, blood, and kidney homogenate was determined. Kidneys were weighed and homogenized at 4°C in 2 ml of TSB with a tissue homogenizer. Serial 10-fold dilutions were made of each sample in TSB, 10 μl of each dilution was plated onto TSA plates and CFUs were enumerated after 18 h of incubation. The leukocytes in the PLF were counted as described elsewhere [22]. PLF supernatant and plasma were stored at -20°C until measurement of the cytokines.

Ethics statement
All animal experiments were performed in compliance with the German animal protection law (TierSchG). The mice were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. The animal welfare committees of the University of Freiburg (Regierungspresidium Freiburg Az 35/9185.81/G-07/15) approved all animal experiments. The institutional review board of the University of Freiburg approved the study protocol.

Statistical analysis
Statistical significance for two-way comparisons was determined by an unpaired t-test as indicated. Analysis of variance (ANOVA) for multigroup comparisons was used on log-transformed data, and the Tukey’s multiple-comparison test was used for posthoc analysis for pairwise comparisons. Survival data were compared using the log-rank (Mantel-Cox) test. To identify the up- and downregulated proteins of the mutant compared to the wild-type, the log2 ratio of each protein quantification was calculated, subtracting the median log2 ratio of the replicates of the wild-type from that of the mutant. The relative quantification of proteins by spectral counting was deemed reliable when its ratio was ascertained in two replicates with not less than two peptides in at least one of the replicates [35]. Statistical results were calculated using the Prism 3 software package. Statistical significance for the NSAF values (mutant versus wild-type) was determined by a t-test in the software Scaffold (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR) with a significance threshold alpha = 0.05.

Results
Analysis of enterococcal surface-associated proteins by the proteomics approach
To gain insight into the protein composition of the enterococcal cell-surface, wild-type bacteria and ΔbgsA surface-exposed proteins were biotinylated and purified by affinity chromatography prior to SDS-PAGE followed by in-gel digestion (IGD) and analysis by LC-MS/MS (GeLC-MS). Before surface proteins were selectively coupled to Sulfo-NHS-SS-Biotin, 14N15N metabolic protein labeling was performed during cultivation in CDM. Biotin-labeled proteins were purified by affinity-chromatography and eluted by cleavage of the disulfide bond of the Sulfo-NHS-SS-Biotin by reduction.
This approach led to the unambiguous identification of a total of 210 proteins from wild-type bacteria in 2/3 replicates (S1 Table). Determined by the theoretical prediction rules of subcellular localization (reference 53, 36 and the LocateP DataBase was used (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py)) the surface proteome fraction included 23 lipoproteins, 16 membrane proteins, and seven extracellular proteins containing a signal peptide. One hundred and sixty-four of the biotinylated proteins obtained were annotated with a cytoplasmic subcellular localization.

**In ΔbgsA the production of lipoproteins is upregulated**

The altered composition of cell membrane glycolipids in ΔbgsA led to a profound change in the pattern of surface-associated proteins as compared to isogenic wild-type *E. faecalis*. A total of 88 proteins were significantly up- or downregulated proteins in ΔbgsA according to the statistical testing. Forty showed a significantly increased amount in ΔbgsA compared with wild-type bacteria, while 48 proteins were decreased (Table 3). Twenty-one surface-associated proteins were upregulated in ΔbgsA. Of those, 13 were predicted to be lipoproteins, six cell membrane proteins and two extracellular proteins (Fig 1). Only six of the downregulated proteins were surface-associated proteins. Strikingly, lipoproteins represented 35.8% of the surface-associated proteins of ΔbgsA compared to only 9.4% in wild-type bacteria as quantified by spectral abundance factors (detailed information see S2 Table). Of the five most overexpressed surface-associated proteins, all were lipoproteins and they were upregulated 1.95–12.22-fold in ΔbgsA compared to wild type bacteria (Table 3). Altogether, our data suggest that the inactivation of *bgsA* disturbs the equilibrium in the cell membrane that in consequence leads to an increased lipoprotein content.

**ΔbgsA promotes vigorous activation of RAW 264.7 mouse macrophages**

To evaluate the effect of the altered cell-surface composition of ΔbgsA on inflammatory responses in vitro, we stimulated RAW 264.7 macrophages in the presence of live and heat-fixed *E. faecalis* and measured TNF-α in the cell culture supernatant after 3 h (live bacteria) and 16 h (heat-fixed bacteria and supernatant). In addition to ΔbgsA and wild-type bacteria, we used ΔbgsB as a second mutant defective in glycolipid biosynthesis in these experiments. At multiplicities of infection between 1:1 and 10:1, ΔbgsA induced significantly higher TNF-α concentrations compared to wild-type bacteria and ΔbgsB, respectively (Fig 2A and 2B).

*Staphylococcus aureus* lipoproteins are known to be released during growth into the culture medium [12]. We therefore also stimulated RAW 264.7 macrophages with dialyzed culture supernatant of *E. faecalis* strains. Supernatant from ΔbgsA was a potent inducer of TNF-α production of RAW 264.7 cells in vitro (Fig 2C). While culture supernatant of wild-type bacteria was inactive even at concentrations as high as 10,000 ng/ml (dry weight per volume), ΔbgsA stimulated TNF-α production at concentrations a 100-fold lower (Fig 2). Low amounts of TNF-α were also induced by cell culture supernatant from ΔbgsB (Fig 2). Together with the proteome analysis, these results demonstrate that lack of DGlcDAG in the cell membrane of ΔbgsA not only changes the composition of cell-surface proteome, but also enhances the activation of innate immunity.

**Lipoprotein-enriched cell membrane fractions of ΔbgsA but not wild-type cells stimulate TNF-α production of RAW 264.7 macrophages**

Due to their amphiphilic properties, lipoproteins can be purified from cell membrane fractions by the detergent Triton X-114 [11,26]. MS-shotgun analysis of Triton-extracted total
Table 3. Surface-associated proteins present in significantly different amounts in \( \Delta bgsA \) compared to the wild-type. The log2ratio depicts the change of \( \Delta bgsA \) compared to the wild-type.

| Protein accession number | log2 \((\Delta bgsA/wt)\) | Annotation | Localizationa |
|--------------------------|--------------------------|------------|---------------|
| EF3041                   | 12.222                   | Pheromone binding protein | lipoprotein   |
| EF1641                   | 3.114                    | Iron compound ABC transporter, iron compound binding protein | lipoprotein   |
| EF1534                   | 2.870                    | Peptidyl prolyl cis/trans isomerase | lipoprotein   |
| EF3198                   | 2.258                    | Lipoprotein, YaeC family | lipoprotein   |
| EF1354                   | 2.230                    | Pyruvate dehydrogenase complex. E1 component. beta subunit | cytoplasmic   |
| EF1353                   | 2.207                    | Pyruvate dehydrogenase complex. E1 component. alpha subunit | cytoplasmic   |
| EF2191                   | 1.983                    | dTDP-4-dehydrohamnose reductase | cytoplasmic   |
| EF3082                   | 1.955                    | Iron compound ABC transporter. substrate binding protein | lipoprotein   |
| EF1111                   | 1.842                    | Signal peptidase I | cell membrane |
| EF1212                   | 1.828                    | Transcriptional regulator LytR | cell membrane |
| EF2156                   | 1.813                    | Uncharacterized protein | extracellular |
| EF3062                   | 1.768                    | Cell shape determining protein MreC | extracellular |
| EF3120                   | 1.740                    | Serine threonine protein kinase | cytoplasmic   |
| EF3037                   | 1.602                    | Glutamyl aminopeptidase | cytoplasmic   |
| EF1677                   | 1.601                    | Lipoprotein, putative | lipoprotein   |
| EF1340                   | 1.562                    | Pheromone cAM373 lipoprotein | lipoprotein   |
| EF1191                   | 1.526                    | DegV family protein | cytoplasmic   |
| EF1759                   | 1.491                    | Phosphate ABC transporter. phosphate binding protein | lipoprotein   |
| EF3027                   | 1.420                    | Serine protease DO | cell membrane |
| EF1523                   | 1.324                    | Conserved domainl protein | cytoplasmic   |
| EF2656                   | 1.315                    | Flavoprotein family protein | cytoplasmic   |
| EF0176                   | 1.300                    | Basic membrane protein family | lipoprotein   |
| EF1416                   | 1.201                    | Glucose-6-phosphate isomerase | cytoplasmic   |
| EF2697                   | 1.159                    | Conserved domain protein | cell membrane |
| EF1753                   | 1.141                    | Uncharacterized protein | cytoplasmic   |
| EF1045                   | 1.111                    | 6-phosphofructokinase | cytoplasmic   |
| EF0761                   | 1.097                    | Amino acid ABC transporter. amino acid binding permease protein | cell membrane |
| EF2496                   | 1.085                    | Lipoprotein | lipoprotein   |
| EF0685                   | 1.082                    | Foldase protein PrsA | cell membrane |
| EF0784                   | 1.079                    | S-adenosylmethionine synthase | cytoplasmic   |
| EF0949                   | 1.040                    | Phosphotransacetylase | cytoplasmic   |
| EF2608                   | 0.904                    | ATP synthase subunit beta | cytoplasmic   |
| EF2610                   | 0.903                    | ATP synthase subunit alpha | cytoplasmic   |
| EF0863                   | 0.887                    | Glycine betaine carnitine choline ABC transporter. glycine betaine carnitine choline binding protein | lipoprotein   |
| EF2609                   | 0.794                    | ATP synthase gamma chain | cytoplasmic   |
| EF1355                   | 0.793                    | Pyruvate dehydrogenase complex E2 component. dihydrolipoamide acetyltransferase | cytoplasmic   |
| EF3255                   | 0.637                    | Thiamin biosynthesis lipoprotein ApbE. putative | lipoprotein   |
| EF1961                   | 0.478                    | Enolase | cytoplasmic   |
| EF2549                   | 0.473                    | Uracil phosphoribosyltransferase | cytoplasmic   |
| EF2903                   | 0.190                    | ABC transporter. substrate binding protein | lipoprotein   |

(Continued)
Table 3. (Continued)

| Protein accession number | log2 (ΔbgsA/wt) | Annotation | Localization |
|--------------------------|-----------------|------------|--------------|
| EF3256                   | -0.569          | Pheromone cAD1 lipoprotein | lipoprotein |
| EF1548                   | -0.577          | Ribosomal protein S1 | cytoplasmic |
| EF1402                   | -0.580          | Conserved domain protein | cytoplasmic |
| EF1193                   | -0.592          | DNA binding response regulator VicR | cytoplasmic |
| EF2932                   | -0.600          | AhpC TSA family protein | cytoplasmic |
| EF1584                   | -0.607          | Cysteine synthase | cytoplasmic |
| EF0997                   | -0.696          | Cell division protein FtsZ | cytoplasmic |
| EF1138                   | -0.871          | Oxidoreductase. aldo-keto reductase family | cytoplasmic |
| EF2355                   | -0.934          | Chaperone protein ClpB | cytoplasmic |
| EF1744                   | -0.956          | General stress protein. putative | extracellular |
| EF1963                   | -0.992          | Phosphoglycerate kinase | cytoplasmic |
| EF1560                   | -1.012          | Uncharacterized protein | cytoplasmic |
| EF3054                   | -1.051          | Lipoprotein. putative | lipoprotein |
| EF0944                   | -1.059          | protein. putative | extracellular |
| EF0715                   | -1.107          | Trigger factor | cytoplasmic |
| EF1522                   | -1.118          | RNA polymerase sigma factor RpoD | cytoplasmic |
| EF1764                   | -1.171          | Ribosomal subunit interface protein | cytoplasmic |
| EF3230                   | -1.174          | 30S ribosomal protein S9 | cytoplasmic |
| EF2607                   | -1.236          | ATP synthase epsilon chain | cytoplasmic |
| EF2397                   | -1.314          | Elongation factor Ts | cytoplasmic |
| EF0228                   | -1.380          | Adenylate kinase | cytoplasmic |
| EF2612                   | -1.382          | ATP synthase subunit b | cytoplasmic |
| EF2866                   | -1.384          | Probable transcriptional regulatory protein | cytoplasmic |
| EF0671                   | -1.412          | Xaa-his dipeptidase | cytoplasmic |
| EF0287                   | -1.442          | Elongation factor P | cytoplasmic |
| EF2718                   | -1.468          | 50S ribosomal protein L1 | cytoplasmic |
| EF0453                   | -1.605          | mC-Ohr family protein | cell membrane |
| EF0105                   | -1.613          | Ornithine carbamoyltransferase. catabolic | cytoplasmic |
| EF0770                   | -1.628          | Uncharacterized protein | cytoplasmic |
| EF0020                   | -1.639          | PTS system. mannose specific IIAB components | cytoplasmic |
| EF2715                   | -1.642          | 50S ribosomal protein L7 L12 | cytoplasmic |
| EF2729                   | -1.689          | Transcription termination antitermination protein nusG | cytoplasmic |
| EF0709                   | -1.708          | Phosphocarrier protein HPr | cytoplasmic |
| EF2415                   | -1.738          | Uncharacterized protein | cytoplasmic |
| EF0220                   | -1.846          | 30S ribosomal protein S8 | cytoplasmic |
| EF2719                   | -1.847          | 50S ribosomal protein L11 | cytoplasmic |
| EF0079                   | -1.896          | Gls24 protein | cytoplasmic |
| EF0820                   | -1.993          | 50S ribosomal protein L25 | cytoplasmic |
| EF2552                   | -2.036          | Sua5 YciO YrdC YwLC family protein | cytoplasmic |
| EF2395                   | -2.093          | Ribosome recycling factor | cytoplasmic |
| EF0394                   | -2.117          | Secreted antigen. putative | extracellular |
| EF1307                   | -2.132          | Protein GrpE | cytoplasmic |
| EF0012                   | -2.132          | 50S ribosomal protein L9 | cytoplasmic |
| EF0080                   | -2.160          | Gls24 protein | cytoplasmic |
| EF2925                   | -2.233          | Cold shock domain family protein | cytoplasmic |

(Continued)
membrane protein fractions from ΔbgsA and wild-type bacteria confirmed a high concentration of lipoproteins of 85% and 63%, respectively, in the extracts (data not shown). The Triton-X114 extracts from ΔbgsA induced an increased TNF-α production compared to the wild-type extracts (Fig 3). Taken together, cell membrane fractions of ΔbgsA highly enriched in lipoprotein are more potent inducers of TNF-α than those from the wild-type strain.

Inactivation of lipoprotein acylation abrogates induction of TNF-α by wild-type E. faecalis and ΔbgsA

In total, 90 genes that harbor the type II signal sequence typical for lipoproteins have been identified in E. faecalis, representing about 2.7% of the genome [36]. Similar to other Gram-positive bacteria, inactivation of the prolipoprotein diacylglycerol transferase (lgt) in E. faecalis causes the arrest of lipoprotein maturation at the stage of acylation of the protein, yielding non-acylated lipoproteins [37,38]. In E. faecalis, the deletion of lgt had no effect on bacterial morphology, growth rate, and sensitivity to sodium chloride, different pH condition, or exposure to antibiotics [38]. To corroborate our findings, we constructed a deletion mutant of the lgt gene in E. faecalis 12030 and created the double-deletion mutants EF 12030ΔbgsA_lgt and EF 12030ΔbgsB_lgt. Activation of TNF-α production in macrophages stimulated with lgt-mutants or wild-type E. faecalis was analyzed (Fig 2). Similar to S. aureus and group B streptococci [2,12,39], impaired protein-lipidation in E. faecalis 12030 led to a reduction of macrophage activation (Fig 2). Likewise, deletion of lgt in ΔbgsA and ΔbgsB decreased the production of TNF-α by RAW 264.7 macrophages compared to the respective single mutant (Fig 2).

Table 3. (Continued)

| Protein accession number | log2 (ΔbgsA/wt) | Annotation | Localization |
|--------------------------|-----------------|------------|--------------|
| EF2633                   | -2.306          | 60 kDa chaperonin | cytoplasmic |
| EF1308                   | -2.341          | Chaperone protein DnaK | cytoplasmic |
| EF0466                   | -2.345          | Glucosamine-6-phosphate deaminase | cytoplasmic |

a Transmembrane domains were predicted with the TMHMM 2.0 algorithm [53]. Lipoproteins were classified according to Reffuveille et al. [36]. For prediction of cell wall, cytoplasmatic and extracellular proteins Locate P was used (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py). For detailed data see S1 Table.

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Fig 2. Induction of TNF-α in RAW 264.7 mouse macrophages by *E. faecalis* strains. Macrophages were stimulated with live and heat-fixed bacteria at multiplicities of infection (MOI) as indicated (A and B) or with cell-free, dialyzed *E. faecalis* supernatants (C). RAW 264.7 macrophages were seeded at a density of 1 x 10^5 cells/ml in 24-well dishes in endotoxin-free DMEM containing 10% fetal calf serum. Cultures were stimulated at 37°C in a 5% humidified CO2 environment for 3 h (A) and 16 h (B), respectively, and supernatant from macrophage culture was analyzed for TNF-α by ELISA. The strains used for stimulation are indicated in the legend. LPS was used as positive control. Data represent mean ± SEM of triplicates. ND = not detected. 

* p < 0.001 12030ΔbgsA versus 12030 WT, ** p < 0.001 12030ΔbgsA versus 12030ΔbgsB, * p < 0.001 12030ΔbgsB versus 12030 WT, § p < 0.001 12030Δlgt, § p < 0.001 12030ΔbgsA versus 12030ΔbgsA_lgt, § p < 0.001 12030ΔbgsB versus 12030ΔbgsB_lgt. Results were compared by 2-way ANOVA with Bonferroni post-test for pairwise comparisons.

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Inactivation of the lgt-gene in ΔbgsA had even stronger effects on TNF-α production in the RAW 264.7 macrophage activation assay if culture supernatant was used as stimulant (Fig 2).

ΔbgsA induces a strong activation of TLR2

Next, we wanted to identify the cognate receptor for lipoproteins in ΔbgsA. To this end, wild-type bacteria, ΔbgsA and ΔbgsB were analyzed for NF-κB activation in a NF-κB-dependent luciferase reporter assay in epithelial cells (HEK 293) that stably express the human TLR2 receptor [40]. Whole E. faecalis wild-type bacteria did not activate NF-κB even at high concentrations (Fig 4). In contrast, ΔbgsA induced NF-κB activation at concentrations as low as 1 μg/ml (dry weight). ΔbgsB also induced NF-κB, but was a less potent stimulus than ΔbgsA (Fig 4). Dialyzed, cell-free bacterial cell culture supernatant of wild-type bacteria also did not induce NF-κB activation, while supernatant from ΔbgsA was a strong inducer. Cells were also stimulated with bacterial cell envelope compounds extracted by the Bligh-Dyer method. This method extracts lipids and lipid-containing biomolecules from bacterial or eukaryotic cells [41]. Again, extracts of ΔbgsA but not of the wild-type stimulated TLR2 activation (Fig 4). Compared to whole bacterial cells, cell culture supernatant and lipophilic antigens extracts were about 10-fold less potent agonists of the TLR2 receptor (Fig 4). These data establish that E. faecalis ΔbgsA cells and its culture supernatant contain higher levels of TLR2 agonists compared to wild-type bacteria.

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**Fig 3. Stimulation of TNF-α production in RAW 264.7 mouse macrophages by lipoprotein-enriched cell membrane fractions of E. faecalis wild type and ΔbgsA.** RAW 264.7 cells were incubated with lipoprotein-enriched Triton X-114 extracts from total membrane protein fractions derived from the indicated E. faecalis strains. The concentration of lipoprotein extracts was measured photometrically and normalized to a bacterial cfu:RAW 264.7 cell ratio of 10,000:1. At 16 h, supernatants were collected and TNF-α concentrations were quantified by ELISA. LPS at a concentration of 100 ng/ml was used as positive control. Data represent mean ± SEM of triplicates. * p < 0.001 12030 ΔbgsA versus 12030 WT.

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Inactivation of \textit{bgsA} enhances virulence in a mouse bacteremia model

TLR-mediated activation of cellular innate immunity during infection is tightly regulated, since over- and underactivation can have detrimental consequences to the host. Given the strong engagement of TLR2 by \textit{\Delta bgsA}, we were interested in the effects of the deletion of \textit{bgsA} on enterococcal virulence in a mouse peritonitis model. The LD50 in this model is $5.0 \times 10^9$ bacteria, reflecting the relatively low virulence of \textit{E. faecalis} in vivo [22]. For the mouse peritonitis model we employed a bacterial dose slightly below the LD50. One of 14 mice died after infection with $1.3 \times 10^9$ wild-type bacteria (Fig 5). In contrast, the same inoculum of \textit{\Delta bgsA} killed 10 of 14 mice within 24 h (p = 0.0096). There were no subsequent deaths (total observation time five days). No significant difference in mortality between mice infected with \textit{\Delta bgsB} and those infected with wild-type \textit{E. faecalis} was observed (Fig 5A). We repeated the experiment at a lower inoculum of $3 \times 10^8$ bacteria per mouse. With this lower dose, 2 of 8 mice infected with \textit{\Delta bgsA} died within the observation period (5 days), while all mice infected with the wild-type survived (p = 0.14).

Peritonitis caused by EF12030\textit{\Delta bgsA} is associated with increased pro-inflammatory cytokines

To better understand excess mortality in peritonitis induced by \textit{\Delta bgsA}, we infected mice with an inoculum similar to that used in the survival model ($2.0 \times 10^8$) and quantified the bacterial concentration in the peritoneal cavity, blood, and kidneys at 3 and 12 hours after infection (Fig 5B). No significant difference in bacterial load was noted at corresponding time points. Thus, \textit{\Delta bgsA} does not appear to impede bacterial clearance during peritonitis, and differences in bacterial load cannot explain the differences in mortality.

We also measured leukocytes and inflammatory cytokines during \textit{E. faecalis} peritonitis. Three hours after infection, more leukocytes were recruited to the peritoneal cavity in animals infected with \textit{\Delta bgsA} than in those infected with wild-type bacteria or \textit{\Delta bgsB} (Fig 6). To
determine whether a dysregulated inflammatory response was involved in the increased mortality caused by ΔbgsA, we measured cytokine concentrations in the peritoneal fluid and blood. One hour after infection with ΔbgsA, plasma concentrations of TNF-α, IL-6, and MIP-2 were significantly increased in mice infected with ΔbgsA as compared to mice infected with wild-type bacteria or ΔbgsB (Fig 7). Infection with ΔbgsA resulted also in significantly increased TNF-α concentrations in the peritoneal lavage fluid 3 h after infection compared to mice with peritonitis caused by wild-type bacteria or ΔbgsB (Fig 6B). At 12 h after infection, the kinetics of TNF-α production in the peritoneum had reversed: while low amounts of TNF-α were found in mice infected with ΔbgsA, mice infected with the wild-type strain or ΔbgsB displayed
elevated levels of this cytokine (Fig 6B). These results show a correlation between mortality caused by ΔbgsA and increased production of inflammatory cytokines at early time points.

**Discussion**

Our current work demonstrates the consequences of a disturbed cell membrane glycolipid homeostasis on the expression of lipoproteins in the cell envelope and the activation of the innate immune system by *E. faecalis*. Inactivation of the bgsA gene in *E. faecalis* led to a more than 3-fold higher lipoprotein-content in ΔbgsA compared to wild-type bacteria. ΔbgsA antigens were more potent activators of mouse macrophages and much stronger agonists of the TLR2 receptor. Genetic inactivation of the biosynthesis of lipoprotein strongly reduced the potency of ΔbgsA to stimulate macrophages. Conversely, lipoprotein-enriched Triton X114 extracts from total membrane fractions of ΔbgsA were stronger activators of mouse macrophages.
macrophages than wild type extracts. The alteration of the cell-surface proteome by inactivation of \textit{bgsA} also enhanced the virulence of \textit{E. faecalis} in a mouse peritonitis model.

Immune recognition of enterococci during mouse peritonitis has been described in detail by Leendertse and coworkers [20,22]. Peritoneal infection by \textit{Enterococcus faecium} is sensed predominantly by macrophages via TLR2 which then secrete IL6, TNF-\(\alpha\), MIP-2, and KC during early infection [20,22]. A consecutive influx of neutrophils into the peritoneal cavity then leads to the clearance of the bacteria [20,22]. Working with an infection model similar to the one used by Leendertse, we were able to examine the consequences of overstimulation of the TLR2 system on the natural course of peritoneal infection. Peritonitis due to \(\Delta bgsA\) led to an excessive induction of TNF-\(\alpha\), IL6, and MIP-2 accompanied by a higher influx of leukocytes in the peritoneum and to increased mortality of infected mice. Our results confirm previous studies which have shown that the overproduction of chemokines such as MIP-2 contributes to mortality in animal models of polymicrobial sepsis [42–44].

It is interesting that the effects of the inactivation of \textit{bgsA} and a de-repressed lipoprotein production on the virulence of \textit{E. faecalis} are highly dependent on the type of infection. We reported previously that inactivation of \textit{bgsA} reduces biofilm formation and adherence to colonic cells, while the mutation improves the ability of \textit{E. faecalis} to colonize the urinary tract [15,45]. In a mouse bacteremia model, \(\Delta bgsA\) was cleared more rapidly from the bloodstream than the wild-type strain [15]. Yet during peritoneal infection as reported here, inactivation of \textit{bgsA} induced stronger inflammation and led to higher mortality. Because of the pleiotropic phenotype of \(\Delta bgsA\), these results have to be interpreted with caution [15]. Nevertheless, repression of lipoprotein production by \textit{bgsA} may be advantageous in some host compartments to escape detection by the host immune system while in others, like the peritoneum, virulence is suppressed. Since colonization of the gastrointestinal tract is the default ecological niche of \textit{E. faecalis}, repression of lipoprotein production by \textit{bgsA} probably improves overall fitness by evasion of the mucosal immune system. A similar strategy to subvert recognition by the innate immune system has been described by the intracellular pathogen \textit{Francisella novicida} [46]. The \textit{F. novicida} protein FTN\_0757 specifically represses the production of its lipoprotein FTN\_1103 and deletion of FTN\_0757 leads to stronger stimulation of TLR2 and induction of higher levels of inflammatory cytokines compared to wild-type bacteria [46]. Hence, FTN\_0757 was suggested to function as an immune escape mechanism in \textit{F. novicida}. Furthermore, in \textit{S. aureus}, capsule expression and formation of small colony variants has been described as a means to downregulate TLR2 activation [47].

Our study does not reveal a clear mechanism how the glycolipid mix of the cell membrane leads to an upregulation of lipoprotein concentration. Studies on the role of glycolipids in membrane physiology, however, point to secondary adjustments of the lipid composition in \(\Delta bgsA\) to restore cell membrane homeostasis [48]. The biophysical properties of membrane lipids are determined by the size of their polar head groups in relation to the hydrophobic acylglycerol backbone. MGlcDAG with its smaller polar head group forms inverted nonlamellar structures as opposed to the bilayer conformation of DGlcDAG [49]. This results in a larger negative curvature and increased bilayer curvature stress for membranes composed of MGlcDAG as compared to DGlcDAG [49]. Cell membranes of \(\Delta bgsA\) only contain MGlcDAG and the presence of additional, bilayer forming amphiphiles are most likely needed to dilute the concentration of the non-bilayer forming glycolipid. In \textit{Streptococcus pneumoniae}, for example, deletion of glycosyltransferase \textit{cpoA} leads to an arrest of the synthesis of galactosyl-glucosyl-diacylglycerol and to a secondary increase in the proportion of phosphatidylglycerol to cardiolipin [50]. Lipoproteins also contain large polar head groups and could potentially act as bilayer-prone amphiphiles in the cell membrane of \(\Delta bgsA\). Studies in \textit{Acholeplasma laidlawii} suggest that the activities of the interfacial glycosyltransferases that synthesize MGlcDAG and
DGlcDAG are regulated by the physical properties of the membrane containing their substrates \([48,51]\). Hence, biochemical regulation possibly also governs the compensatory increase of synthesis of lipoproteins in \(\Delta bgsA\). This model could also explain why inactivation of \(\Delta bgsB\) does not cause enhanced activation of the innate immunity or promotes virulence in peritonitis. Mutation of \(bgsB\) in \(E. faecalis\) causes a complete arrest of glycolipid synthesis \([25]\) and the mutant therefore does not overproduce toxic non-bilayer forming glycolipids. Hence, fewer adaptive changes to the cell envelope maybe needed to maintain membrane homeostasis.

Another question raised by our study is, if TLR2 activation by \(\Delta bgsA\) is mediated by a global upregulation of lipoproteins or by one or more specific lipoproteins that act as dominant inducers of TLR2. Upregulation clearly affected lipoproteins unevenly in \(\Delta bgsA\). While a total of 12 lipoproteins were upregulated between 0.19 and 3.11-fold compared to wild-type levels, the expression of EF3041 was increased over 12-fold. Theoretical considerations as well as experimental studies, however, do not support the theory that certain lipoproteins act as dominant inducers of TLR2. Modeling studies of the crystallized structures of the TLR2-TLR6-lipopeptide complex suggest that TLR2 activation by lipopeptides is mediated only by a small and highly conserved structural motif of two ester-bound acyl-chains of at least 12 carbons each and the first two N-terminal amino acids of the polypeptide chain \([52]\). According to this model, the structure of the polypeptide chain beyond the second amino acid is negligible for the potency of lipopeptide or lipoprotein to engage TLR2. Furthermore, in vitro studies of the \(FTN_0757\) deletion mutant in \(F. novicida\) show that equimolar concentrations of lipoproteins from the wild-type strain and \(\Delta FTN_0757\) had a similar potency as TLR2 activators \([46]\).

Taken together, our findings reveal an intimate interplay between the concentration of the bilayer-forming glycolipid DGlcDAG, the expression of lipoproteins, and activation of the host immune system. We show that deletion of \(bgsA\) leads to upregulation of bacterial lipoproteins and strongly enhances host inflammatory response and virulence in enterococcal peritonitis.

Supporting Information

**S1 Table. List of identified proteins by proteomic analysis.** The table includes all protein quantification data in “summary”-tab as well as the number of peptides identified in each single analysis in the tabs WT 1/2/3 and \(bgsA\) 1/2/3. Protein identifications were considered significant for the biological system if the protein was identified in at least two out of three samples in wild type or the mutant.

(XLSX)
S2 Table. List of proteins quantified by spectral abundance factors. (XLSX)

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Author Contributions
Conceived and designed the experiments: CT AKD IS YB AO JH PH DB. Performed the experiments: CT AKD IS DW YB KH MB. Analyzed the data: CT AKD AO IS PH DB JH. Wrote the paper: CT AKD PH JH.

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