Innate Immunity

Original Article

Innate Immunity
2016, Vol. 22(4) 284–293
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DOI: 10.1177/1753425916639669
ini.sagepub.com
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# Deletion of *fabN* in *Enterococcus faecalis* results in unsaturated fatty acid auxotrophy and decreased release of inflammatory cytokines

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## Abstract

The Gram-positive bacterium *Enterococcus faecalis* can cause life-threatening infections and is resistant to several commonly used antibiotics. The type II fatty acid pathway in bacteria is discussed as a potential target for antimicrobial therapy. However, it was shown that inhibition or deletion of its enzymes can be rescued in Gram-positive bacteria by supplementation with fatty acids. Here we show that by deletion of the *fabN* gene, which is essential for unsaturated fatty acid (UFA) synthesis in *E. faecalis,* growth is impaired but can be rescued by supplementation with oleic acid or human serum. Nonetheless, we demonstrate alterations of the UFA profile after supplementation with oleic acid in the Δ*fabN* mutant using a specific glycolipid. In addition, we demonstrate that cytokine release *in vitro* is almost abolished after stimulation of mouse macrophages by the mutant in comparison to the wild type. The results indicate that *fabN* is not a suitable target for antimicrobials as UFA auxotrophy can be overcome. However, deletion of *fabN* resulted in a decreased inflammatory response indicating that *fabN* and resulting UFA synthesis are relevant for virulence.

## Keywords

*Enterococcus faecalis*, *fabN*, fatty acid uptake, virulence, unsaturated fatty acid auxotrophy

Date received: 24 September 2015; revised: 7 February 2016; accepted: 17 February 2016

## Introduction

The type II fatty acid synthesis (FASII) pathway is discussed as potential target for antimicrobial development,[1,2] as there are significant structural differences to the type I fatty acid synthesis pathway in mammals.[2] The antimicrobials platencin and platensimycin inhibit the fatty acid acyl carrier protein synthase II and the fatty acid acyl carrier protein synthase III of the FASII pathway, and have been tested in preclinical trials against methicillin-resistant *Staphylococcus aureus* strains.[3] Platensimycin is also discussed as a potential anti-tuberculosis drug.[3] The selective antistaphylococcal enoyl-acyl carrier protein (ACP) reductase (FabI) inhibitor AFN-1252 is even already under investigation in human trials.[4] The *fabN* gene from *Enterococcus faecalis* encodes for a 3-hydroxyacyl-[acyl-carrier-protein]-dehydratase (3-hydroxyacyl-ACP-dehydratase) (EC: 4.2.1.59) that is also an enzyme in the FASII pathway. The FabN enzyme was first described by Wang and

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Cronan,[5] and defined as a bifunctional dehydratase/isomerase. It catalyzes the first step in the synthesis of unsaturated fatty acids by dehydration of β-hydroxydecanoyl–ACP and subsequent isomerization to *cis*-3-decenoyl–ACP,[6] which is a substrate for further unsaturated fatty acid (UFA) elongation.[7] In *Streptococcus pneumoniae* and *Streptococcus mutans*, the functional closely related FabM is an isomerase, essential for UFA synthesis in these pathogens.[8] Growth was impaired when *fabM* was deleted but was restored by the addition of UFAs to the media or by functional replacement of the deleted *fabM* by *fabN* from *E. faecalis*.[8] This strategy was also applied for the *fabA* deletion mutant in the Gram-negative bacterium *Escherichia coli*.[5] FabA is the essential enzyme for UFA synthesis in *E. coli* and catalyzes the isomerase/dehydratase reaction to *cis*-3-decenoyl–ACP.[5,9] Upon replacement of *fabA* with *fabN* from *E. faecalis,* only small amounts of UFAs were produced, owing to substrate competition with the enoyl reductase FabI. Altogether, these findings demonstrate the versatility of FabN for the catalysis of unsaturated fatty acid synthesis in different bacterial species. We hypothesized that by deleting *fabN,* growth of *E. faecalis* would be impaired, owing to the described potential essentiality of the enzyme for UFA synthesis. Additionally, we wanted to find out whether the host inflammatory response was altered in the mutant. We have shown before that *E. faecalis* 12030 is a strong biofilm producer,[10,11] and that glycolipids are involved in this process.[11,12] In addition, we have detected a strong inflammatory reaction *in vitro* and *in vivo*, when the glycosyltransferase BgsA, transferring the second Glc moiety yielding diglycosyl-diacylglycerol (DGlcDAG) was deleted and the glycolipid monoglycosyl-diacylglycerol (MGlcDAG), containing unsaturated fatty acids, accumulated.[11,13] Therefore, we wanted to investigate whether there is a connection between microbial UFAs and the immune response.

## Materials and methods

### Bacterial strains, plasmids and culture conditions

The pMAD Gram-positive, temperature-sensitive mutagenesis shuttle vector has been described previously.[14] *Enterococcus faecalis* 12030 strain was grown in tryptic soy broth (TSB; Carl Roth, Karlsruhe, Germany) medium or on TSA plates (Carl Roth) at 37°C for 18 h.[15] For growth of *E. faecalis* 12030Δ*fabN* TSB media or TSA plates were supplemented with 0.1 mM oleic acid (Sigma Aldrich, St. Louis, MO, USA). When required, medium was supplemented with 50 µg/ml erythromycin (Carl Roth). *Escherichia coli* XL-1-blue (Invitrogen, Carlsbad, CA, USA), containing pMAD, was grown in lysogeny broth supplemented with 100 µg/ml ampicillin (Carl Roth) at 30°C with agitation (200 rpm) for 48 h. For blue/white selection, agar plates were supplemented with 80 µg/ml X-gal (Applichem, Chicago, IL, USA).

### Construction of Δ*fabN*

A non-polar deletion of a portion of *fabN* (*ef* 0284 in *E. faecalis* 12030), encoding for the enzyme (*R*)-3-hydroxymyristoyl–ACP dehydratase, was created using the method described previously.[16] Genomic DNA was isolated from *E. faecalis* 12030 via the MasterPure Gram Positive DNA Purification Kit (Illumina, Madison, WI, USA). Primer pair 0284_P1_fw_BamHI (CTCACCA**GGATCC**GGATATGGCAGCAACTGTGATGCTA) and 0284_P2 rv (ACTAGCGCGGCCGCTTGCTCCCGGATAGCGATTAGGAATCATTTCCATA) amplified a 528-bp fragment upstream of *fabN* and a part of the terminal sequence of *ef* 0283. The primer pair 0284_P3 fw (GGAGCAAGCGGCCGCGCTAGTGACGTTTATTGTGGGACGATAAGAATCA) and 0284_P4_rv_SalI (GAGTGGT**GTCGAC**GCAAGCCCATTGAGTTAATACTTCCTA) were used to amplify a 389-bp fragment downstream of *ef* 0284, and the beginning of *ef* 0285 (restriction enzyme sites are indicated in bold). Primers 2 and 3 contain a 21-bp complementary sequence (underlined). Overlap extension PCR was used to create a PCR product lacking 411 bp of the *ef* 0284 gene. The overlap construct was cloned into the multiple cloning site of the shuttle vector pMAD.[14] Restriction and modifying enzymes were obtained from ThermoScientific (Waltham, MA, USA) and used following the manufacturer's instructions. The resulting plasmid pMAD::Δ*fabN* was transformed into *E. coli* XL-1-Blue chemocompetent cells. For selection of positive clones, 100 µg/ml ampicillin and 80 µg/ml X-gal were used. Successful transformation was confirmed by PCR with primer pair pMAD-1F (TCTAGCTAATGTTACGTTACAC) and pMAD-1R (TCATAATGGGGAAGGCCATC), and by digestion with the appropriate restriction enzymes. The generated recombinant plasmid was transformed into electro-competent *E. faecalis* 12030 cells prepared as described by Cruz-Rodz and Gilmore.[17] Gene replacement was performed as described previously,[18] with additional supplementation to the media by 0.1 mM oleic acid. Deletion of the gene was verified by sequencing (GATC Biotech AG, Konstanz, Germany).

### Growth kinetics

Overnight cultures of *E. faecalis* 12030 and the *E. faecalis* 12030Δ*fabN* mutant were adjusted to an $OD_{600}$ of 0.1 in TSB medium without or with supplementation with 0.1 mM oleic acid (TSBO). For determination of growth by the mutant in human serum, medium was prepared out of 50% TSB and 50% human serum (filtered sterile). As before, 0.1 mM oleic acid was added to half of the samples. Again, the starting $OD_{600}$ was adjusted with overnight cultures, grown for 18 h at 37°C, to 0.1. The cultures supplemented with human serum or only oleic acid were grown at same conditions at 37°C at 200 rpm. $OD_{600}$ was measured hourly over a time span of 6 h until stationary phase was reached.

### Membrane lipid extraction

Crude lipid extraction was done following the method of Bligh and Dyer,[19] with a few modifications. *Enterococcus faecalis* 12030 or *E. faecalis* 12030Δ*fabN* were incubated for 18 h at 37°C without agitation in TSB or TSBO, respectively. The cultures were spun down (1800 *g*, 20 min, 4°C) and the cell pellet was re-suspended in 20 ml NaAc-buffer [0.1% sodium acetate (w/v); pH 4.7] and pelleted again. The cell pellet was re-diluted in 10 ml NaAc buffer and glass beads with a diameter of 0.1 mm (Carl Roth) were added 1:1 to the suspension. With a BeadBeater (Glenn Mills, Clifton, NJ, USA) or, alternatively, by vortexing, cells were disrupted. After the glass beads had settled or had been spun down (42,000 *g*, 20 min, 4°C) the cell suspension was transferred to a new tube and centrifuged (7000 *g*, 20 min, 4°C). The supernatant was discarded and NaAc buffer, methanol and chloroform were added in ratios of 0.8:2:1 to the cell pellet and incubated for lipid extraction for 2 h at room temperature (RT; 22°C) while stirring. The suspension was centrifuged in polytetrafluoroethylene tubes (ThermoFisher Scientific, Waltham, MA, USA) (1800–7000 g, 15 min, 4°C). The supernatants, containing the extracted lipids, were combined and the cell pellet was re-diluted using the same ratio as before, stirred for an additional hour and separated again by centrifugation. The supernatants were combined and chloroform and NaAc buffer were added in volumes of 1:1:1 to the supernatant, mixed briefly and the organic and aqueous phase were separated again by centrifugation for 15 min. The lower organic phase containing the lipids was combined and separated again by centrifugation until only the organic solvent, containing the lipids, remained. By evaporation, using a rotary vacuum evaporator, the chloroform was separated from the extract. It was important that the temperature did not exceed 40–50°C, to decrease oxidation of double bonds. Subsequently, the lipid extract was dried under a fluent stream of nitrogen. Until use, lipids were stored in chloroform or under nitrogen atmosphere at −20°C.

### RAW 264.7 mouse macrophage stimulation

For determination of cytokine formation, RAW264.7 mouse macrophages (a generous gift from the laboratory of Philip Bufler, Children's Hospital, Munich, Germany) were seeded at a density of $1 \times 10^5$ cells/ml in 24-well dishes in DMEM (high Glc, GlutaMAX$^{TM}$, supplemented with 10% FCS and 100 U/ml Pen/Strep). The adherent cells were washed once with PBS and stimulated with sterile filtered (0.22 µm) supernatants of overnight cultures from mutant and wild type, adjusted to a protein concentration of 300 µg/ml by photometric determination. Cultures were incubated for 16 h at 37°C in a 5% humidified $CO_2$ environment. Stimulation assays were performed at cell passages 12–15. The TLR2 agonist Pam2CSK4 (R&D Systems, Minneapolis, MN, USA) was used as positive control. Cytokines were measured by ELISA using commercially available kits (eBioscience, San Diego, CA, USA). Statistical significance for two-way comparisons was determined by an unpaired *t*-test as indicated.

### Visualization of lipids

Lipid extracts, diluted in chloroform, were applied onto a thin-layer chromatography (TLC) plate (0.1 mM Silica gel 60 $F_{254nm}$; Merck, Darmstadt, Germany), which was developed in a TLC development chamber in $CHCl_3/MeOH/H_2O$ [65:25:4; (v/v/v)] running buffer. To visualize total lipid extract, the plate was stained with molybdenum [5% $H_2SO_4$ (97%) (v/v); 0.1% $Ce(SO_4) \times 4\ H_2O$ (w/v); 5% $(NH_4)_6Mo_7O_{24} \times H_2O$ (w/v)],[20] air-dried and developed at 150°C for 5 min. For visualization of glycolipids, the plate was stained with Molisch's reagent [82% MeOH (v/v), 10% $H_2SO_4$ (97%) (v/v); 3.2% α-naphthol $C_{10}H_8O$ (w/v)] and developed at 150°C for 5 min.

### Purification of MGlcDAG via silica gel column chromatography

A glass column (NS 14/23 porosity: 0; Carl Roth) was filled with 7–10 ml silica gel (0.04–0.063 mm silica gel 60; Merck). The lipid extract, solved in chloroform, was added slowly onto the dry, even surface of the silica gel. By increasing the polarity of the eluent via decreasing the $CHCl_3/MeOH$ (v/v) ratio (100:0; 97:3; 95:5; 92:8; 90:10; 50:50) fractionation of the lipids occurred. The fractions were each eluted with a total volume of 100 ml. The eluents were removed again by rotary evaporation. Via TLC the combined fractions were analyzed for purity.

### Determining the fatty acid composition of MGlcDAG

The fatty acid composition of MGlcDAG of the wild type and the ΔFabN mutant was determined after methanolysis (2 M HCl/MeOH, 85°C, 2 h), acetylation (85°C, 10 min) and detection by GC/MS. GC/MS analyses of all samples were performed on an Agilent

Technologies 7890A gas chromatograph equipped with a dimethylpolysiloxane column [HP Ultra 1, 12 m × 0.2 mm × 0.33 µm film thickness and 5975C series MSD detector with electron impact ionization (EI) mode under autotune condition at 70 eV (Agilent, Santa Clara, CA, USA)]. The temperature program was 70°C for 1.5 min, then 60°C $\text{min}^{-1}$ to 110°C and 5°C $\text{min}^{-1}$ to 320°C for 10 min. A reference probe (Bacterial Acid Methyl Ester Mix; Sigma Aldrich) with known lipid composition and elution profile was measured under the same conditions.

### Preparation of picolinyl esters

For the exact determination of the position of the double bond in 18:1 fatty acid, fatty acids of MGlcDAG from the wild type and ΔFabN mutant were derivatized to 3-pyridylcarbinol ('picolinyl') esters.[21] The fatty acids were released (1 M NaOH–MeOH, 1 h, 85°C), recovered in $CHCl_3$, treated with trifluoroacetic anhydride (1 h, 50°C) and subsequently with 20% (w/v) 3-pyridinemethanol solution in tetrahydrofuran (1 h, 50°C) and injected in GC/MS as described above.

### Biofilm formation assay

Biofilm formation was measured as described previously.[22] Briefly, TSB media supplemented with 1% Glc (w/v) and 0.1 mM oleic acid (TSBGO) was inoculated and incubated at 37°C for 18 h. Polystyrene tissue culture plates were filled with 198 µl TSBGO media and 2 µl of the overnight culture. The plate was incubated 18 h at 37°C. After the incubation period $OD_{630}$ was measured. Supernatants were discarded and wells were washed twice with 200 µl PBS. The plate was dried for 1.5 h at 50–60°C and subsequently 100 µl crystal violet solution (Sigma Aldrich) was added and incubated for 2 min at RT. The solution was discarded, the plate rinsed thoroughly with tap water and dried again at 50–60°C for 15 min. $OD_{630}$ was measured and normalized to growth with the biofilm index [biofilm index = OD biofilm × (0.5)/(OD growth)].

## Results

### Deletion of EF0284

The described *fabN* (*ef*0284) gene from *E. faecalis* V583 shares 99.08% nucleotide identity to its homologue gene in *E. faecalis* 12030.[5] We hypothesized that by deleting *fabN*, *E. faecalis* 12030 would not be able to synthesize UFAs including vaccenic acid (18:1 $\Delta^{11}$). To characterize the role of *fabN*, we created a non-polar deletion mutant using targeted mutagenesis.[16] Deletion mutants could only be successfully isolated by supplementation of TSB media with 0.1 mM oleic acid during the second cross-over event to overcome UFA auxotrophy. Without supplementation the *fabN* mutant had a pronounced growth defect and formed small colonies on tryptic soy agar plates approximately two-thirds smaller than the corresponding wild type.

### Deletion of fabN results in UFA auxotrophy

Growth of the mutant in un-supplemented TSB resulted in a maximal (±SEM) growth to an $OD_{600}$ of $0.52 \pm 0.01$, whereas maximum growth for the wild type was three times higher with an $OD_{600}$ of $1.73 \pm 0.03$ (Figure 1). With addition of 0.1 mM oleic acid the growth defect was partly overcome. Maximal growth for Δ*fabN* doubled to an $OD_{600}$ of $1.13 \pm 0.04$. However, growth of the wild type was not altered by supplementation of oleic acid ($OD_{600}$ $1.73 \pm 0.01$ vs. $1.73 \pm 0.03$ in the un-supplemented culture) (Figure 1). This shows that *fabN* is essential for growth, when no exogenous UFAs are available. As was also shown previously,[23] *E. faecalis* is capable of using exogenous UFAs.

### UFA auxotrophy can be overcome by growth in human serum

UFA auxotrophy in deletion mutants of *Streptococcus agalactiae* or by inhibition of the FASII pathway of Gram-positive pathogens was overcome under *in vivo* conditions by adding human serum to the growth media. Human serum contains usually about 1.88 mM oleic acid, which is the third most abundant fatty acid.[24] Therefore, we tested if growth of the *fabN* deletion mutant could also be restored by adding human serum (Figure 2). Growth of the Δ*fabN* mutant increased to similar levels ($1.24 \pm 0.02$ with and $1.33 \pm 0.11$ without oleic acid) as the wild type ($1.30 \pm 0.11$ with and $1.20 \pm 0.01$ without oleic acid).

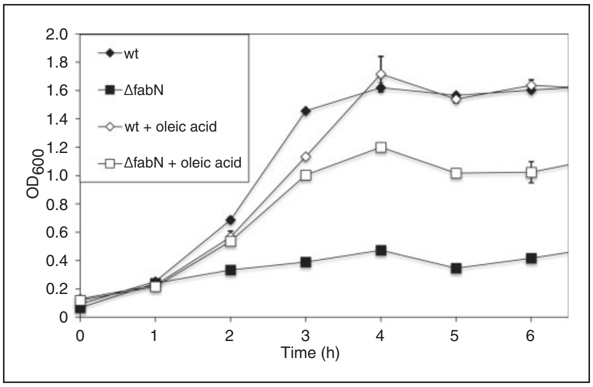

**Figure 1.** Growth of wild type (wt) and *E. faecalis* 12030Δ*fabN* strain in TSB or TSBO, respectively. Values are represented as averages ± SEM (*n* = 3). Growth curve measurements were performed independently in triplicate.

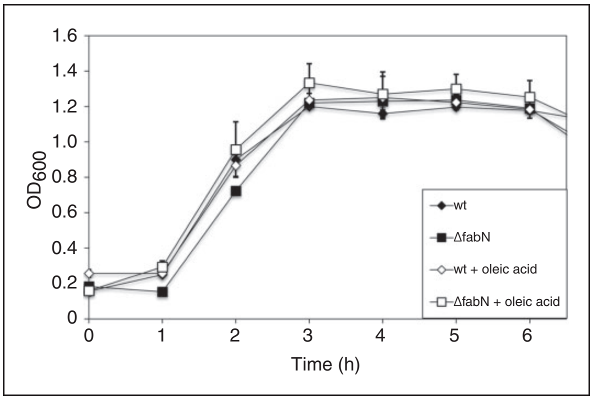

**Figure 2.** Growth of wild type (wt) and *E. faecalis* 12030Δ*fabN* strain in 50% (v/v) human serum in TSB or TSBO, respectively. Values are represented as averages ± SEM ($n = 3$). Growth curve measurements were performed independently in triplicate.

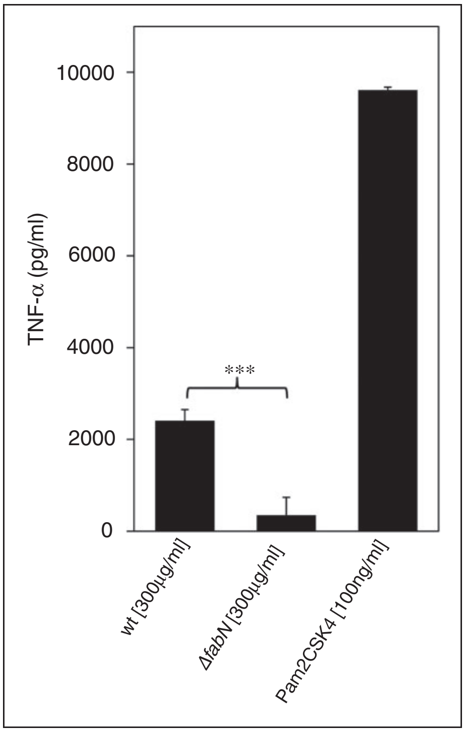

**Figure 3.** Induction of TNF-α in RAW 264.7 mouse macrophages by *E. faecalis* 12030 wild type (wt) and Δ*fabN* mutant. Macrophages were stimulated with cell-free, sterile *E. faecalis* supernatants adjusted to a protein concentration of 300 µg/ml. After 16 h of incubation, supernatant from macrophage culture was analyzed for TNF-α by ELISA. Pam2CSK4 was used as positive control. Data represent mean ± SEM of triplicates. $^{***}P < 0.001$ *E. faecalis* 12030 wt vs. *E. faecalis* 12030 Δ*fabN* with *t*-test for unpaired comparisons. The assay was performed in duplicate independently.

Altogether, these results show that the effect of *fabN* deletion and subsequent UFA auxotrophy can be overcome under *in vivo* conditions.

### *Deletion of* fabN *almost completely abolishes induction of TNF-α in RAW 264.7 mouse macrophages*

To evaluate the biological effects of the deletion and the potentially changed lipid membrane composition, we stimulated mouse macrophages with supernatant from the wild type and mutant adjusted to same protein concentrations of 300 µg/ml (Figure 3). As in our previous work we observed that *E. faecalis* 12030 sheds cell membrane lipids into the media (personal observation),[25] we argued that changed lipid content in the supernatant would resemble changed membrane lipid composition. After 16 h, we measured the release of the cytokine TNF-α in the cell culture supernatant. TNF-α production in RAW264.7 cells was almost completely abrogated after stimulation with the mutant and, overall, sevenfold lower than the wild type strain. Therefore, our data suggest that impairment of UFA synthesis reduces the capability of culture supernatants to induce TNF-α production by macrophages. This indicates that microbial unsaturated acids potentially play a role in the induction of an inflammatory response.

### *Fatty acid composition of MGlcDAG*

In order to prove to what extent the mutation in the *fabN* gene changed the chemical composition of the fatty acids using the example of a single glycolipid, total lipids of the wild type and the Δ*fabN* mutant were extracted and fractionated as described. The α-naphthol stain (Figure 4B, D) shows that a glycolipid with an Rf ~ 0.75 was eluted with 95:5 (Figure 4A, B, lane 3) or 92:8 $CHCl_3$/MeOH (Figure 4C, D, lane 4) for the wild type or Δ*fabN* mutant, respectively, and was identified as MGlcDAG compared with the lipid profile of *E. faecalis* 12030Δ*bsgA* strain overproducing MGlcDAG (Figure 4A, B, lane 1).[11] The composition of the fatty acids of MGlcDAG from the wild type, as determined by GC/MS with the use of authentic standard of bacterial acid methyl ester (Sigma Aldrich), revealed the presence of glycerol, hexose, 16:1, 16:0, 18:1 and 18:0 (Figure 5A). This analysis, however, did not allow us to differentiate completely between 18:1 $\Delta^9$ and 18:1 $\Delta^{11}$, as methyl esters of these fatty acids partially elute with the same retention time (21.753 min). Thus, picolinyl ester derivatives were prepared (data not shown) and it was shown that in the glycolipid from wild type, mainly $\Delta^{11}$ of 18:1 was present, with only traces of 18:1 $\Delta^9$. The exact position (sn-1 or sn-2) of the different fatty acids on the glycerol backbone remains to be elucidated. The methanolysis of MGlcDAG from Δ*fabN* showed the presence of glycerol, hexose, 16:0, 18:1 $\Delta^9$, 18:1 $\Delta^{11}$ and 18:0 (Figure 5B). The picolinyl derivates (data not shown) revealed the presence of both forms of 18:1, with the ratio 2:1 18:1 $\Delta^9$ and 18:1 $\Delta^{11}$, respectively. Taken

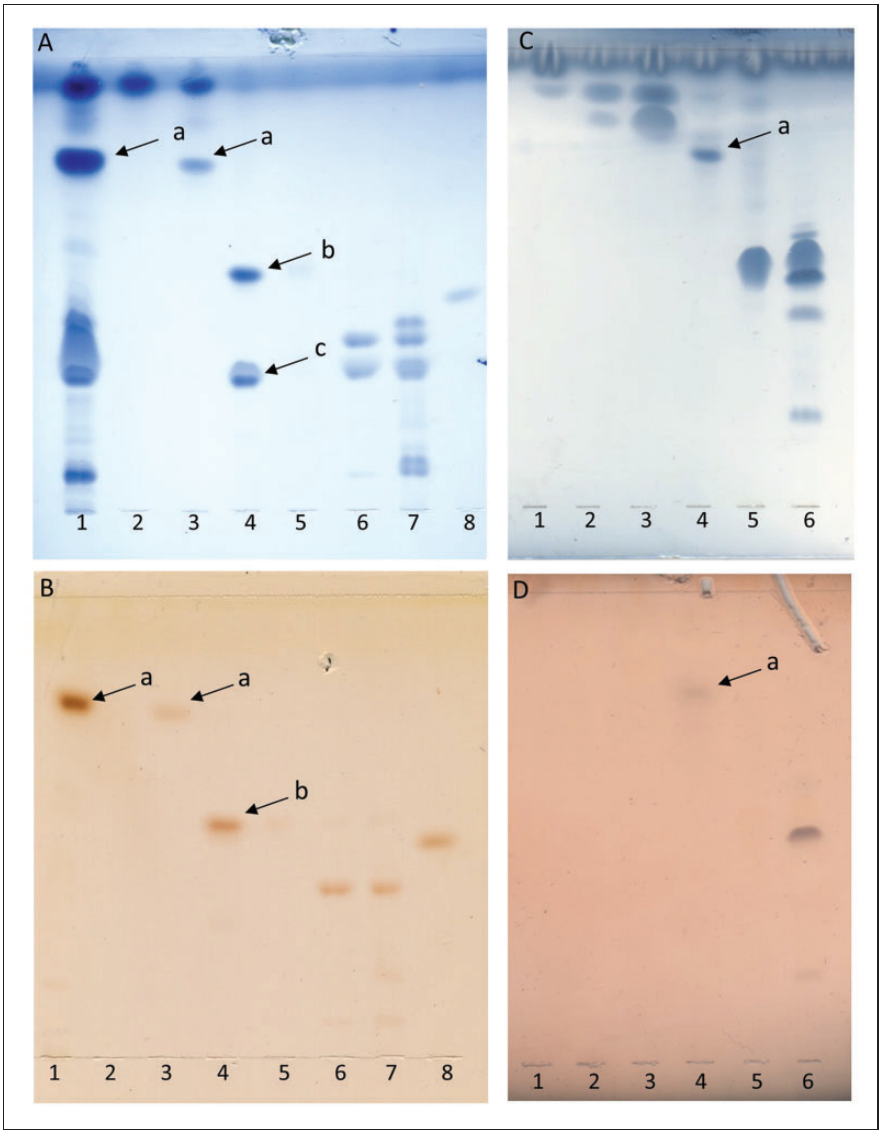

**Figure 4.** TLC of fractionated lipid extracts of *E. faecalis* 12030 wild type (A, B). From silica gel column eluted fractions with $CHCl_3$ (100) (lane 2), $CHCl_3$/MeOH (95:5) (lane 3), $CHCl_3$/MeOH (90:10) (lane 4), $CHCl_3$/MeOH (85:15) (lane 5), $CHCl_3$/MeOH (80:20) (lane 6) and $CHCl_3$/MeOH (50:50) (lane 7). Twenty μg crude lipid extract of *E. faecalis* 12030Δ*bgsA* accumulating MGlcDAG (lane 1) was loaded as ladder. Twenty μg DGlcDAG (lane 8) as positive control. Fractions are concentrated, applied to a TLC plate, developed in $CHCl_3$/MeOH/$H_2O$ [65:25:4 (v/v/v)] and visualized with (A) molybdenum or (B) α-naphthol. Arrows are indicating the glycolipid bands and the cardiolipin contamination. MGlcDAG; Rf ~ 0.74 (a), DGlcDAG; Rf ~ 0.49 (b) and cardiolipin (verified by MS) (c). TLC of fractionated lipid extracts of *E. faecalis* 12030Δ*fabN* (C, D). Fractions eluted with $CHCl_3$ (100) (lane 1), $CHCl_3$/MeOH (97:3) (lane 2), $CHCl_3$/MeOH (95:5) (lane 3), $CHCl_3$/MeOH (92:8) (lane 4), $CHCl_3$/MeOH (90:10) (lane 5) and $CHCl_3$/MeOH (50:50) (lane 6). Fractions were concentrated, applied to a TLC plate, developed in $CHCl_3$/MeOH/$H_2O$ [65:25:4 (v/v/v)] and visualized with molybdenum (C) or α-naphthol (D). Arrows are indicating the MGlcDAG spots.

together the analyses showed that by deletion of *fabN* the composition of unsaturated fatty acid structures in *E. faecalis* changed (detection of supplemented 18:1 $\Delta^9$); however, the mutant still possessed a decreased amount of 18:1 $\Delta^{11}$.

### *Biofilm formation of* ΔfabN

*Enterococcus faecalis* mutant 12030Δ*fabN* did not show significant differences compared with the wild type in regard to biofilm formation on plastic surfaces (Figure 6).

## Discussion

Here we analyzed how deletion of the enzyme FabN, catalyzing the first step in unsaturated fatty acid synthesis in *E. faecalis*, affects growth kinetics, the inflammatory response of mouse macrophages and composition of fatty acids, using the glycolipid

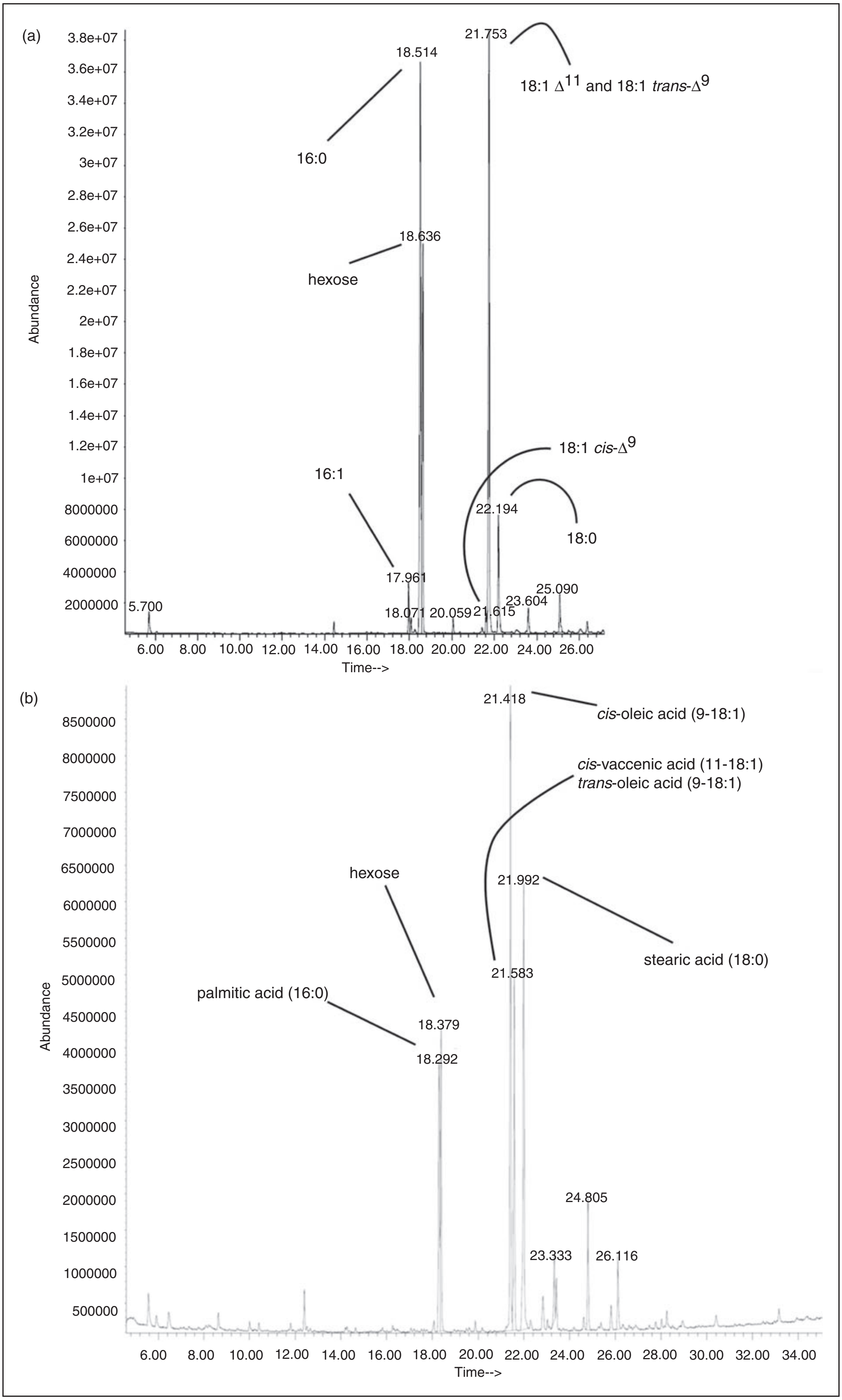

**Figure 5.** GC/MS chromatogram of fatty acid methyl ester of MGlcDAG purified from wild type (a) and methyl esters MGlcDAG purified from the *E. faecalis* 12030Δ*fabN* mutant (b).

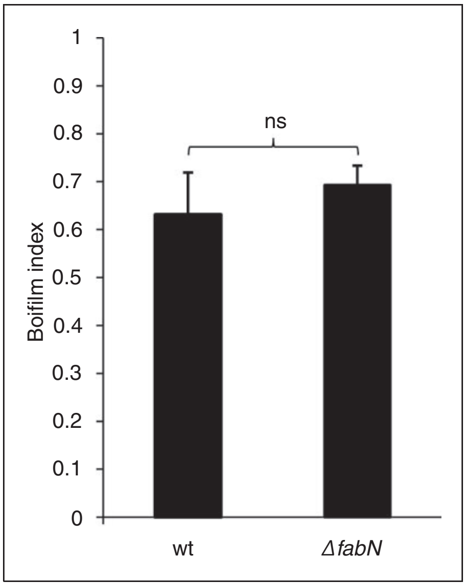

**Figure 6.** Biofilm formation on plastic surfaces by *E. faecalis* 12030 wild type (wt) and Δ*fabN* grown in TSBGO for 18 h at 37°C. Biofilm index was calculated correlating growth to biofilm [biofilm index = OD biofilm × (0.5)/(OD growth)]. Data represent mean ± SEM ($n = 6$). ns: not significant; *E. faecalis* 12030 wild type vs. *E. faecalis* 12030Δ*fabN* with *t*-test for unpaired comparisons. The assay was performed in duplicate independently.

MGlcDAG as example. The deletion of *fabN* resulted in UFA auxotrophy, which was partly overcome by supplementation of the culture medium with the exogenous unsaturated fatty acid oleic acid or human serum. This phenotype was also previously reported for *S. mutans*, *S. pneumoniae* and *S. agalactiae fabM*-null mutants.[8,26] It has been previously reported that UFA auxotrophy caused by deletion or inhibition through antimicrobials of different enzymes in the FASII pathway of Gram-positive pathogens could be overcome by supplementation with unsaturated fatty acids.[26] Our findings corroborate previous studies by Zhu et al.,[27] who suggested that enzymes of the FASII pathway in *E. faecalis* are not a suitable target for antimicrobials. They reported that UFA auxotrophy in the enoyl-ACP reductase (FabI) deletion mutant was rescued by supplementation with oleic acid.[27] Similar observations were made in *S. pneumoniae*, in which exogenous fatty acids could replace *de novo* synthesized fatty acids.[28] Nevertheless, we could demonstrate that deletion of *fabN* resulted in a reduced inflammatory response by macrophages. Although growth cannot be inhibited by antimicrobials targeting the enzyme, they might possibly reduce the inflammatory potency of the pathogen. Targeting bacterial virulence (e.g. with Abs neutralizing toxins) reduces the pressure for drug-resistant mutations and protects the host microbiota against detrimental changes.[29] However, there are only a few antimicrobials that inhibit *fabN* homologs, which, to our knowledge, have not yet been tested in enterococci.[1,30,31] Integration of UFAs into immunogenic compounds have an influence on their inflammatory potency. It has been shown recently that incorporation of exogenous unsaturated fatty acids into lipoproteins of *S. aureus* was connected to an enhanced inflammatory response by human monocytes and HEK–TLR2 cells.[32] Lipoproteins are the most pro-inflammatory compounds in the Gram-positive cell wall.[33,34] Additionally, in some glycolipids the importance of the double bond of the fatty acids for recognition by *i*NKT cells and subsequent initiation of an inflammatory response was reported.[35,36] Therefore, an altered unsaturated fatty acid profile in the Δ*fabN* mutant likely affects its inflammatory characteristics. We hypothesized that by deletion of *fabN* the structure of the glycolipid MGlcDAG would change from vaccenic acid to the supplemented oleic acid. We found recently that an *E. faecalis* 12030 deletion mutant, accumulating the glycolipid MGlcDAG, showed a strongly increased inflammatory phenotype due to an up-regulated lipoprotein content,[13] making the glycolipid an interesting target for analysis of a potential altered lipid composition. We found, in fact, a changed lipid profile for the glycolipid synthesized by the Δ*fabN* mutant, with oleic acid and palmitic acid being the most prominent fatty acids, but there was still vaccenic acid present. Analysis of the fatty acid composition of *fabM* mutants in *S. pneumoniae* and *S. mutans* showed that vaccenic acid is not detected when oleic acid is supplemented.[8] Taken together, these results suggest that in *E. faecalis*, FabN is not the only enzyme that is capable of synthesizing 18:1 $\Delta^{11}$. Another enzyme, designated as FabZ (*ef*2878) with 58.7% identical residues to FabN,[5] was identified in *E. faecalis* V583. Wang and Cronan showed that replacement with *fabN* by the functional homolog dehydratase/isomerase in the *E. coli* Δ*fabA* mutant restored unsaturated fatty acid synthesis. However, FabZ from *E. faecalis* could not restore synthesis of unsaturated fatty acids in the *fabA* deletion mutant, but traces of unsaturated fatty acids could be detected. Therefore, FabZ seems to be capable of synthesizing unsaturated fatty acids but with a rather low activity. The active site residues of FabA[8] are mostly conserved in FabN and FabZ. Domain swapping of FabN and FabZ[6] showed that β-sheets direct the form of the α-helix forming the substrate-binding tunnel. β-Sheets in FabN are differently positioned than in FabZ and are placing the substrate in the suitable position for isomerization.[6] Based on these findings it is conceivable that FabZ is able, with a rather low activity and affinity, to synthesize traces of unsaturated fatty acids.

In conclusion, we could demonstrate that the deletion of *fabN* in *E. faecalis* results in UFA auxotrophy, which can be complemented by supplementation either with oleic acid or human serum. Deletion of *fabN* results in a changed lipid profile in the glycolipid MGlcDAG, although the UFA vaccenic acid was still detected, suggesting another enzyme is capable of synthesizing vaccenic acid with lower activity than FabN. Cytokine release by stimulation of mouse macrophages with the mutant was almost completely abrogated. Hence, although UFA auxotrophy can be overcome and FabN is not a target for antimicrobials killing *E. faecalis*, inhibition of FabN could result in a less pathogenic phenotype and be an objective for anti-virulence strategies, which are a promising alternative to treat bacterial infections.[29] Further *in vivo* studies are required to explore the suitability of FabN and orthologue enzymes as targets for anti-virulence strategies.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

## References

1. Zhang Y-M, White SW and Rock CO. Inhibiting bacterial fatty acid synthesis. *J Biol Chem* 2006; 281: 17541–17544.
2. Wright HT and Reynolds KA. Antibacterial targets in fatty acid biosynthesis. *Curr Opin Microbiol* 2008; 10: 447–453.
3. Allahverdiyev AM, Bagirova M, Abamor ES, et al. The use of platensimycin and platencin to fight antibiotic resistance. *Infect Drug Resist* 2013; 6: 99–114.
4. Parsons JB, Yao J, Frank MW, et al. FabH mutations confer resistance to FabF-directed antibiotics in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2015; 59: 849–858.
5. Wang H and Cronan JE. Functional replacement of the FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues. *J Biol Chem* 2004; 279: 34489–34495.
6. Lu Y-J, White SW and Rock CO. Domain swapping between *Enterococcus faecalis* FabN and FabZ proteins localizes the structural determinants for isomerase activity. *J Biol Chem* 2005; 280: 30342–30348.
7. Parsons JB and Rock CO. Bacterial lipids: metabolism and membrane homeostasis. *Prog Lipid Res* 2013; 52: 249–276.
8. Altabe S, Lopez P and de Mendoza D. Isolation and characterization of unsaturated fatty acid auxotrophs of *Streptococcus pneumoniae* and *Streptococcus mutans*. *J Bacteriol* 2007; 189: 8139–8144.
9. Zhu L, Cheng J, Luo B, et al. Functions of the *Clostridium acetobutylicium* FabF and FabZ proteins in unsaturated fatty acid biosynthesis. *BMC Microbiol* 2009; 9: 119.
10. Fabretti F, Theilacker C, Baldassarri L, et al. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. *Infect Immun* 2006; 74: 4164–4171.
11. Theilacker C, Sanchez-Carballo P, Toma I, et al. Glycolipids are involved in biofilm accumulation and prolonged bacteraemia in *Enterococcus faecalis*. *Mol Microbiol* 2009; 71: 1055–1069.
12. Theilacker C, Sava I, Sanchez-Carballo P, et al. Deletion of the glycosyltransferase bgsB of *Enterococcus faecalis* leads to a complete loss of glycolipids from the cell membrane and to impaired biofilm formation. *BMC Microbiol* 2011; 11: 67.
13. Theilacker C, Diederich A-K, Otto A, et al. *Enterococcus faecalis* glycolipids modulate lipoprotein-content of the bacterial cell membrane and host immune response. *PLoS One* 2015; 10: e0132949.
14. Arnaud M, Chastanet A and De M. New vector for efficient allelic replacement in naturally Gram-positive bacteria. *Appl Environ Microbiol* 2004; 70: 6887–6891.
15. Huebner J, Wang Y, Krueger WA, et al. Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. *Infect Immun* 1999; 67: 1213–1219.
16. Bao Y, Sakinc T, Laverde D, et al. Role of mprF1 and mprF2 in the pathogenicity of *Enterococcus faecalis*. *PLoS One* 2012; 7: e38458.
17. Cruz-Rodz AL and Gilmore MS. High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. *Mol Gen Genet* 1990; 224: 152–154.
18. Hébert L, Courtin P, Torelli R, et al. *Enterococcus faecalis* constitutes an unusual bacterial model in lysozyme resistance. *Infect Immun* 2007; 75: 5390–5398.
19. Bligh EG and Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; 37: 911–917.
20. Peschel A, Jack RW, Otto M, et al. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med* 2001; 193: 1067–1076.
21. Christie WW, Brechany EY, Johnson SB, et al. A comparison of pyrrolidide and picolinyl ester derivatives for the identification of fatty acids in natural samples by gas chromatography-mass spectrometry. *Lipids* 1986; 21: 657–661.
22. Baldassarri L, Cecchini R, Bertuccini L, et al. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. *Med Microbiol Immunol* 2001; 190: 113–120.
23. Saito HE, Harp JR and Fozo EM. Incorporation of exogenous fatty acids protects *Enterococcus faecalis* from membrane-damaging agents. *Appl Environ Microbiol* 2014; 80: 6527–6538.
24. Nakamura T, Azuma A, Kuribayashi T, et al. Serum fatty acid levels, dietary style and coronary heart disease in three neighbouring areas in Japan: the Kumihama study. *Br J Nutr* 2003; 89: 267–272.
25. Diederich AK, Wobser D, Spiess M, et al. Role of glycolipids in the pathogenesis of *Enterococcus faecalis* urinary tract infection. *PLoS One* 2014; 9: e96295.
26. Brinster S, Lamberet G, Staels B, et al. Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. *Nature* 2009; 458: 83–86.
27. Zhu L, Bi H, Ma J, et al. The two functional enoyl-acyl carrier protein reductases of *Enterococcus faecalis* do not mediate triclosan resistance. *MBio* 2013; 4: e00613–13.
28. Parsons JB, Frank MW, Subramanian C, et al. Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. *Proc Natl Acad Sci U S A* 2011; 108: 15378–15383.
29. Cegelski L, Marshall GR, Eldridge GR, et al. The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* 2008; 6: 17–27.
30. Sharma SK, Kapoor M, Ramya TNC, et al. Identification, characterization, and inhibition of *Plasmodium falciparum* beta-hydroxyacyl-acyl carrier protein dehydratase (FabZ). *J Biol Chem* 2003; 278: 45661–45671.
31. Gratraud P, Surolia N, Besra GS, et al. Antimycobacterial activity and mechanism of action of NAS-91. *Antimicrob Agents Chemother* 2008; 52: 1162–1166.
32. Nguyen MT, Hanzelmann D, Härtner T, et al. Skin-specific unsaturated fatty acids boost the *Staphylococcus aureus* innate immune response. *Infect Immun* 2015; 84: 205–215.

33. Martinez de Tejada G, Heinbockel L, Ferrer-Espada R, et al. Lipoproteins/peptides are sepsis-inducing toxins from bacteria that can be neutralized by synthetic anti-endotoxin peptides. *Sci Rep* 2015; 5: 14292.
34. Zähringer U, Lindner B, Inamura S, et al. TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* 2008; 213: 205–224.
35. Brennan PJ, Brigl M and Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* 2013; 13: 101–117.
36. Kinjo Y, Illarionov P, Vela JL, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* 2011; 12: 966–974.