Incorporation of Exogenous Fatty Acids Protects *Enterococcus faecalis* from Membrane-Damaging Agents

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Enterococcus faecalis is a commensal bacterium of the mammalian intestine that can persist in soil and aquatic systems and can be a nosocomial pathogen to humans. It employs multiple stress adaptation strategies in order to survive such a wide range of environments. Within this study, we sought to elucidate whether membrane fatty acid composition changes are an important component for stress adaptation. We noted that *E. faecalis* OG1RF was capable of changing its membrane composition depending upon growth phase and temperature. The organism also readily incorporated fatty acids from bile, serum, and medium supplemented with individual fatty acids, often dramatically changing the membrane composition such that a single fatty acid was predominant. Growth in either low levels of bile or specific individual fatty acids was found to protect the organism from membrane challenges such as high bile exposure. In particular, we observed that when grown in low levels of bile, serum, or the host-derived fatty acids oleic acid and linoleic acid, *E. faecalis* was better able to survive the antibiotic daptomycin. Interestingly, the degree of membrane saturation did not appear to be important for protection from the stressors examined here; instead, it appears that a specific fatty acid or combination of fatty acids is critical for stress resistance.

Enterococcus faecalis is a Gram-positive bacterium and a member of the intestinal flora of mammals. Since enterococci are commensals of many mammals and capable of survival in water sources, they are used as indicators of fecal contamination (1, 2). *E. faecalis* is also linked to the fermentation of specific cheeses and sausages, and its production of bacteriocins has led to the use of the organism and its by-products in commercial products (1). Although *E. faecalis* is a human commensal bacterium, this organism frequently causes costly nosocomial infections that are difficult to eradicate. Additionally, enterococci possess efflux pumps and undergo cell wall alterations that contribute to routine resistance to antibiotics, including vancomycin (3, 4). Given that enterococci are capable of conjugation, there is growing concern that they may pass their resistance genes to other organisms (4–6).

While enterococci are considered intestinal commensals, these bacteria are clearly well adapted to survive a wide variety of environmental conditions and stressors. These include low pH, high osmotic stress, a wide range of temperatures, and desiccation (reviewed in reference 1). Its survival over a range of pH values and salt concentrations enables the organism to colonize, and thus cause disease, systemically. Additionally, the ability to survive a wide range of temperatures and desiccation contributes to its persistence in the environment, which is important for the transfer of resistant strains between hosts.

Given that enterococci naturally inhabit mammalian intestines, they must survive growth in bile. In particular, bile salts can be highly damaging to bacterial membranes (7). Studies with organisms such as *Listeria monocytogenes* and *Escherichia coli* have identified efflux pumps that are activated by bile salts (8, 9). Many efflux pumps are also linked to drug resistance (10). Studies with *E. faecalis* have shown that this organism alters gene expression and protein synthesis when grown in the presence of bile (11, 12), and resistance to bile stress also provides cross-protection against other sources of stress (13). However, the exact mechanisms for enterococci's resistance to bile stress have not been completely elucidated.

Many bacteria alter their membrane fatty acid composition

and/or membrane phospholipids as a protective measure against environmental stressors (14). In response to temperature fluctuations, numerous bacterial species will shift the ratio of saturated to unsaturated fatty acids within their membranes to increase or decrease fluidity as needed (15). Chemical inhibition or deletion of specific enzymes involved in either cyclopropane fatty acid synthesis or unsaturated fatty acid biosynthesis rendered those mutant strains more susceptible to low-pH stress than the parental strains or the genetically complemented mutant strains (16–18). *E. faecalis* also produces cyclopropane fatty acids, and strains deficient in the ability to produce cyclopropane fatty acids have been shown to be more sensitive to low pH (19). Furthermore, *E. faecalis* can modify its membrane phospholipids with the addition of amino acids, which affects the overall membrane charge and aids in resistance to antimicrobial compounds (20, 21).

The transportation and utilization of extracellular fatty acids have been well studied in the model organism *E. coli* but are largely unexplored in Gram-positive organisms (22). Recent data demonstrated that both *Staphylococcus aureus* and *Streptococcus pneumoniae* are capable of incorporating exogenous fatty acids from the host (23). However, in-depth analysis has suggested that incorporation of specific fatty acids has different metabolic effects on these organisms (24). Little work, though, has been conducted to examine the effects of exogenously supplied fatty acids on the physiology of *E. faecalis*.

Considering all of these previous studies, we hypothesized that

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modification of the membrane fatty acid content would be important for resistance to membrane damage for *E. faecalis*. As *E. faecalis* also has access to fatty acids while residing in the human host, we hypothesized that the organism could incorporate extracellular fatty acids, thereby altering its membrane content and its resistance to membrane stress. To test these hypotheses, we examined how the membrane fatty acid content changed when growth parameters were altered or exogenous fatty acids were added to the growth medium and whether these alterations contributed to survival when *E. faecalis* was exposed to membrane-damaging agents, including the antibiotic daptomycin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Enterococcus faecalis* OG1RF (gift from J. Lemos, University of Rochester Medical Center) was grown statically in brain heart infusion medium (BHI; BD Difco) at 37°C unless indicated otherwise. Overnight cultures were used to inoculate medium to an optical density (OD₆₀₀) value at 600 nm of 0.01. Unless indicated, culture supplements were purchased from Sigma-Aldrich. Cultures were supplemented with bovine bile, pooled human serum (ICN Biomedicals), fatty acids, bile salts, sodium dodecyl sulfate (SDS), and antibiotics as indicated in the text and in Table 2.

GC-FAME preparation and analysis. *E. faecalis* was grown as indicated in the text and in Table 2. When the appropriate cellular concentration was reached, 10 to 20 ml of cells was harvested by centrifugation and extensively washed twice with an equal volume of 1× phosphate-buffered saline (PBS) to ensure no carryover of fatty acids from the cell culture. Cell pellets were stored at -80°C prior to gas chromatography-fatty acid methyl ester (GC-FAME) analysis performed by Microbial ID, Inc. (Newark, DE), as described previously (25). Briefly, resuspended cell pellets were subjected to saponification using a sodium hydroxide-methanol mixture. This was followed by a methylation step and extraction with hexane prior to the analysis. Averages and standard deviations of three independently grown cultures are shown for each condition.

Challenge assays. Cells (2 ml) were harvested from mid-log-phase cultures (OD₆₀₀ of ~0.4). The cells were centrifuged and resuspended in 2 ml of a “challenge” medium. Challenge medium consisted of BHI with 10% bile, 0.1% sodium dodecyl sulfate (SDS), or 30 µg/ml of daptomycin and 100 mM CaCl₂ or an equal volume of 100% ethanol as a solvent control. Prior to treatment with daptomycin, cultures were grown in BHI with 100 mM CaCl₂. Serial dilutions were plated onto BHI agar at 0, 15, 30, and 60 min after centrifugation and resuspension in a challenge medium. Each challenge study was performed in triplicate.

Scanning electron microscopy (SEM). Cells were grown in BHI supplemented as indicated until late log phase (OD₆₀₀ of ~1.0). Aliquots (1 ml) were harvested and washed twice with 1× PBS to remove medium. Following the washing steps, a primary fixation was performed at room temperature with 1× PBS and 3% glutaraldehyde for at least 1 h. Cells were rinsed in 1× PBS three times for 10 min (or a single overnight rinse); then, a secondary fixation in 2% osmium tetroxide and 1× PBS was completed at room temperature for a minimum of 1 h. Fixed cells were rinsed in water a minimum of two times for 10 min. During the last rinse, cells were allowed to settle onto a 3- by 4-mm silicon chip. Samples were dehydrated with a series of increasing ethanol concentrations (25%, 50%, 70%, 95%, and 100%) for 10 min at each grade. Samples were then visualized using a Hitachi SU 3500 or Zeiss Auriga 40 microscope at the Microscopy and Imaging Center at the University of Tennessee. For unsupplemented and bile-grown cultures, four independent cultures were analyzed; for oleic acid-grown cells, two independent cultures were analyzed.

Flow cytometry analysis. Cells were grown as indicated above to an OD₆₀₀ of ~0.4. Cells were then treated with daptomycin (30 µg/ml) (Sigma) or ethanol (95%, control). At 0, 5, 15, 30, and 60 min post-daptomycin addition, 100-µl aliquots were taken, flooded with 4 ml of 1×

TABLE 1 Generation times for *E. faecalis* OG1RF with various supplements

Medium constituent	Concn	Generation time (min)	Growth rate (h ⁻¹)
BHI ^a	NA ^c	36.0 ± 3.0	1.2 ± 0.07
Bile	0.2% bile	49.5 ± 4.5	0.8 ± 0.07
C _{18:1 cis 11} (<i>cis</i> -vaccenic acid)	10 µg/ml	34.9 ± 1.2	1.2 ± 0.04
C _{18:2 cis 9,12} (linoleic acid)	10 µg/ml	60.5 ± 9.8	0.7 ± 0.1
C _{18:1 cis 9} (oleic acid)	10 µg/ml	29.7 ± 1.0	1.4 ± 0.05
C _{18:1 cis 9} (oleic acid)	20 µg/ml	34.1 ± 1.0	1.2 ± 0.03
C _{18:1 cis 9} (oleic acid)	100 µg/ml	34.0 ± 1.5	1.2 ± 0.05
C _{16:0} (palmitic acid)	5 µg/ml	79.3 ± 8.2	0.5 ± 0.06
C _{18:0} (stearic acid)	10 µg/ml	36.5 ± 1.9	1.1 ± 0.06
C _{18:0} (stearic acid)	15 µg/ml	54.0 ± 3.0	0.7 ± 0.04
Serum	15% serum	36.1 ± 0.8	1.2 ± 0.02
Cholic acid	10 µg/ml	41.1 ± 2.2	1.0 ± 0.05
Bile salt mixture ^b	Each 10 µg/ml	39.4 ± 1.6	1.1 ± 0.04

^a For all other conditions, BHI was supplemented as listed.

^b Bile salt mixture contained 10 µg/ml each of the following: deoxycholic acid, lithocholic acid, glycocholic acid hydrate, and taurocholic acid sodium hydrate.

^c NA, not applicable.

PBS, and centrifuged. Cell pellets were resuspended in 1 ml of 1× PBS and stained with 10 µg/ml DiBAC₄ (3) (Life Technologies). After 20 min of incubation in the dark at room temperature, cells were washed with 4 ml of 1× PBS and analyzed by flow cytometry in an LSR II flow cytometer (Becton Dickinson) with a 488-nm laser. Samples were run at an event rate of 3,000 events per second. Green fluorescence was collected in the fluorescein isothiocyanate (FITC) channel. Differences in daptomycin-treated cells stained with DiBAC₄ (3) were obtained using geometric mean fluorescence for six independent experiments.

RESULTS

Growth in low levels of bile protects *E. faecalis* OG1RF from severe bile stress. *Enterococcus faecalis* inhabits the proximal region of the intestine, where it encounters the membrane-damaging effects of high levels of bile and, in particular, bile salts (26). Previously, it was shown that growth in low levels of bile salts or bovine bile could protect *E. faecalis* from severe membrane stress (11, 13). We wanted to establish the mechanism for this protection. We first began by supplementing the growth medium (BHI) of *E. faecalis* OG1RF with 0.2% bovine bile and noted that the growth rate was slightly reduced compared to that of unsupplemented cultures (Table 1); however, the bile-grown cultures reached an optical density similar to that of unsupplemented cultures in stationary phase (data not shown). We then tested the ability of our bile-grown and unsupplemented cultures to survive membrane damage using 10% bile or 0.1% sodium dodecyl sulfate (SDS). As shown, when challenged with either 10% bile (Fig. 1A) or 0.1% SDS (Fig. 1B), there were significantly more survivors for cultures grown in low bile than for those without ($P < 0.005$ for all time points).

We wanted to verify that the resistance observed in our 0.2% bile cultures was due to acclimation and not a selection for a genetic mutant(s). Following bile challenge, cells were grown overnight at 37°C and then used to inoculate fresh cultures containing BHI alone. The challenge with 10% bile was then repeated. Cells from these rechallenged cultures returned to the same level of sensitivity as that of cells not grown in bile (data not shown). Therefore, we attributed this protection to severe stress as an environmental acclimation and not a selection for resistant mutants.

FIG 1 Fatty acid supplementation protects OG1RF from membrane-damaging agents. (A) Challenge with 10% bovine bile. Cells were grown in BHI or BHI supplemented with 0.2% bovine bile, oleic acid, or ethanol (solvent control) as indicated in Table 2. Cultures ($n = 3$) were then challenged with 10% bile, and the log ratios of viable cells were determined. * indicates statistical significance between BHI and the supplemented culture ($P < 0.005$ as determined by Student's t test for all time points). There was no statistically significant difference in survival between BHI and the ethanol control. (B) Challenge with 0.1% SDS. Cells were grown as described for panel A and challenged with 0.1% SDS, and the log ratio of viable cells was determined ($n = 3$). * indicates statistical significance between supplemented and unsupplemented cultures as determined by Student's t test ($P < 0.0001$). Note that the log ratio of viable cells grown in bile was significantly higher than that of cells grown in oleic acid ($P < 0.005$).

Bile salts fail to provide protection from sudden stress. Bile is a complex mixture of various proteins, ions, fatty acids, and bile salts (27). However, it is the amphiphilic properties of bile salts that cause membrane damage. Work by other groups has suggested that growth in the presence of low levels of bile salts can induce changes in *E. faecalis* that enhance its survival under severe membrane stress (13). Other enteric organisms possess and activate efflux pumps in response to bile salts that may also contribute to drug resistance. For example, *L. monocytogenes* upregulates *mdrT* specifically in response to cholic acid (8) and *E. coli* utilizes ArcAB to pump out unconjugated bile salts and decanoate (9).

To determine whether bile salts contributed to the enhanced stress resistance in *E. faecalis*, OG1RF was grown in cholic acid (10 $\mu\text{g/ml}$) as well as a bile salt mixture consisting of 10 $\mu\text{g/ml}$ each of deoxycholate, lithocholic acid, taurocholic acid, and glycocholic acid hydrate (8). The addition of the salts did not noticeably impact growth rates (Table 1). Growth in the bile salt mixture (Fig. 2A) or cholic acid (Fig. 2B) alone did not significantly enhance survival when cultures were challenged with 10% bile or 0.1% SDS (data not shown). Solvent control experiments also showed no impact of the solvent on cell survival or growth (Fig. 2; data not shown). These findings suggested that another component within bile contributes to protection from membrane-damaging agents.

Membrane fatty acid content of *E. faecalis* is dependent upon growth conditions. Fatty acids are another major component found within bile. Many bacteria, including streptococcal, staphylococcal, and lactococcal species, are known to incorporate exogenous fatty acids into their membrane, altering their membrane content (24, 28–30). We hypothesized that *E. faecalis* may incorporate fatty acids from bile, and this could provide protection from membrane stress. However, we first needed to establish the fatty acid content of OG1RF from cultures grown under standard laboratory conditions. Gas chromatography of fatty acid methyl esters (GC-FAME; Microbial ID, Inc.) was performed on BHI medium to confirm that there were no detectable fatty acids in the medium (data not shown). Following this, the fatty acid content from cells grown in BHI to exponential (OD_{600} of ~ 0.4) and stationary (after 24 h of growth) phases (Table 2; see also Table S3 in the supplemental material) was analyzed in the same fashion. We note that for all of the membrane composition analyses presented in this report, those fatty acids found in minor amounts ($\leq 5\%$) can have inflated standard deviations. This variation was often because a fatty acid was not detected in one of the replicates. However, the fatty acid content tended to be well conserved across replicates, especially for those fatty acids that make up the majority of the membrane composition.

FIG 2 Growth in bile salts does not protect from acute bile stress. (A) Cells were grown in BHI or in BHI supplemented with 10 $\mu\text{g/ml}$ of each of the following: deoxycholic acid, lithocholic acid, glycocholic acid hydrate, taurocholic acid sodium hydrate, or solvent control. Cells were then treated with 10% bile as described in Materials and Methods ($n = 3$). (B) Cells were grown in BHI or BHI supplemented with 10 $\mu\text{g/ml}$ of cholic acid or methanol control. Cultures were challenged with 10% bile as described in Materials and Methods, and the log ratios of viable cells were determined. In both panels A and B, there was no statistically significant difference in survivors, as determined by Student's t test, between BHI and the supplemented cultures or the solvent control.

TABLE 2 *E. faecalis* OG1RF membrane fatty acid composition during exponential-phase growth

Fatty acid	% of total membrane content (avg ± SD) ^a							
	BHI	Bile ^b	C _{18:1 cis 9} ^c (oleic acid)	C _{18:1 cis 11} ^d (<i>cis</i> -vaccenic acid)	C _{18:2 cis 9,12} ^d (linoleic acid)	C _{16:0} ^e (palmitic acid)	C _{18:0} ^f (stearic acid)	Serum ^g
C _{12:0}	0.8 ± 0.3	ND	0.7 ± 0.2	0.6 ± 0.5	2.3 ± 1.5	0.1 ± 0.1	0.2 ± 0.0	ND
C _{14:0}	5.1 ± 0.9	0.5 ± 0.1	2.0 ± 1.5	0.4 ± 0.4	3.4 ± 0.6	2.4 ± 0.2	2.0 ± 0.2	3.3 ± 0.1
C _{15:1}	<0.1 ± 0.1	ND	ND	ND	2.0 ± 3.5	ND	ND	ND
C _{16:1}	8.1 ± 2.2	0.8 ± 0.1	1.5 ± 0.7	0.8 ± 0.1	2.7 ± 0.4	3.5 ± 0.2	2.3 ± 0.4	4.9 ± 0.1
C _{16:0}	36.0 ± 3.8	51.2 ± 1.4	10.3 ± 8.3	2.0 ± 0.2	10.0 ± 1.5	69.0 ± 3.3	14.9 ± 4.4	40.6 ± 0.2
C _{17:0 iso 3OH}	ND	ND	ND	ND	ND	ND	ND	1.5 ± 0.2
C _{17:1}	ND	0.5 ± 0.1	0.8 ± 0.5	0.3 ± 0.6	1.4 ± 2.5	0.2 ± 0.4	0.2 ± 0.2	0.7 ± 0.0
C _{17:0}	<0.1 ± 0.05	1.6 ± 0.0	ND	ND	ND	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.0
C _{17:0 2OH}	2.5 ± 0.4	0.1 ± 0.1	0.3 ± 0.3	1.9 ± 0.4	0.3 ± 0.5	1.3 ± 0.4	1.2 ± 0.7	0.6 ± 0.1
C _{18:1 cis 9}	ND	19.3 ± 0.6	71.9 ± 12.6	ND	ND	0.6 ± 0.2	0.1 ± 0.1	17.5 ± 0.6
C _{18:1 cis 11}	40.6 ± 1.9	2.7 ± 0.2	4.0 ± 3.5	91.9 ± 1.3	5.3 ± 0.2	17.3 ± 2.6	13.9 ± 4.6	9.7 ± 0.5
C _{18:0}	4.2 ± 1.7	20.8 ± 0.9	1.4 ± 1.0	0.9 ± 0.1	2.2 ± 2.6	4.0 ± 0.3	64.6 ± 10.4	7.5 ± 0.3
C _{20:1}	0.1 ± 0.2	ND	0.3 ± 0.2	ND	2.8 ± 2.6	ND	ND	ND
C _{20:0}	ND	0.2 ± 0.1	5.0 ± 3.3	ND	ND	ND	<0.1 ± 0.1	ND
C _{18:2}	0.1 ± 0.1	ND	ND	0.1 ± 0.2	67.1 ± 5.3	0.2 ± 0.1	ND	12.5 ± 0.3
C _{19 cyclo}	1.0 ± 0.3	0.4 ± 0.7	1.9 ± 0.3	1.1 ± 0.2	0.3 ± 0.4	0.7 ± 0.1	0.5 ± 0.1	ND
Others ^h	1.4 ± 0.8	2.1 ± 0.7	ND	ND	0.2 ± 0.4	0.6 ± 0.7	<0.1 ± 0.1	0.9 ± 0.2
Saturated/unsaturated ratio	0.9 ± 0.2	3.2 ± 0.2	0.3 ± 0.1	<0.1 ± 0.0	0.2 ± 0.1	3.5 ± 0.5	5.0 ± 2.9	1.1 ± 0.0

^a Percentage of total membrane content as determined by GC-FAME by Microbial ID, Inc.; shown are averages ± standard deviations of three independent experiments. ND, not detected.

^b Bovine bile was added to a final concentration of 0.2%.

^c Oleic acid was added to a final concentration of 20 µg/ml.

^d *cis*-vaccenic or linoleic acid was added to a final concentration of 10 µg/ml.

^e Palmitic acid was added to a final concentration of 5 µg/ml.

^f Stearic acid was added to a final concentration of 10 µg/ml.

^g Human serum was added to a final concentration of 15%.

^h "Others" indicates the total of all fatty acids comprising <1% of the total membrane content individually.

In both exponential and stationary phases of growth, the dominant fatty acids, palmitic acid (C_{16:0}) and *cis*-vaccenic acid (C_{18:1 cis 11}), comprised over 60% of the total membrane content (Table 2; see also Table S3 in the supplemental material). The most significant change observed between growth phases was an increase in C_{19:1 cyclo 11} from 1% in exponential phase to nearly 11% in stationary phase with a corresponding decrease in *cis*-vaccenic acid (C_{18:1 cis 11}). Unsaturated fatty acids are known to be converted to cyclopropane fatty acids during stationary phase, and so the decrease in *cis*-vaccenic acid may be attributed to this pathway (31, 32).

Growth at low temperatures has been shown to lead to a decrease in the proportion of saturated fatty acids and a concomitant increase in the amount of unsaturated fatty acids to help maintain membrane fluidity in other organisms (15). Given that *E. faecalis* can grow at a range of temperatures, we sought to determine whether OG1RF undergoes similar changes. There was a noticeable shift toward a lower saturated/unsaturated ratio that occurred with cultures grown at 25°C versus 37°C in unsupplemented medium (see Tables S4 and S5 in the supplemental material), suggesting that *E. faecalis* increased the proportion of unsaturated membrane fatty acids to acclimate to decreasing temperatures.

When supplemented with 0.2% bile, the membrane content of OG1RF was significantly altered compared to that of cultures grown in medium alone. Analysis of the bile-supplemented medium indicated that it contained many fatty acids (see Table S1 in the supplemental material), including oleic acid (C_{18:1 cis 9}). Oleic acid is abundant in human tissues through dietary consumption

of plant and animal oils. As noted in Table 2, *E. faecalis* did not produce detectable levels of oleic acid when grown in BHI. However, when the medium was supplemented with bile, 19% of the total membrane fatty acid content was composed of oleic acid (Table 2). This suggests that *E. faecalis* incorporates oleic acid from bile into its membrane. On the other hand, the fatty acid *cis*-vaccenic acid (C_{18:1 cis 11}) comprised approximately 40% of the total membrane fatty acid content of OG1RF grown in BHI medium. But, in bile-grown cells, *cis*-vaccenic acid comprised only about 3% of the total membrane fatty acid content (Table 2). Thus, not only was there a reduction in the total amount of C_{18:1} fatty acid but the type of C_{18:1} (i.e., the position of the double bond) was significantly different when OG1RF was grown in the presence of bile.

Additional changes were observed when *E. faecalis* was grown with bile. There was a considerably greater percentage of saturated fatty acids found in cultures supplemented with bile during exponential growth in comparison to those without, as is indicated in the saturated/unsaturated ratio (Table 2; see also Table S2 in the supplemental material). In exponential phase, there was approximately a 5-fold increase in stearic acid (C_{18:0}) in bile-grown cells and higher levels of palmitic acid (C_{16:0}). When differences in the saturated fatty acid compositions from exponential and stationary phases were examined, there was very little fluctuation in cultures grown without the fatty acid source. However, for cultures supplemented with bile in stationary phase, there were decreases in the dominant saturated fatty acids, palmitic acid and stearic acid, which are indicated in the decrease in the saturated/unsaturated ratio for bile-grown cells (Table 2; see also Tables S2 and S3).

FIG 3 Morphology of OG1RF grown under different conditions. OG1RF was grown as described in Materials and Methods, and cells were prepared and analyzed via SEM. Shown are figures representative of four independently grown BHI and BHI-plus-0.2% bile cultures; also shown are figures representative for two independently grown cultures supplemented with oleic acid.

Exogenously supplied oleic acid provides protection from acute membrane stress. From the above analysis, we noted that OG1RF incorporated fatty acids found within bile into its own membrane. In particular, the organism replaced the predominant unsaturated fatty acid *cis*-vaccenic acid ($C_{18:1 \text{ cis } 11}$) with oleic acid ($C_{18:1 \text{ cis } 9}$). We hypothesized that the incorporation of oleic acid may be impacting cell survival and decided to examine this by supplementation of the growth medium with oleic acid alone. We noted that medium supplementation with oleic acid did not significantly impact generation time, even when supplemented at high levels (100 $\mu\text{g/ml}$) of oleic acid; thus, we chose 20 $\mu\text{g/ml}$ for subsequent membrane analysis and survival assays (Table 1).

Oleic acid comprised more than 70% of the membrane fatty acid composition in exponential-phase cultures when the growth medium was supplemented solely with this fatty acid. Thus, as was seen for bile-grown cells, *E. faecalis* readily incorporates oleic acid into its membrane from its growth medium. Concomitantly, the percentages of palmitic and *cis*-vaccenic acids, which in combination comprised approximately 76% of the total fatty acid content of unsupplemented cultures, were greatly reduced (Table 2). In stationary phase, the level of oleic acid was reduced to approximately 35% and the level of palmitic acid increased to about 30%, nearly the same level as that seen in BHI-grown cultures (see Table S3 in the supplemental material). This decrease in oleic acid content in the membranes could be due to exhaustion of the supply added to the medium. To support this, supplementation with a

larger amount of oleic acid (100 $\mu\text{g/ml}$) maintained a higher percentage of this fatty acid in stationary-phase cultures (approximately 52%, data not shown).

When challenged with 10% bile, cells grown in oleic acid survived significantly better than the unsupplemented control group (Fig. 1A; P value of <0.005 for all time points). Thus, incorporation of exogenously supplied oleic acid alone can provide protection from severe bile stress. Interestingly, growth in oleic acid did provide cross-protection against the detergent SDS (Fig. 1B). However, cells grown in bile survived SDS challenge far better than those grown in oleic acid ($P < 0.005$). Thus, bile offered better cross-protection against SDS damage than did oleic acid (Fig. 1B).

In addition, cells grown in BHI alone or supplemented with oleic acid exhibited typical pair or chain morphology characteristic of *E. faecalis*, while cells grown in 0.2% bile were more likely to be arranged in a curved, long-chain formation. Some daptomycin-resistant enterococcal strains have altered cell morphology and aberrant placement of cell septa (33); therefore, we were interested to know if the location of cell septa was shifted in bile-grown cells. Scanning electron microscopy (SEM) confirmed that chain formation was clearly favored in bile cultures but not for unsupplemented or oleic acid-grown cultures (Fig. 3). However, transmission electron microscopy (TEM) analysis revealed no aberrant placement of cell septa for bile-grown cells (data not

shown). We concluded that morphological changes observed did not impact membrane stress survival under the conditions tested.

Not all exogenously supplied unsaturated fatty acids provide protection from membrane damage. We wanted to know if protection from membrane damage was unique to the addition of oleic acid to OG1RF cultures or if the addition of other unsaturated fatty acids could result in protection. Thus, we examined the effects of two other C_{18} -length unsaturated fatty acids. Linoleic acid ($C_{18:2}$ *cis* $9,12$) is a fatty acid found in bile and serum (see Table S1 in the supplemental material); *cis*-vaccenic acid ($C_{18:1}$ *cis* 11) is a prominent component of OG1RF membranes (Table 2; see also Table S3). The growth rate of *E. faecalis* was affected by increasing concentrations of linoleic acid, so 10 $\mu\text{g/ml}$ was chosen for membrane analysis and stress challenge assays for both fatty acids (Table 1).

The addition of these fatty acids significantly altered the membrane fatty acid content of OG1RF (Table 2). For cultures supplemented with *cis*-vaccenic acid, this particular fatty acid comprised 90% of the total membrane content in cultures grown to exponential phase. When cultures were supplemented with linoleic acid, more than two-thirds of the membrane content consisted of linoleic acid. This result was similar to what we observed when cultures were supplemented with oleic acid, where the total membrane fatty acid content was predominantly this single fatty acid (approximately 72%; Table 2). For cultures supplemented with either oleic, *cis*-vaccenic, or linoleic acid, there were decreased amounts of palmitic acid, leading to saturated/unsaturated ratios far lower than those for unsupplemented cultures (Table 2; also see Table S2 in the supplemental material). In stationary-phase cultures, we observed an increase in saturated fatty acids and a significant decrease in the supplemented unsaturated fatty acids (see Table S3), which may be due to exhaustion of the supply provided. Lastly, in stationary-phase cultures, there were increases in the levels of cyclopropane fatty acids; however, these increases were not as high as those in unsupplemented cultures.

After confirmation that exogenous linoleic and *cis*-vaccenic acid impacted the overall membrane content, the ability of these fatty acids to improve membrane stress survival was tested. Although not as robust as oleic acid supplementation (Fig. 1A), supplementation with *cis*-vaccenic acid improved cell survival after exposure to 10% bile for all time points compared to unsupplemented cultures (Fig. 4) ($P < 0.05$). Linoleic acid-grown cells were more variable in their ability to survive 10% bile stress; however, we observed an increased ability to survive bile stress compared to unsupplemented cultures only after 30 min of bile exposure. In exponential-phase cultures, the portion of the membrane comprised of linoleic acid was similar to the level of oleic acid (Table 2); therefore, the failure of this supplement to rescue cells from bile damage does not appear to be due to inadequate levels of incorporation but likely due to the individual impact that each fatty acid has on cellular physiology.

Exogenous saturated fatty acids impact membrane content but not survival of high bile concentrations. We observed that exogenously supplied unsaturated fatty acids were incorporated into the membrane of OG1RF and that this influenced the ability of the bacterium to survive membrane damage. However, bile-grown cells had a significantly higher ratio of saturated to unsaturated fatty acids during exponential phase. We wanted to ascertain if survival was at all linked to the saturated/unsaturated ratio or was due to incorporation of a specific fatty acid or combination

FIG 4 Specific membrane fatty acid compositions protect OG1RF from high bile exposure. Cells were grown as indicated in BHI or BHI supplemented with the different fatty acids (as indicated in Table 2) or ethanol (solvent control). Cultures ($n = 3$) were subjected to 10% bile challenge, and the log ratios of survivors were determined. As determined by Student's *t* test, there were significantly more survivors in cultures supplemented with *cis*-vaccenic acid than in unsupplemented cultures at all time points ($P < 0.05$). Survival of cultures supplemented with linoleic acid versus that of cultures grown only with BHI was significantly different only at 30 min ($P < 0.005$). No statistical difference in survivors was observed between any other supplementation and unsupplemented cells at any time point.

of fatty acids. To examine this, we supplemented *E. faecalis* with either palmitic acid ($C_{16:0}$) or stearic acid ($C_{18:0}$), since these are the dominant saturated fatty acids found in the membrane of *E. faecalis* OG1RF and, in particular, stearic acid is of the same chain length as oleic acid (both C_{18}).

The addition of the selected saturated fatty acids greatly decreased the growth rates of OG1RF (Table 1). The addition of 5 $\mu\text{g/ml}$ of palmitic acid doubled the generation time compared to growth in BHI alone (79 versus 36 min); therefore, 5 $\mu\text{g/ml}$ was used for subsequent experiments. This observed toxicity is consistent with the published observations for *S. pneumoniae* (23). The addition of stearic acid alone to cultures also increased generation time. For example, when the concentration of stearic acid was increased from 10 to 15 $\mu\text{g/ml}$, the exponential-phase doubling time increased from 36 min to 54 min (Table 1); thus, we used 10 $\mu\text{g/ml}$ for our analysis.

Exponential-phase cultures supplemented with 5 $\mu\text{g/ml}$ of palmitic acid ($C_{16:0}$) had a membrane composition that consisted of approximately 70% of this sole fatty acid (Table 2). The ratio of saturated to unsaturated fatty acids was similar to that of cells grown in bile and much higher than that of unsupplemented cultures (Table 2; see also Table S2 in the supplemental material). The proportion of *cis*-vaccenic acid in the membrane, however, was far less (approximately 17%) than that for growth without palmitic acid (40%). The addition of stearic acid ($C_{18:0}$) also altered the membrane content, with approximately 65% of the total membrane being comprised of this one fatty acid during exponential phase; cells without this supplement contained only approximately 4% stearic acid in their membrane. Levels of both palmitic (decreased from 36 to 14%) and *cis*-vaccenic (40 to 14%) acid were lower than those in unsupplemented cultures during growth with stearic acid. The ratio of saturated to unsaturated fatty acids was much greater for stearic acid-grown cells than for unsupplemented cultures (Table 2).

After challenge with 10% bile, cultures grown in the presence of 5 $\mu\text{g/ml}$ of palmitic acid or 10 $\mu\text{g/ml}$ of stearic acid did not survive better than the controls (Fig. 4). Interestingly, cultures

FIG 5 Growth in specific fatty acid sources can protect cells from daptomycin-induced killing. (A) Cells were grown in BHI or BHI supplemented with 0.2% bovine bile or 15% human serum and challenged with 30 $\mu\text{g}/\text{ml}$ of daptomycin as indicated in the text. The log ratio of survivors was statistically significant between unsupplemented cultures and cultures supplemented with bile or human serum using Student's *t* test ($P < 0.0005$ at all time points, $n = 3$). (B) Cultures ($n = 3$) were supplemented with the indicated fatty acids as described in the text and challenged with daptomycin. There were statistically significant differences in survivors between cultures grown in BHI and those supplemented with either linoleic acid or oleic acid; for cultures supplemented with linoleic acid, P was < 0.0005 for all time points, and for cultures supplemented with oleic acid, P was < 0.005 for all time points. For stearic acid, statistical analysis showed significantly more survivors than those for unsupplemented cultures only at 30 min ($P < 0.05$).

supplemented with either palmitic or stearic acid had a saturated/unsaturated fatty acid ratio similar to that observed in bile-grown cells, yet bile-grown cells survived the high bile concentration far better. These data, along with the results presented above, suggest that the individual fatty acid composition, not the overall ratio of saturated to unsaturated fatty acids, contributes to membrane stress survival.

Membrane fatty acid changes interfere with killing by the antibiotic daptomycin. The antibiotic daptomycin targets Gram-positive bacteria through a calcium-dependent mechanism to form pores within the membrane (34–37). This lipopeptide is approved for treating *S. aureus* infections (38) but has also been used to treat cases involving *E. faecalis*. Recent studies examining daptomycin-resistant strains of *E. faecalis* reported morphological changes and aberrant placement of cell septa (33, 39). Studies have also described daptomycin-resistant strains that have mutations in genes related to phospholipid synthesis (33, 39–41). Given these previously published results and our own observations with bile-grown cell morphology (Fig. 3), we tested the ability of OG1RF grown in BHI medium or medium supplemented with fatty acid sources to survive daptomycin treatment.

When OG1RF was pregrown in low levels of bovine bile and then challenged with daptomycin (30 $\mu\text{g}/\text{ml}$), there were significantly more survivors than those for cells grown without bile (Fig. 5A) ($P < 0.005$). Given that daptomycin inserts into membranes and that bile-grown cells have an altered membrane fatty acid profile, we examined whether growth supplementation with individual fatty acids could protect *E. faecalis* from daptomycin-mediated killing. Indeed, cells pregrown with 20 $\mu\text{g}/\text{ml}$ of oleic acid survived better than cells grown without the fatty acid (Fig. 5B), implying that the altered membrane fatty acid content is protective against the antibiotic. Each culture was also challenged with an equivalent volume of ethanol (solvent used for daptomycin resuspension) to confirm that the ethanol solvent did not contribute to cell death.

Since *E. faecalis* can cause a myriad of infections and because human serum is a source of exogenous fatty acids, we wished to determine if growth in human serum could protect *E. faecalis* from daptomycin challenge. We supplemented our cultures with 15% human serum and noted no significant differences in growth

rate (Table 1). Analysis of cells grown in serum revealed an altered membrane profile compared to cells grown without serum (Table 2; see also Table S3 in the supplemental material). The saturated/unsaturated fatty acid ratio in exponential-phase cultures was similar to that seen in cells grown without supplementation, and in fact, the levels of individual saturated fatty acids were similar between the two conditions (Table 2; see also Table S2). In contrast, the unsaturated fatty acid composition was different from what was observed in either BHI or bile-supplemented cultures. The total percentage of 18-carbon unsaturated fatty acids (41%) was similar to that under other conditions tested (41% $\text{C}_{18:1 \text{ cis } 11}$ in BHI; 34% total $\text{C}_{18:1 \text{ cis } 9}$ and $\text{C}_{18:1 \text{ cis } 11}$ in bile). However, membranes of serum-grown cells contained a different combination of 18-carbon unsaturated fatty acids: 10% *cis*-vaccenic acid, 18% oleic acid, and 13% linoleic acid ($\text{C}_{18:2 \text{ cis } 9,12}$). FAME analysis of serum-supplemented medium detected the presence of these fatty acids, indicating that they were incorporated into the membrane from the medium (see Table S1).

When subjected to daptomycin challenge, there was a higher percentage of survival for those cells grown in serum (Fig. 5A) than those grown without the supplement. This was similar to cells grown with either bile or oleic acid, even though there were significant membrane fatty acid differences among the three groups (Table 2). This suggests that a variety of altered fatty acid compositions are able to protect against damage from daptomycin.

Given that serum-grown cells had a significant amount of linoleic acid within their membranes, we examined its effects on protection from daptomycin. Similar to what was observed with cultures pregrown with bile or oleic acid, cells grown with linoleic acid were significantly better at surviving daptomycin challenge than was the control (Fig. 5B, $P < 0.0005$). Thus, a variety of fatty acid supplements are capable of enhancing resistance to daptomycin.

Another major component in both bile and serum was stearic acid ($\text{C}_{18:0}$; see Table S1 in the supplemental material). We examined whether supplementation of this fatty acid could protect cells from daptomycin-induced damage. Stearic acid gave the weakest level of protection of all supplements tested (Fig. 5B). The only significant difference between BHI and supplementation with ste-

FIG 6 Membrane depolarization can occur quickly upon daptomycin exposure. Cells were grown as described in Table 2: BHI (no supplementation) (A), bile (B), oleic acid (C), linoleic acid (D), and stearic acid (E). Five minutes (–5) prior to daptomycin exposure, cultures were split and stained with DiBAC₄ (3) to determine basal permeability. At 0 min, daptomycin (gray) or solvent control (black) was added, and immediately following, aliquots were taken for analysis. Aliquots were removed and stained at indicated time points. Shown are averages and standard deviations (n is 6 for all but stearic acid, for which n is 5); * indicates a P value of <0.05 as determined by Student's t test for daptomycin-treated cells versus the untreated control.

aric acid was found 15 min postchallenge. The other fatty acid sources (bile, serum, linoleic acid, and oleic acid) provided more robust protection and had statistically more survivors 15, 30, and 60 min postchallenge ($P < 0.005$). These findings were interesting because, despite the vast differences in the individual membrane compositions of the cells grown with the different supplements, their ability to protect from bile stress was not indicative of their ability to protect from daptomycin.

Depolarization rates upon daptomycin treatment are dependent upon membrane composition. Addition of daptomycin leads to rapid membrane depolarization (36). We further examined membrane permeability of selected samples to better examine the kinetics of this damage. We utilized DiBAC₄ (3), which enters cells upon membrane depolarization and results in increased fluorescence (42, 43). Immediately upon the addition of daptomycin ($t = 0$), the sample grown in BHI alone had a statistically significant increase in fluorescence versus its paired control sample (no daptomycin) as determined by flow cytometry

(Fig. 6). For cultures grown with oleic or linoleic acid, measurable differences in permeability did not occur until 5 min posttreatment, and gradually those differences became more severe over time (Fig. 6C and D). Surprisingly, both bile- and stearic acid-supplemented cultures showed no major differences in depolarization from the control until 60 min post-daptomycin treatment, despite the fact that there was cell loss as indicated by our colony count data (Fig. 5 and 6B and E). For stearic acid, there seems to be significant depolarization over time even for the control, implying that the supplement may have negative effects overall (see Discussion). Although cultures supplemented with linoleic acid had a log ratio of survivors after daptomycin treatment similar to that of bile-grown cells (Fig. 5), cells supplemented with bile showed far fewer changes in membrane permeability (Fig. 6B and D). Thus, there may be additional factors contributing to the protective mechanism of fatty acids other than preventing rapid changes in membrane permeability. However, this analysis indicates that the rapid membrane depolarization experienced by BHI-grown cells

can be slowed if oleic or linoleic acid is provided, which may explain the enhanced survival of these cultures as well (Fig. 5B).

DISCUSSION

Here, we confirmed that *E. faecalis* OG1RF alters its membrane fatty acid composition depending on growth environment and through the incorporation of exogenous fatty acids from a variety of sources. These membrane changes have a significant impact on cellular survival when cells are challenged with membrane-damaging agents. Specifically, growth in bile, oleic acid, or linoleic acid leads to membrane alterations that can enhance resistance to the antibiotic daptomycin. The precise mechanism of protection still remains to be elucidated.

Membrane changes: *de novo*. Many studies have shown that bacteria can alter their membrane fatty acid composition depending upon growth temperature and phase of growth as well as changing environmental conditions (14, 15). As we have illustrated here, the membrane profile of *E. faecalis* does change throughout its growth cycle: specifically, when grown at 37°C, cells in exponential phase have a membrane profile dominated by palmitic acid and *cis*-vaccenic acid. When those cells are in stationary phase, we and others report a significant increase in cyclopropane fatty acids (Table 2; see also Table S3 in the supplemental material) (31). This increase in cyclopropane fatty acids could be a protective measure against acidification of the medium as the culture enters stationary phase. *E. coli* synthesizes cyclopropane fatty acids in stationary phase, which serves to increase its resistance to low pH (18). Other Gram-positive bacteria, such as lactobacilli, also increase the proportion of membrane cyclopropane fatty acids in response to low pH (44). Many oral streptococci do not make cyclopropane fatty acids but instead increase the proportion of unsaturated fatty acids in their membranes in response to acidification (16, 44, 45).

Additionally, we confirmed that OG1RF increases the amount of unsaturated fatty acids within its membrane when the growth temperature is decreased. This likely maintains membrane fluidity and is a common cold-temperature adaptation for many bacteria. This mechanism has been well studied in *E. coli* and *Bacillus subtilis*, and yet each organism achieves the same outcome through a different route. *E. coli* increases the amount of unsaturated fatty acids at the biosynthetic level; *B. subtilis* actually modifies fatty acid tails already within the membrane using a membrane phospholipid desaturase encoded by the *des* gene (46). We have not identified a *des* gene homolog in OG1RF; however, it is also not clear if OG1RF utilizes the *E. coli* pathway to increase its unsaturated fatty acids in response to low growth temperature. Further experimentation is needed to determine the exact mechanism that *E. faecalis* employs to maintain membrane fluidity in response to temperature changes.

Membrane changes: incorporation of exogenous fatty acids.

As we show in this report, *E. faecalis*, like many bacteria, can incorporate exogenous fatty acids into its membrane. However, OG1RF does not include genes for beta-oxidation, based on homology searches; thus, it cannot degrade fatty acids for use as a carbon source (6, 47). *Vibrio cholerae* changes its phospholipid profile in response to growth in bile; these changes are attributed to exogenous fatty acids and not to other bile components (29). *Streptococcus agalactiae* incorporates a variety of fatty acids into its membrane after growth in serum (28). *S. aureus* and *S. pneumoniae* can incorporate a variety of exogenous fatty acids, notably

oleic acid, and use these to replace a significant amount of the membrane fatty acid content (23, 24). Our data demonstrate that exogenous long-chain fatty acids ($\geq 16:0$) are incorporated into the membrane of *E. faecalis* without elongation and can significantly alter the membrane content (Table 2; see also Table S3 in the supplemental material). This finding is further validated by work showing that an *E. faecalis* fatty acid auxotroph could be maintained when the culture medium was supplemented by oleic acid (48).

In addition to membrane changes, exogenous fatty acids had various effects on growth rates and stress responses. Medium supplementation with bile, saturated fatty acids, and linoleic acid, a C_{18:2} fatty acid, impacted growth rates, while C_{18:1} fatty acids had a less noticeable effect. Although the growth rates were reduced when OG1RF was supplemented with either palmitic acid or stearic acid, the organism was not more resistant to the membrane-damaging agents tested here. Slow-growing bacteria are often better protected from antibiotics because many antibiotics target actively growing cells by inhibiting protein synthesis, RNA synthesis, etc. The agents examined in this study are membrane damaging; they lead to dissipation of proton motive force and ion imbalance, which will eventually cause cellular death. This “non-specific” nature of damage may explain why reduced growth rates did not enhance resistance to these agents.

A previous study reported the impact of a variety of different fatty acids on the growth of *S. aureus* and *S. pneumoniae* (23). In contrast to these organisms, *E. faecalis* did not follow the same sensitivity pattern. It is important to note that the natural niche of *E. faecalis* is the intestine, which is a far different environment than the nares or nasal pharynx for *S. pneumoniae* and *S. aureus*; it is likely that organisms have adapted to the most abundant host-derived fatty acids in their normal habitat. These various responses to different fatty acids between the organisms are likely linked to alterations in membrane fluidity, membrane protein content, and/or possible negative feedback on the fatty acid biosynthetic genes.

Exogenous fatty acids and stress resistance. For organisms like *L. monocytogenes*, bile salts are known triggers of efflux pumps, which lead to membrane stress resistance (8). Other groups have shown that growth of *E. faecalis* within bile salts can protect cells from membrane-damaging agents. We did not observe a similar protection with OG1RF. It is important to note that the strain that we examined was not the same one used in the previous study, and the concentration of bile salts also varied, which may explain the opposing finding; furthermore, there is large variation in sequenced strains of *E. faecalis*, which could also contribute to the different results (13, 47, 49).

Nonetheless, growth in oleic acid alone can significantly increase resistance to bile (Fig. 1A). Given that oleic acid is present in bile, it is often encountered by *E. faecalis* and may be preferentially used by the bacterium in the host. In fact, a recent report indicated that a small-colony variant isolated from a patient grew significantly better when provided with oleic acid (oleate) *in vitro* (48).

Supplementing cultures with 0.2% bile protected against both bile and SDS challenge. Compared to that for oleic acid-grown cells, protection from SDS was significantly better for bile-grown cultures. This suggests that there are other components within bile, besides oleic acid, that are responsible for the strong cross-protection against SDS. Indeed, previous studies have shown that bile treatment can impact the expression of numerous genes and

proteins (11, 13); however, the global impact of exogenous fatty acids on gene expression or protein levels has not been explored. It may be that growth in bile induces the expression of additional genes versus growth in fatty acids (oleic) alone or even bile salts, and induction of these additional genes is responsible for the cross-resistance against detergents such as SDS.

When examining resistance to the lipopeptide daptomycin, an array of membrane fatty acid compositions protected the cells. Specifically, growth in bile, serum, oleic acid, or linoleic acid could provide protection from daptomycin challenge. Upon examination of the individual membrane fatty acid profiles, we could not identify a specific increase or decrease in a particular fatty acid that correlated with protection across all conditions (Table 2). Levels of *cis*-vaccenic acid were lowered for all protected compositions compared to unsupplemented controls, but reduced levels of *cis*-vaccenic acid were also observed for cultures grown in stearic acid, which were not significantly daptomycin resistant.

Supplementation with bile gives a more saturated membrane fatty acid profile that is protective against daptomycin, while the unsaturated fatty acids oleic acid and linoleic acid were also beneficial. How such different compositions protect *E. faecalis* from daptomycin challenge is not clear. Perhaps, exogenous fatty acids induce changes in gene expression, and those changes may be critical for the protection. However, stearic acid did not show a similar protection and was not well tolerated by the organism at high concentrations. The membrane depolarization data for stearic acid-grown cells showed that even untreated cells experienced a fairly large amount of depolarization compared to cells grown with other fatty acids (Fig. 6). The stearic acid-supplemented cultures had a saturated/unsaturated ratio even higher than that of bile-grown cells. As these findings indicate that there is no correlation between membrane saturation and resistance, other aspects of the membrane content, including phospholipid composition and/or membrane proteins and their activities, may be responsible for the observed resistance.

Growth in bile and specific fatty acids resulted in an altered morphology of OG1RF. In particular, cells were found in long chains that had curvature to them (Fig. 3). However, this altered morphology did not necessarily confer resistance to the membrane-damaging agents that we tested, including the antibiotic daptomycin. For example, growth in oleic acid protected cells from both bile challenge and daptomycin compared to control, and yet these cells had a “normal” morphology. Previous studies of daptomycin-resistant enterococcal strains showed aberrant placement of cell septa and altered cell morphology (33, 39). Our findings, though, suggest that while altering the membrane composition may lead to morphological changes, these did not correlate with the increased daptomycin resistance that we observed. Genetic analysis of daptomycin-resistant strains also revealed mutations related to cell membrane changes (33, 40, 41). Among these are mutations to cardiolipin synthase. While this mutation may enhance survival of daptomycin challenge, this mutation alone was not enough to confer resistance in all strains (40). Our results support this observation and suggest that resistance to daptomycin may be enhanced by supplementation with host-derived fatty acids.

Role of exogenous fatty acids in microbial killing. The ability to utilize exogenous fatty acids has significant therapeutic relevance. Bacterial fatty acid biosynthesis has long been considered an attractive drug target because each step occurs through the

action of an individual enzyme, unlike the eukaryotic approach (50, 51). However, studies have shown that *S. pneumoniae* could completely utilize exogenous fatty acids to support its growth, while *S. aureus* could not, indicating that inhibition of fatty acid biosynthesis may not be effective against all pathogens (24). In the same study, there were significant impacts upon cellular physiology when either organism was grown in the presence of exogenous fatty acids, and the same fatty acid affected each organism differently (23, 24). As noted previously, we have also observed growth alterations attributed to exogenous fatty acids (Table 1). These data show that both the effects of supplementation with exogenous fatty acids on the ability to overcome fatty acid biosynthetic inhibition and the specific consequences of exogenous fatty acid supplementation on cellular physiology are unique for each pathogen.

Our data demonstrate that *E. faecalis* can utilize host fatty acids to enhance its resistance to specific membrane-damaging agents. The combined effects of fatty acid uptake and utilization may contribute to the resilience of *E. faecalis* within the host and external environment. Further experiments to reveal the mechanism and consequences of fatty acid incorporation would provide information toward understanding the persistence of this organism.

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