Characterization of Monolaurin Resistance in *Enterococcus faecalis*<sup>V</sup>

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There is increasing concern regarding the presence of vancomycin-resistant enterococci in domestically farmed animals, which may act as reservoirs and vehicles of transmission for drug-resistant enterococci to humans, resulting in serious infections. In order to assess the potential for the use of monolaurin as a food preservative, it is important to understand both its target and potential mechanisms of resistance. A Tn<sub>917</sub> mutant library of *Enterococcus faecalis* AR01/DGVS was screened for resistance (MIC, >100 μg/ml) to monolaurin. Three mutants were identified as resistant to monolaurin and were designated DGRM2, DGRM5, and DGRM12. The gene interrupted in all three mutants was identified as *traB*, which encodes an *E. faecalis* pheromone shutdown protein and whose complementation in *trans* restored monolaurin sensitivity in all three mutants. DGRM2 was selected for further characterization. *E. faecalis* DGRM2 showed increased resistance to gentamicin and chloramphenicol (inhibitors of protein synthesis), while no difference in the MIC was observed with the cell-wall-active antibiotics penicillin and vancomycin. *E. faecalis* AR01/DGVS and DGRM2 were shown to have similar rates (30% cell lysis after 4 h) of cell autolytic activity when activated by monolaurin. Differences in cell surface hydrophobicity were observed between the wild type and the mutant, with the cell surface of the parent strain being significantly more hydrophobic. Analysis of the cell wall structure of DGRM2 by transmission electron microscopy revealed an increase in the apparent cell wall thickness and contraction of its cytoplasm. Taken together, these results suggest that the increased resistance of DGRM2 was due to a change in cell surface hydrophobicity, consequently limiting the diffusion of monolaurin to a potential target in the cytoplasmic membrane and/or cytoplasm of *E. faecalis*.

Monolaurin, a food grade glycerol monoester of lauric acid, has been reported to have the greatest antimicrobial activity of all of the monoglycerides (50). Monolaurin, like any fatty acid ester, is a lipophilic compound and hence its inhibitory activity is probably through interactions with the cytoplasmic membrane. Although the mechanism of antibacterial action of fatty acids and their derivatives is not defined, it has been suggested to involve disruption of the cell membrane permeability barrier and inhibition of amino acid uptake (29, 51). The activity of monolaurin against gram-negative bacteria has been shown to be enhanced when combined with high temperatures (30), freezing (54), acidulants (8, 29, 43, 53), and chelating agents such as EDTA (5), treatments believed to increase the ability of the monoglyceride to access the cytoplasmic membrane (51). Glycerol monolaurate has been shown to inhibit the production of exoenzymes and virulence factors in *Staphylococcus aureus* (41), to block the induction of vancomycin resistance in *Enterococcus faecalis* (46), and to modulate T-cell proliferation (62), all of which involve membrane-bound signal transduction systems. Dodecylglycerol (corresponding ether of monolaurin) has been shown to activate the proteolytic enzyme responsible for the activation of autolysin in the cell wall of *E. faecium* (40, 56, 57) and to inhibit glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans* (6).

Generally, the activity of fatty acids and their derivatives against bacteria is affected by the presence of starch, proteins such as serum albumin, lipids such as phospholipids, and other surface-active agents such as cholesterol (27, 49). There have been few studies investigating the mechanism(s) of resistance to fatty acids and their derivatives. Lee and Shafer (32) studied the resistance of gonococci to long-chain fatty acids and discovered that resistance was mediated by an efflux pump encoded by *farAB* (fatty acid resistance). To et al. (55) also reported that the resistance of *Listeria monocytogenes* to the surfactant benzalkonium chloride was due to efflux pumps.

Enterococci can survive some types of food processing and have been implicated in outbreaks of food-borne illnesses and in the spoilage of processed cooked meat, raw meat, milk, and milk products (1, 14, 28). Enterococci are not important food-borne pathogens, but livestock and poultry can serve as reservoirs for drug-resistant strains of *Enterococcus*, which may then enter the human food chain and cause serious infections (4, 14, 15, 37, 44). There is increasing concern about the emergence of multiple-antibiotic-resistant enterococci and the presence of vancomycin-resistant enterococci in nonhuman reservoirs (1, 14, 18, 20, 52). In order to assess the utility of monolaurin as a food preservative against enterococci, it was important to determine its cellular target(s) and potential mechanisms of resistance to this compound.

In this communication, we report on the isolation and characterization of monolaurin-resistant *E. faecalis* mutants. We propose that monolaurin resistance in these mutants is medi-
ated by changes in their cell surface hydrophobicity limiting the access of monolaurin to a potential target in the cytoplasmic membrane and/or in the cytoplasm of the bacterium.

**MATERIALS AND METHODS**

**Chemical stocks.** Unless otherwise stated, all of the chemicals used were purchased from Sigma Chemical Co., St. Louis, MO, and stock solutions were filter sterilized (0.2 μm Supor Acrodisc; Gelman Sciences, Ann Arbor, MI). Monolaurin and Lauricidin (90% lauric acid, 8% myristic acid, 2% capric acid; Med-Chem Labs) were prepared as stock solutions of 50 mg/ml in 95% ethanol.

**Media, bacterial strains, and plasmids.** The media (Difco, Fort Richard Laboratories Ltd., Auckland, New Zealand) used in the present study were prepared according to the manufacturer’s specifications. The bacterial strains and plasmids used in the present study are listed in Table 1. For routine cultivation, *E. faecalis* and *Escherichia coli* strains were propagated for 24 h at 37°C in brain heart infusion (BHI) and Luria broth, respectively. Strains in regular use were subcultured on either Luria agar or BHI agar every 2 weeks and maintained at 4°C. Stock cultures were stored at −80°C in 10% skim milk containing 20% glycerol.

**Effect of monolaurin on the growth of *E. faecalis* AR01/DGVS.** An overnight culture of *E. faecalis* AR01/DGVS was diluted to an optical density at 595 nm (OD<sub>595</sub>) of 0.01 in BHI and 100–μl volumes were dispensed into the wells of a flat-bottom 96-well microtiter plate (Nalgene Nunc GmbH & Co. KG, Wiesbaden, Germany). The stock solution of monolaurin was diluted to attain final concentrations of 800, 400, 200, 100, 50, 25, 10, and 5 μg/ml in BHI, and 100 μl was added to each well when the bacterial cells reached an OD<sub>595</sub> of approximately 0.4. Control wells received 100 μl of broth or 100 μl of 0.1% ethanol, and all tests were conducted in triplicate. The cultures were incubated at 37°C for 24 h in a plate reader (Multiskan Ascent Microtiter Plate Reader; LabSystems, Vantaa, Finland) with absorbance readings (595 nm) taken every 2 h. The MIC was determined by Southern blot hybridization of HindIII-digested (National Center for Biotechnology Information, Los Alamos, NM), available (DNASTAR, Inc.) and the programs BLASTN, BLASTP, BLASTX, and CDD on the internet. The sites of Tn<sub>917</sub> insertions in the selected monolaurin-resistant mutants were mapped by inverse PCR with Tn<sub>917</sub>-derived primers ErmP2 (5′-TACAA ATTCCTCTTGGACGC-3′) and HindIII (5′-GACATTTAAAGCGGCTTGCG-3′). Total DNA from Tn<sub>917</sub> mutants was digested with HindIII and self-ligated with T4 DNA ligase (Roche), and an inverse PCR was carried out with an Expand Long Template PCR system (Roche) under the conditions recommended by the manufacturer.

**Nucleotide sequencing and sequence analysis.** PCR products and plasmids were sequenced directly. Sequencing reactions were carried out with a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom) and a model ABI377 automated DNA sequence (Applied Biosystems). The nucleotide sequences were assembled with Seqman (DNASTAR, Inc.). Sequence analyses were carried out with Editsq (DNASTAR, Inc.) and the programs BLASTN, BLASTP, BLASTX, and CDD (National Center for Biotechnology Information, Los Alamos, NM), available via the internet. The sites of Tn<sub>917</sub> insertions were determined by Southern blot hybridization of HindIII-digested (Roche, Mannheim, Germany) genomic DNA and a radioactively labeled, HindIII-digested pTV1-OK probe (47).

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> MCI061</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD139 (ara-lexa)7696 galE15 galK16 (lac)X74 rpsL (Str&lt;sup&gt;R&lt;/sup&gt;) hsdR2 (r&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;−&lt;/sup&gt;) mcrA mcrB1</td>
<td>7</td>
</tr>
<tr>
<td>AR01/DGVS</td>
<td>AR01/DG cured of pJM02 (V&lt;sup&gt;R&lt;/sup&gt; E&lt;sup&gt;R&lt;/sup&gt;) Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>DGRM12</td>
<td>AR01/DGVS traB:Tn&lt;sub&gt;917&lt;/sub&gt; Em&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; Mk&lt;sup&gt;R&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>DGRM5</td>
<td>AR01/DGVS traB:Tn&lt;sub&gt;917&lt;/sub&gt; Em&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; Mk&lt;sup&gt;R&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>DGRM12</td>
<td>AR01/DGVS harboring pAM401 Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; M&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>DGRM2/pAMCL6</td>
<td>DGRM2 harboring pAMCL6 Em&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; Sp&lt;sup&gt;R&lt;/sup&gt; M&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>DGRM5/pAMCL6</td>
<td>DGRM5 harboring pAMCL6 Em&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; Sp&lt;sup&gt;R&lt;/sup&gt; M&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>DGRM12/pAMCL6</td>
<td>DGRM12 harboring pAMCL6 Em&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; Bc&lt;sup&gt;R&lt;/sup&gt; Sp&lt;sup&gt;R&lt;/sup&gt; M&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>JH2-2</td>
<td>Glycopeptide-susceptible strain commonly used for gene transfer experiments with <em>Enterococcus</em></td>
<td>37</td>
</tr>
<tr>
<td>JH2-7349</td>
<td>JH2-2 harboring pPIT7349 Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>JH2-7013</td>
<td>JH2-2 harboring pPIT7013 Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>Plasmids</td>
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<tr>
<td>pTV1-OK</td>
<td>repA(Ts)-pWW01Ts aphA3 Tn&lt;sub&gt;917&lt;/sub&gt; Km&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pCL1921</td>
<td>Low-copy-number vector; St&lt;sup&gt;R&lt;/sup&gt; Sp&lt;sup&gt;R&lt;/sup&gt; 4.6 kb</td>
<td>33</td>
</tr>
<tr>
<td>pCL1921</td>
<td>pCL1921 harboring 6.8-kb insert from AR01/DGVS containing traB; St&lt;sup&gt;R&lt;/sup&gt; Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pAM401</td>
<td><em>E. coli</em>- <em>E. faecalis</em> shuttle vector; Cm&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt; 10.4 kb</td>
<td>61</td>
</tr>
<tr>
<td>pAMCL6</td>
<td>pAM401 harboring pCL1921 and 6.8-kb insert from AR01/DGVS containing traB; Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>pPIT7349</td>
<td>pPD1::Tn&lt;sub&gt;917&lt;/sub&gt; derivative with insertion in traB</td>
<td>16</td>
</tr>
<tr>
<td>pPIT7013</td>
<td>pPD1::Tn&lt;sub&gt;917&lt;/sub&gt; derivative with insertion in orfY</td>
<td>16</td>
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</tbody>
</table>
overnight in 20 ml of THBG (Todd-Hewitt broth containing 2% glucose) and E. Jonge et al. (12). Cells of JH2-2 (no pPD1 plasmid) and JH2-7013 (a non-DGRM2, the MICs and cell surface hydrophobicity of DGRM2 were compared to those of another trb mutant, E. faecalis JH2-7348. The controls included were JH2-2 (no pPD1 plasmid) and JH2-7013 (a non-trb mutant). E. faecalis strains JH2-7348 and JH2-7013 were obtained by electroporating plasmids pTP7349 and pTP1703, respectively (provided by Shuhei Fujimoto [16]) into E. faecalis JH-2.

Autoysis assay. Cell autoysis was assayed by modification of the method of de Jonge et al. (12). Cells of E. faecalis AR01/DGVs and DGRM2 were grown overnight in 20 ml of THBG (Todd-Hewitt broth containing 2% glucose) and THBG/Erm10 (THBG containing 10 μg/ml erythromycin), respectively. One milliliter of THBG was used to inoculate THBG or THBG/Erm10 which was incubated at 37°C until an OD500 of 0.3 was reached, at which point the cells were chilled on ice for 15 min. The cells were washed twice in ice-cold MiliQ water and resuspended in 50 mM Tris-HCl (pH 8.0) to an OD500 of 1.0. The cell suspensions were incubated at 37°C for 6 h, and autoysis was determined by reading the OD500 at regular intervals. Two concentrations of monolaurin were tested for the ability to induce autoysis, 50 μg/ml AR01/DGVs) and 100 μg/ml DGRM2). Also included were 0.015% Triton X-100 and MiliQ water as positive and negative controls, respectively.

Fatty acid analysis. The fatty acid compositions of E. faecalis AR01/DGVs and DGRM2 cells and cell membranes were established when the bacteria were grown in the presence (50 μg/ml) or absence of monolaurin. Overnight cultures of E. faecalis AR01/DGVs and DGRM2 were grown in BH1 and BH1/Erm10, respectively.

For the preparation of whole-cell samples, bacterial cells were collected by centrifugation (7,000 × g, 15 min at 4°C). The bacterial cells were washed three times in ice-cold MiliQ water and cell pellets (0.5 g wet weight) were stored at −20°C until required.

For the preparation of cell membranes, cells (5 g, wet weight) were resuspended in 5 ml of 50 mM morpholinopropanesulfonic acid (MOPS) buffer (pH 7.5) containing cacodylate buffer (pH 7.0) with 3 mg/ml ruthenium red. After 20 min at 4°C, the samples were washed three times with the same buffer and the cells were embedded in 3% glutaraldehyde. The fixed samples were washed three times in the same buffer and then postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.0) containing ruthenium red. After 1 h at room temperature, the samples were removed and then dried for 2 h on a rotator (2 rpm) at room temperature. The samples were then dehydrated with increasing concentrations of ethanol, and finally embedded in Quetol 651 resin. The samples were visualized using a Philips CM100 TEM (FEI/Philips, Eindhoven, The Netherlands).

Nucleotide sequence accession number. The DNA sequence data from E. faecalis AR01/DGVs, located on pJM01, has been deposited in GenBank under accession number EF035487.

RESULTS

Effect of monolaurin on the growth of E. faecalis AR01/DGVs. The Enterococcus strain used in this study was derived from E. faecalis AR01/DG, isolated from a dog with mastitis. AR01/DG harbors two plasmids carrying antibiotic resistance genes, pJM01 (tetracycline and bacitracin resistance) and pJM02 (vancomycin and erythromycin resistance). E. faecalis AR01/DGVs was obtained by curing AR01/DG of plasmid pJM02 (36). Plasmid pJM01 is 72 kb in size and has a frequency of conjugation of 7.2 × 10−4 in broth mating (36).
The MIC of monolaurin for *E. faecalis* AR01/DGVS was 100 μg/ml (Table 2), but 200 μg/ml was required to inhibit exponentially growing cells (Fig. 1). Monolaurin added to exponentially growing cells at high concentrations caused a rapid decrease in OD$_{595}$ suggestive of cell lysis (Fig. 1). AR01/DGVS partially growing cells at high concentrations caused a rapid decrease in OD$_{595}$ suggestive of cell lysis (Fig. 1). Monolaurin added to exponentially growing cells (Fig. 1). Monolaurin added when the OD$_{595}$ reached 0.4 to final concentrations of 0 μg/ml (■), 1 μg/ml (○), 5 μg/ml (▲), 10 μg/ml (●), 25 μg/ml (▲), 50 μg/ml (□), 100 μg/ml (●), 200 μg/ml (○), 400 μg/ml (◇), and 800 μg/ml (+). Each symbol represents the mean of triplicate samples. Error bars represent standard deviation. The vertical arrow indicates the point at which monolaurin was added.

### TABLE 2. Phenotypic characterization of the *E. faecalis* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>% Hydrophobicity</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monolaurin</td>
</tr>
<tr>
<td>JH2-2</td>
<td>Wild type</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>JH2-7013</td>
<td>Tn917::orfY</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>JH2-7349</td>
<td>Tn917::traB</td>
<td>69</td>
<td>300</td>
</tr>
<tr>
<td>AR01/DGVS</td>
<td>Wild type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DGRM2</td>
<td>Tn917::traB</td>
<td>65</td>
<td>300</td>
</tr>
<tr>
<td>DGRM5</td>
<td>Tn917::traB</td>
<td>65</td>
<td>300</td>
</tr>
<tr>
<td>DGRM12</td>
<td>Tn917::traB</td>
<td>70</td>
<td>300</td>
</tr>
</tbody>
</table>

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The resistance of *E. faecalis* AR01/DGVS and the mutants DGRM2, DGRM5, and DGRM12 to other antimicrobial compounds was investigated to establish if the resistance to monolaurin also conferred resistance to antimicrobial compounds with diverse targets. The MICs of gentamicin and chloramphenicol for DGRM2, DGRM5, and DGRM12 were twofold higher than for AR01/DGVS (Table 2). An identical pattern of change in sensitivity to gentamicin and chloramphenicol was observed between JH2-2 and JH2-7349. The MICs of gentamicin and chloramphenicol for JH2-2 and JH2-7013 (transposon inserted in a non-traB gene) were identical. No differences in the MICs of the cell wall-targeting antibiotics penicillin and vancomycin were observed between the parent and mutant strains (Table 2).

The cell surfaces of *E. faecalis* DGRM2, DGRM5, DGRM12, and JH2-7349 were much more hydrophilic than those of *E. faecalis* AR01/DGVS, JH2-2, or JH2-7013 (Table 2). The fatty acid composition of *E. faecalis* AR01/DGVS and DGRM2 cells was analyzed when the bacterial cells were grown in the presence or absence of monolaurin. Myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1, cis), C16:1 isomer, stearic (C18:0), and vaccenic (C18:1) acids were identified as the major fatty acids in the *E. faecalis* strains (Table 3). The fatty acid profiles of AR01/DGVS and DGRM2 were similar for cultures grown in either the presence or the absence of monolaurin (Table 3). The addition of monolaurin had a minor influence on the total level of fatty acids, which increased on average by 21% (27.5% for AR01/DGVS and 14.6% for DGRM2), but had a major impact on the fatty acid ratios, with lauric acid accounting for 50% of the total fatty acid in both AR01/DGVS and DGRM2 (Table 3). The presence of monolaurin changed the cellular saturated-to-unsaturated fatty acid ratio from 1.4 (averages of 1.26 and 1.54) to 4.35 (averages of 4.26 and 4.43) (Table 3).

The fatty acid analysis of *E. faecalis* AR01/DGVS and DGRM2 cell membranes revealed the same pattern, i.e., no difference in profiles, irrespective of whether cells were grown in the presence or absence of monolaurin (Table 4). However, it did identify C16:0 and C18:1 as the major saturated and unsaturated fatty acids, respectively (Table 4). Addition of monolaurin to test cultures resulted in the incorporation of lauric acid into the membranes, which was accompanied by a similar decrease in C18:1 levels (Table 4). On average, lauric acid accounted for 10% of the membranes fatty acids (Table 4).

TEM showed no obvious differences between the cell walls of *E. faecalis* AR01/DGVS and DGRM2 grown in the absence of monolaurin (Fig. 4A and C). However, when grown in the...
presence of monolaurin, DGRM2 showed an apparent increase in the thickness of the cell wall (Fig. 4D). No changes were noted in the cell cytoplasm of E. faecalis AR01/DGVS; by contrast, E. faecalis DGRM2 showed contraction of its cytoplasm (Fig. 4D). E. faecalis DGRM2 cells were also observed to form small clumps of cells when grown in the presence of monolaurin (data not shown).

**DISCUSSION**

Although studies on the antimicrobial properties of fatty acids and their derivatives, or surface-active anionic detergents, date back to 1899 (10), there is little information on the mechanisms of resistance to such compounds. Because of the potential spread of vancomycin-resistant enterococci from animal to humans through the food chain (4, 14, 37, 44), E. faecalis was used as a model to study bacterial resistance to monolaurin. In preliminary studies on the inhibitory effect of monolaurin (data not shown).

To confirm that monolaurin resistance was due to inactivation of the *traB* gene, complementation of the *E. faecalis* mutants DGRM2, DGRM5, and DGRM12 was carried out. In all three monolaurin-resistant mutants, sensitivity to monolaurin was restored by complementation with *traB* in *trans*, suggesting that the *traB* mutation alone conferred resistance to monolaurin. Since all three mutants were shown to be interrupted in *traB*, a single mutant, *E. faecalis* DGRM2, was selected for further characterization. *E. faecalis* mutants resistant to monolaurin also showed increased resistance to gentamicin and chloramphenicol, suggesting a lack of penetration by these compounds and thus an altered cytoplasmic membrane. This observation is supported by other studies where it was shown that dodecylglycerol (corresponding ether of monolaurin) in-

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**TABLE 3. Fatty acid composition of *E. faecalis* cells**

<table>
<thead>
<tr>
<th>Strain, condition</th>
<th>Total fatty acids (μg/g [wet wt])</th>
<th>SFA/UFA ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fatty acid (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C&lt;sub&gt;12:0&lt;/sub&gt;</td>
</tr>
<tr>
<td>WT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.647 (13.3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.26</td>
<td>4.7 (11.1)</td>
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<td>WT, ML&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.883 (9.4)</td>
<td>49.7 (11.8)</td>
<td>2.7 (6.7)</td>
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<td>WT, ML&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>52.8</td>
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<tr>
<td>DGRM2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.610 (14.3)</td>
<td>1.54</td>
<td>5.4</td>
</tr>
<tr>
<td>DGRM2, ML&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.845 (0.8)</td>
<td>51.6 (0.9)</td>
<td>3.8 (1.1)</td>
</tr>
<tr>
<td>DGRM2, ML&lt;sup&gt;d&lt;/sup&gt;</td>
<td>893&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.8</td>
<td>7.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild-type *E. faecalis* AR01/DGVS.

<sup>b</sup> Average for two cultures, two extractions per culture, duplicate FAME analyses (n = 8).

<sup>c</sup> ML, grown in the presence of 50 μg/ml monolaurin.

<sup>d</sup> Average for one culture, two extractions, duplicate FAME analyses (n = 4).

<sup>e</sup> Values in parentheses are coefficients of variation (%).

<sup>f</sup> Total fatty acids, excluding lauric acid.

<sup>g</sup> Ratio of saturated fatty acids (SFA) to unsaturated fatty acids (UFA).

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**TABLE 4. Fatty acid composition of *E. faecalis* cell membranes**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Fatty acid composition of cell membranes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;12:0&lt;/sub&gt;</td>
<td>0.3</td>
</tr>
<tr>
<td>C&lt;sub&gt;14:0&lt;/sub&gt;</td>
<td>4.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>37.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;&lt;sup&gt;isomer&lt;/sup&gt;</td>
<td>1.8</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1&lt;sup&gt;Δ9cis&lt;/sup&gt;&lt;/sub&gt;</td>
<td>5.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:0&lt;/sub&gt;</td>
<td>6.5</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:1&lt;sup&gt;n9c&lt;/sup&gt;&lt;/sub&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:1&lt;/sub&gt;</td>
<td>41.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:2&lt;/sub&gt;</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild-type.

<sup>b</sup> ML, grown in the presence of 50 μg/ml monolaurin.
hibited glycerolipid and lipoteichoic acid biosynthesis in *S. mutans* (6).

Fatty acids and their derivatives have been reported to target the cytoplasmic membrane (17, 24–27), and it was therefore possible that the increased resistance of the *traB* mutant was linked to changes in the composition of the cytoplasmic membrane. However, there was no difference in the fatty acid composition (percentage of each identified fatty acid) between *E. faecalis* AR01/DGVS and DGRM2, irrespective of the growth conditions. We did observe a change in the fatty acid profile (ratio of saturated to unsaturated fatty acids) between AR01/DGVS and DGRM2 when they were grown in the presence of monolaurin, with the ratio of saturated to unsaturated fatty acids increasing in both cases. The increase in saturated fatty acid (C14:0, C16:0, and C18:0) and the decrease in unsaturated fatty acid (C18:1) suggest that AR01/DGVS and DGRM2 were adapting to the presence of monolaurin by attempting to make the cell membrane more rigid and therefore potentially less permeable to monolaurin. Rigidification of the membrane has been suggested as a mechanism of resistance for membrane-active compounds such as nisin (34, 38, 55). It should be noted that when the cells were grown in the presence of monolaurin, lauric acid was incorporated into the cell membranes, resulting in changes in the fatty acid profile of the *E. faecalis* strains. Juneja and Davidson (23) reported that growing *L. monocytogenes* in the presence of exogenous fatty acids resulted in the incorporation of and consequently an increase in the percentage of these particular fatty acids in the cell membrane. This suggests that the incorporation of lauric acid into the cell membrane was responsible for the fatty acid profile changes observed and that this incorporation is eventually toxic to the cells.

The cell surface of the parent strain was hydrophobic, while that of the monolaurin-resistant mutant was more hydrophilic in nature. Monolaurin, a hydrophobic compound, would be less able to penetrate a highly hydrophilic cell surface; thus, it is possible that the resistance of DGRM2 to monolaurin was a direct consequence of its low cell surface hydrophilicity. It has been reported that the cell surfaces of organic-solvent-tolerant mutants isolated from *E. coli* were more hydrophilic than those of their parent strain (3). Low cell surface hydrophilicity has been reported to serve as a defensive mechanism which prevents the accumulation of organic solvent molecules in the cytoplasmic membrane (3). The same change in cell surface hydrophilicity was observed in JH2-7349, a *traB* mutant (16). The lack of difference in cell surface hydrophilicity between JH2-2 and JH2-7013 indicated that the observed changes were not induced by the presence of Tn917. Although the AR01/DGVS and JH2-7349 *traB* sequences have no homology to each other or any other *traB* sequence, the degree of predicted amino acid similarity, the presence of the conserved COG1916 domain, and the fact that the two strains had identical patterns of hydrophobicity and permeability to antibiotics provide strong evidence that the functions of these two proteins are similar.

The possibility cannot, however, be excluded that the low cell surface hydrophilicity observed in the *traB* mutants is not directly linked to the inactivation of *traB* since JH2-2 also
showed high cell surface hydrophobicity in the absence of traB (JH2-2 does not contain a copy of traB). It is possible that Tn917 inactivation of traB had a downstream effect on the expression of another protein such as the aggregation substance. Aggregation substance, a bacterial adhesin, mediates the contact between donor and recipient cells, thus facilitating plasmid conjugation (2). Various studies (21, 58) have reported that the expression of aggregation substance on the cell surface of *E. faecalis* cells resulted in a significant increase in cell surface hydrophobicity. The role of TraB is to shut down the pheromone response and/or to prevent self-induction, which results in part in the shutting down of aggregation substance production (2). Waters et al. (59) have shown that the expression of aggregation substance on the cell wall of AR01/DGVS results in low cell surface hydrophobicity when the bacteria are grown in the presence of monolaurin. This remains to be further investigated.

There was no significant difference between the cell wall structure of *E. faecalis* AR01/DGVS and that of DGRM2 viewed by TEM when bacteria were grown in the presence or absence of monolaurin. However, in the presence of monolaurin, the cytoplasm of *E. faecalis* DGRM2 seemed to have shrunk drastically and the space between the cell membrane and cell wall markedly increased. The effects of monolaurin on *E. faecalis* DGRM2 cell morphology could be explained by decreased cell surface permeability preventing proper ethanol fixation. This correlates with the findings by Aono and Kobayashi (3), who reported that low cell surface hydrophobicity prevented the access of organic solvent to the cytoplasmic membrane.

In conclusion, monolaurin-resistant mutants of *E. faecalis* were isolated by transposon mutagenesis. The monolaurin-resistant mutants were disrupted in the pheromone shutdown protein TraB. The characterization of one of these monolaurin-resistant mutants, *E. faecalis* DGRM2, suggests that its resistance is linked to a decrease in cell surface hydrophobicity that limits the diffusion of monolaurin into the cell. This, in turn, suggests that monolaurin requires access to the cytoplasmic membrane and/or the cytoplasm to exert its antimicrobial activity.

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REFERENCES


