

Characterization of Monolaurin Resistance in *Enterococcus faecalis*[∇]

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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 Tn917 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-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>E. coli</i> MC1061	F ⁻ <i>araD139 (ara-leu)7696 galE15 galK16 (lac)X74 rspL (Str^r) hsdR2 (r_k⁻ m_k⁺) mcrA mcrB1</i>	7
<i>E. faecalis</i>		
AR01/DGVS	AR01/DG cured of pJM02 (Vm ^r Em ^r) Tc ^r Bc ^r	36
DGRM2	AR01/DGVS <i>traB::Tn917</i> Em ^r Tc ^r Bc ^r MI ^r	36
DGRM5	AR01/DGVS <i>traB::Tn917</i> Em ^r Tc ^r Bc ^r MI ^r	36
DGRM12	AR01/DGVS <i>traB::Tn917</i> Em ^r Tc ^r Bc ^r MI ^r	36
AR01/DGVS/pAM401	AR01/DGVS harboring pAM401 Tc ^r Bc ^r Cm ^r	This study
DGRM2/pAMCL6	DGRM2 harboring pAMCL6 Em ^r Tc ^r Bc ^r Sp ^r MI ^s	This study
DGRM5/pAMCL6	DGRM5 harboring pAMCL6 Em ^r Tc ^r Bc ^r Sp ^r MI ^s	This study
DGRM12/pAMCL6	DGRM12 harboring pAMCL6 Em ^r Tc ^r Bc ^r Sp ^r MI ^s	This study
JH2-2	Glycopeptide-susceptible strain commonly used for gene transfer experiments with <i>Enterococcus</i>	22
JH2-7349	JH2-2 harboring pPIT7349 Em ^r	This study
JH2-7013	JH2-2 harboring pPIT7013 Em ^r	This study
Plasmids		
pTV1-OK	<i>repA</i> (Ts)-pWV01Ts <i>aphA3</i> Tn917 Km ^r Em ^r	19
pCL1921	Low-copy-number vector; St ^r Sp ^r , 4.6 kb	33
pCL6	pCL1921 harboring 6.8-kb insert from AR01/DGVS containing <i>traB</i> ; St ^r Sp ^r	This study
pAM401	<i>E. coli-E. faecalis</i> shuttle vector; Cm ^r Tc ^r , 10.4 kb	61
pAMCL6	pAM401 harboring pCL1921 and 6.8-kb insert from AR01/DGVS containing <i>traB</i> ; Tc ^r	This study
pPIT7349	pPD1::Tn917 derivative with insertion in <i>traB</i>	16
pPIT7013	pPD1::Tn917 derivative with insertion in <i>orfY</i>	16

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 μ m 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. Stock solutions of monolaurin, Lauricidin, and antibiotics were stored at -20°C until required.

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_{595}) of 0.01 in BHI, and 100- μ l 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, 5, and 1 μ g/ml in BHI, and 100 μ l was added to each well when the bacterial cells reached an OD_{595} of approximately 0.4. Control wells received 100 μ l of broth or 100 μ l 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 defined as the lowest concentration of monolaurin to show complete inhibition of bacterial growth (OD_{595} , <0.1) after 24 h of incubation at 37°C .

Transposon mutagenesis and isolation of monolaurin-resistant mutants. To identify genes involved in the development of resistance to monolaurin in *E. faecalis*, an *E. faecalis* AR01/DGVS Tn917 mutant library (36) was screened for resistance by the following protocol. A Lauricidin stock solution was diluted to 500 μ g/ml in BHI/Erm10 (BHI containing 10 μ g/ml erythromycin), and 40 μ l was added to the wells of microtiter plates containing 100 μ l of BHI/Erm10. A 60- μ l volume of an overnight culture of each mutant strain was added to each well, and

the OD_{595} was read at 0 h with the plate reader. Following 24 h of incubation at 37°C , the absorbance was read again and the change in OD_{595} (ΔOD) was calculated by subtracting the absorbance value at time zero from that at 24 h.

Genomic DNA extraction, transformation, and genetic techniques. *E. faecalis* chromosomal DNA was obtained as described previously (35). Transformation of *E. faecalis* with glycine-grown cells was performed as described by Shepard and Gilmore (48). DNA manipulations were carried out according to standard molecular biology protocols (47). Purified plasmid DNA was prepared with a QIAprep spin miniprep kit (QIAGEN, Hilden, Germany) for high-copy plasmid extraction or a plasmid midi kit (QIAGEN) for low-copy vectors. Restriction endonucleases, ligases, and polymerases were used according to the manufacturer's instructions. PCR were performed in accordance with the manufacturer's instructions, by using the PCR program described previously (31). Radiolabeled PCR products and plasmids were prepared by incorporation of [α - ^{32}P]dCTP-labeled deoxynucleotides with Ready-to-Go DNA labeling beads (Amersham, Buckinghamshire, England). Southern transfer and hybridization were performed as previously described (35).

Mapping of transposon inserts. The presence of single Tn917 insertions was determined by Southern blot hybridization of HindIII-digested (Roche, Mannheim, Germany) genomic DNA and a radioactively labeled, HindIII-digested pTV1-OK probe (47).

The sites of Tn917 insertions in the selected monolaurin-resistant mutants were mapped by inverse PCR with Tn917-derived primers ErmP2 (5'-TACAA ATTCTCGTAGGC-3') and HindIII (5'-GACATTATAAGCCGCTGTGCG-3'). Total DNA from Tn917 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 sequencer (Applied Biosystems). The nucleotide sequences were assembled with Seqman (DNASTAR, Inc.). Sequence analyses were carried out with Editseq (DNASTAR, Inc.) and the programs BLASTN, BLASTP, BLASTX, and CDD (National Center for Biotechnology Information, Los Alamos, NM), available via the internet.

Cloning of the *traB* gene and plasmid construction for complementation studies. The *traB* gene of *E. faecalis* AR01/DGVS was cloned as a 6.8-kb EcoRI (Roche) fragment into pCL1921, creating plasmid pCL6. The resulting construct was electroporated into *E. coli*, and blue/white selection was used to screen the transformants. The DNA insert of randomly selected transformants was se-

quenced to confirm the presence of the *traB* gene. To enable complementation, plasmid pAMCL6 was constructed by ligating pCL6 into *E. coli-E. faecalis* shuttle vector pAM401. *E. coli* transformants were selected for tetracycline resistance and chloramphenicol sensitivity. The size of pAMCL6 was confirmed by enzyme digestion and gel electrophoresis. pAMCL6 was electroporated into *E. faecalis*, and its presence was confirmed by enzyme digests and PCR.

The *traB::Tn917* mutants were originally selected on the basis of their increased resistance to monolaurin compared to the *E. faecalis* parent strain. It was therefore necessary to determine if complementation of the *traB* mutants with the *traB* gene would restore their sensitivity to monolaurin. Plasmids extracted from the complemented *E. faecalis* mutants were used as template DNA for amplification of the *traB* gene with the TraBF22 (5'-CGGAGAGACACCGTCAGGGG-3') and TraBR1121 (5'-CCTATAGCTCCTCTAAATT-3') primers. The MICs of monolaurin and other selected antimicrobials (i.e., vancomycin, gentamicin, penicillin, and chloramphenicol) for *E. faecalis* strains were established as described above.

***E. faecalis* JH2-7349.** To further characterize our *traB* mutant, *E. faecalis* DGRM2, the MICs and cell surface hydrophobicity of DGRM2 were compared to those of another *traB* mutant, *E. faecalis* JH2-7349. The controls included were JH2-2 (no pPD1 plasmid) and JH2-7013 (a non-*traB* mutant). *E. faecalis* strains JH2-7349 and JH2-7013 were obtained by electroporating plasmids pPIT7349 and pPIT7013, respectively (provided by Shuhei Fujimoto [16]) into *E. faecalis* JH2-2.

Autolysis assay. Cell autolysis 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 overnight culture was used to inoculate THBG or THBG/Erm10, which was incubated at 37°C until an OD₆₀₀ 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 MilliQ water and resuspended in 50 mM Tris-HCl (pH 8.0) to an OD₆₀₀ of 1.0. The cell suspensions were incubated at 37°C for 6 h, and autolysis was determined by reading the OD₆₀₀ at regular intervals. Two concentrations of monolaurin were tested for the ability to induce autolysis, 50 µg/ml (AR01/DGVS) and 100 µg/ml (DGRM2). Also included were 0.01% Triton X-100 and MilliQ 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 BHI and BHI/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 MilliQ 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 morpholinepropanesulfonic acid (MOPS) buffer (pH 7.5) containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.2 mg of lysozyme, and 2,500 U of mutanolysin. Following 60 min of incubation at 37°C with gentle stirring, 15 mM magnesium chloride containing 2 mg of DNase was added to the suspensions. The suspensions were incubated for a further 15 min at 37°C and then lysed by two passages through a French pressure cell at 20,000 lb/in². The crude lysate was centrifuged at 10,000 × g for 20 min at 4°C to remove unbroken cells. The supernatant was centrifuged for 1 h at 180,000 × g to harvest the cell membranes. The membranes were washed and resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM magnesium chloride, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Samples were stored at -20°C prior use.

To extract lipids, the samples (cells and cell membranes) were transferred to a Kimax tube and the final volume was adjusted to 4.5 ml with MilliQ water. The internal standard (tridecanoic acid; Nu-Check Prep Inc., Elysian, MN), 60 µl of a 10-mg/ml solution in 1:1 (vol/vol) CHCl₃-methanol (MeOH) (GR for analysis and LiChrosolv; Merck, Darmstadt, Germany) was added to the tubes (series A tubes). To each sample, 12 ml of CHCl₃ and 6 ml of MeOH were added to give a final medium composition of 12:6:4.5 (vol/vol) CHCl₃-MeOH-water. The suspensions were shaken at room temperature (120 cycles/min, 30 min) and centrifuged (3,000 × g, 10 min). The organic layer was transferred into another Kimax tube (series B tubes), which was centrifuged (1,000 × g, 10 min) to remove any particulate at the surface of the organic layer. The organic layer was transferred to another 20-ml Kimax tube (series C tubes). Series A tubes were reextracted in 2:1 (vol/vol) CHCl₃-MeOH. The organic layer was transferred to series B tubes and centrifuged as described above. The combined organic layer was dried under a constant stream of nitrogen gas (oxygen free) while in a heating block at 30°C.

To series C tubes were added 300 µl of toluene and 1.4 ml of 14% BF₃-MeOH. The tubes were heated at 100°C for 2 h and then left to cool to room temperature. A saturated NaCl solution (6 ml) (NaCl AR grade; BDH, Poole, England) was added to each tube, which was shaken and then centrifuged (1,000 × g, 10 min). An aliquot of the top layer was transferred to a 2-ml autosampler vial containing a 250-µl insert for gas chromatography (GC) analysis.

Analysis of fatty acid methyl esters (FAMES) was carried out on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 series autosampler (Agilent Technologies Inc., Wilmington, DE). The injections were carried out in split mode (40:1) at 250°C. A flame ionization detector at 260°C was used with gas flow rates of air at 300 ml/min, hydrogen at 30 ml/min, and nitrogen at 30 ml/min. For quantitative analysis, separation was carried out on a BPX70 capillary column (50-m length, 330-µm inside diameter, 0.25-µm film thickness; SGE Analytical Products, Melbourne, Australia) with hydrogen gas (2 ml min⁻¹, 33 cm s⁻¹, constant-flow mode). The GC oven was initially held at 35°C for 5 min, increased to 205°C at 2.5°C min⁻¹, and then increased to 230°C at 10°C min⁻¹. For confirmation of peak FAME identities, separation was also carried out on a BPX5 capillary column (30-m length, 250-µm inside diameter, 0.25-µm film thickness; SGE Analytical Products, Melbourne, Australia) with hydrogen gas (1.2 ml/min, 36 cm/s, constant-flow mode). The GC oven started at 60°C and was then increased to 240°C at 2°C/min and held for 20 min. Data integration and computation were performed with HP Chemstation software (Hewlett-Packard).

Identification of fatty acids was carried out with authentic standards, and the retention times were compared on two columns with differing polarities (see above). The authentic standards included a FAME mixture (FAMEQ005) and a set of C_{18:1} isomers (6-*cis*-octadecenoic acid methyl ester, 6-*trans*-octadecenoic acid methyl ester, 11-*cis*-octadecenoic acid methyl ester, and 11-*trans*-octadecenoic acid methyl ester; Nu-Check Prep Inc., Elysian, MN).

Cell surface hydrophobicity. The cell surface hydrophobicity of *E. faecalis* AR01/DGVS and DGRM2 was assayed by the MATH (*microbial adhesion to hydrocarbons*) assay as described by Reifsteck et al. (42), with slight modifications. Bacteria were first washed three times in ice-cold MilliQ water and finally resuspended in phosphate wash solution to an OD₅₀₀ of 0.5. A 4.8-ml volume of each bacterial suspension was mixed with 0.8 ml of *n*-hexadecane in a glass tube and vigorously shaken for 1 min. After the preparations rested for 30 min, the OD₅₀₀ values of the aqueous phase were determined. The affinity of bacteria for the solvent was evaluated by the formula % adherence = (1 - A/A₀) × 100, where A₀ is the OD₅₀₀ of the bacterial suspension before mixing and A is the OD after mixing.

Transmission electron microscope (TEM) analysis of *E. faecalis* cells. To visualize differences occurring in the cell wall structure between *E. faecalis* AR01/DGVS and DGRM2 grown in the presence of monolaurin (AR01/DGV, 50 µg/ml; DGRM2, 100 µg/ml) or its absence, cells were examined by TEM. *E. faecalis* strains AR01/DGVS and DGRM2 were grown overnight in the presence or absence of monolaurin. Bacterial cells were washed in 0.1 M phosphate-buffered saline (pH 7.0) and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) with 3 mg/ml ruthenium red. After 2 h on a rotator (2 rpm) at room temperature, the samples were washed three times with the same buffer and 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 washed three times in the same buffer and the cells were embedded in 3% agarose. The samples were then loaded into a tissue processor, dehydrated with increasing concentrations of ethanol, and finally embedded in Quetol 651 resin. The samples were visualized with a Philips CM100 TEM (FEI/Philips, Eindhoven, The Netherlands).

Nucleotide sequence accession number. The DNA sequence of *traB* 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 7.28 × 10⁻³ in broth mating (36).

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

Strain	Genotype	% Hydrophobicity	MIC ($\mu\text{g/ml}$)				
			Monolaurin	Gentamicin	Chloramphenicol	Penicillin	Vancomycin
JH2-2	Wild type	95	100	50	7.5	2.5	2.5
JH2-7013	Tn917::orfY	92	100	50	7.5	2.5	2.5
JH2-7349	Tn917::traB	69	300	100	15	2.5	2.5
AR01/DGVS	Wild type	100	100	50	7.5	2.5	5
DGRM2	Tn917::traB	65	300	100	15	2.5	5
DGRM5	Tn917::traB	65	300	100	15	2.5	5
DGRM12	Tn917::traB	70	300	100	15	2.5	5

The MIC of monolaurin for *E. faecalis* AR01/DGVS was 100 $\mu\text{g/ml}$ (Table 2), but 200 $\mu\text{g/ml}$ was required to inhibit exponentially growing cells (Fig. 1). Monolaurin added to exponentially growing cells at high concentrations caused a rapid decrease in OD₅₉₅ suggestive of cell lysis (Fig. 1). AR01/DGVS mutants resistant to monolaurin were isolated in an attempt to identify its site of action and potential mechanisms of resistance to this compound.

Isolation of monolaurin-resistant mutants of *E. faecalis* AR01/DGVS. To identify genes involved in the development of resistance to monolaurin in *E. faecalis*, an *E. faecalis* AR01/DGVS Tn917 mutant library (36) was screened for resistance to monolaurin. Eight thousand Tn917 insertion mutants of *E. faecalis* AR01/DGVS were screened for the ability to grow in BHI/Erm10 containing 100 $\mu\text{g/ml}$ Lauricidin. Lauricidin was used in the initial screening because the cost of large amounts of monolaurin was prohibitive. A cell inoculum was added (30%, wt/vol), and Lauricidin was present at the time of inoculation. The average increase in OD₅₉₅ achieved after 24 h by

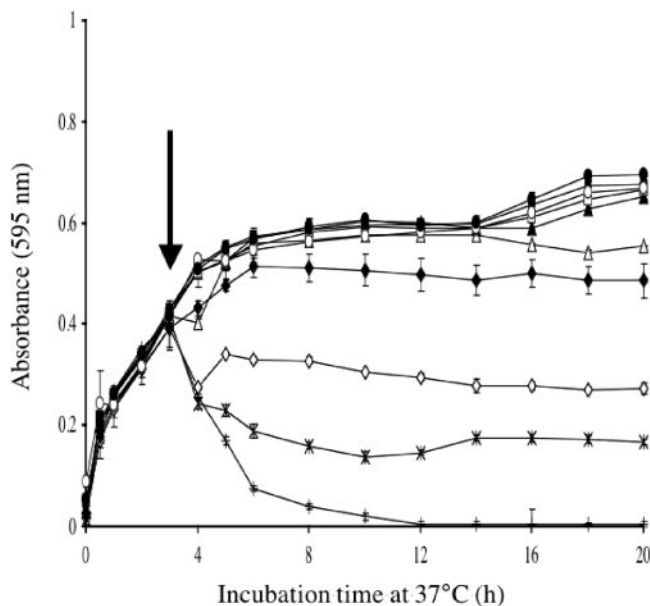


FIG. 1. Effect of monolaurin on the growth of *E. faecalis* AR01/DGVS. Monolaurin was added when the OD₅₉₅ reached 0.4 to final concentrations of 0 $\mu\text{g/ml}$ (●), 1 $\mu\text{g/ml}$ (○), 5 $\mu\text{g/ml}$ (■), 10 $\mu\text{g/ml}$ (□), 25 $\mu\text{g/ml}$ (▲), 50 $\mu\text{g/ml}$ (△), 100 $\mu\text{g/ml}$ (◆), 200 $\mu\text{g/ml}$ (◇), 400 $\mu\text{g/ml}$ (×), and 800 $\mu\text{g/ml}$ (+). Each symbol represents the mean of triplicate samples. Error bars represent standard deviation. The vertical arrow indicates the point at which monolaurin was added.

the 8,000 mutants was 0.355 ± 0.001 . Three mutants, designated DGRM2, DGRM5, and DGRM12, grew to OD₅₉₅s of 0.713, 0.591, and 0.640, respectively. All three monolaurin-resistant mutants had a hybridization profile indicative of a single chromosomal insertion of Tn917 (two bands of variable size and one common fragment of 1.3 kb, internal to Tn917) (Fig. 2A). No hybridization of the pTV1-OK probe with DNA from *E. faecalis* AR01/DGVS was observed. Inverse PCR was used to amplify the DNA sequence flanking the left variable arm of Tn917. The PCR products were sequenced, and one open reading frame (ORF) was identified that encodes a protein having 67% identity to TraB, a pheromone shutdown protein, from *E. faecalis* V583. Figure 2B indicates the sites of Tn917 insertions in the *traB*::Tn917 mutants.

Complementation of *traB*::Tn917 mutants. When pAMCL6, containing functional *traB*, was electroporated into *E. faecalis* DGRM2, DGRM5, and DGRM12, susceptibility to monolaurin was restored (MIC of 100 $\mu\text{g/ml}$), demonstrating that the Tn917 insertion in the *traB* gene was responsible for the observed phenotype (data not shown).

Characterization of monolaurin-resistant mutants. For the characterization of our *E. faecalis* *traB* mutant strains, we decided to use for comparison an *E. faecalis* strain harboring a pheromone-responsive plasmid carrying an inactivated (characterized) *traB* gene. For this purpose, we generated *E. faecalis* strain JH2-7349 carrying a pPD1 (pheromone-responsive plasmid) derivative, which had an inactivated *traB* gene because of a Tn917 insertion (16). The second pPD1 derivative, pPIT7013, had a Tn917 insert mapped in *orfY* and was used as a control to establish if any of the effects observed were due to the presence of Tn917 on plasmid pPD1 (16).

The MIC of monolaurin for strains DGRM2, DGRM5, DGRM12, and JH2-7349 was 300 $\mu\text{g/ml}$, while for AR01/DGVS, JH2-2, and JH2-7013 it was 100 $\mu\text{g/ml}$ (Table 2). In the absence of monolaurin, the growth rate of DGRM2 was comparable to that of the wild-type strain (Fig. 3). The addition of 200 $\mu\text{g/ml}$ monolaurin to actively growing cells resulted in an immediate decrease in OD₅₉₅ and inhibition of growth of *E. faecalis* AR01/DGVS but had no effect on the growth of DGRM2 (Fig. 3). On the basis of the observation that monolaurin induced cell lysis at high concentrations (Fig. 1), the autolytic activities of the wild type and the mutant were compared when they were grown in the presence of monolaurin, and they were found to be identical (30% cell lysis after 4 h), suggesting that the increased resistance of DGRM2 to monolaurin was not linked to altered autolytic activity (data not shown).

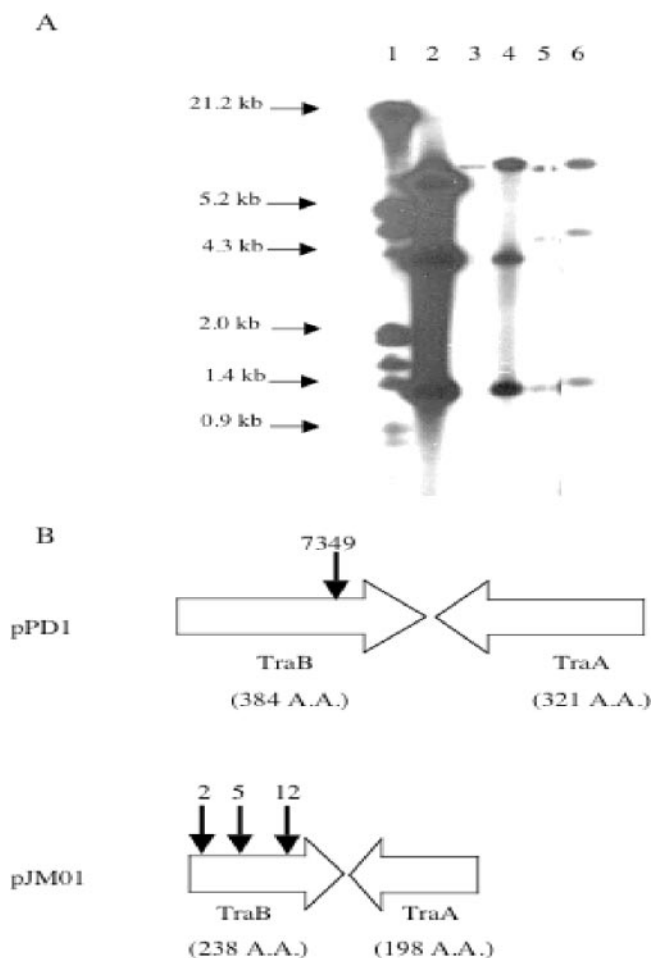


FIG. 2. Sites of Tn917 insertions. (A) Autoradiogram from a Southern blot analysis of the restriction digests of total DNA from *E. faecalis* strains following hybridization with ^{32}P -labeled pTV1-OK. Lane 1, lambda DNA digested with HindIII and EcoRI; lane 2, HindIII-digested pTV1-OK; lane 3, HindIII-digested *E. faecalis* AR01/DGVS. HindIII-digested *E. faecalis* mutants: lane 4, DGRM2; lane 5, DGRM5; lane 6, DGRM12. (B) Downward-pointing arrows indicate the sites of Tn917 insertions for *E. faecalis* strains as follows: 2, DGRM2; 5, DGRM5; 12, DGRM12; 7349, JH2-7349. Tn917 insertions for strains DGRM2, DGRM5, and DGRM12 were located within the *traB* gene of pJM01, while a Tn917 insertion for strain JH2-7349 was located within the *traB* gene of pPD1. The number of amino acids (A.A.) for each ORF is in parentheses.

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).

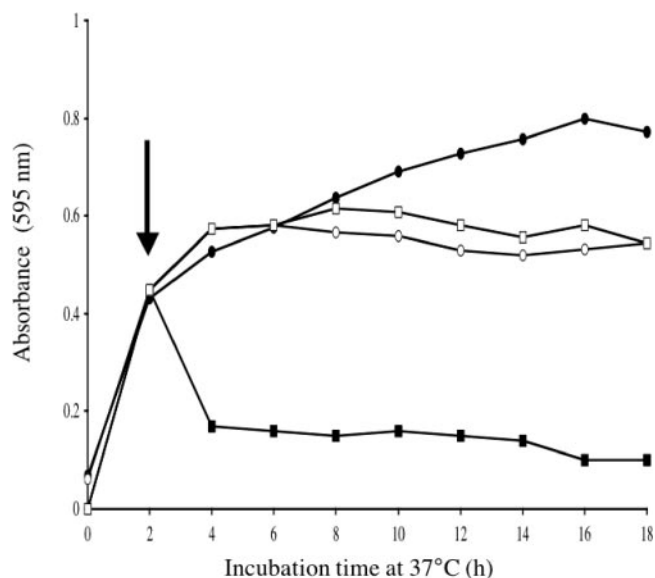


FIG. 3. Growth curves of *E. faecalis* strains grown in the presence (■, □) or absence (●, ○) of monolaurin. Growth curves of *E. faecalis* strains AR01/DGVS (■, ●) and DGRM2 (□, ○) are shown. Each symbol represents the mean of triplicate samples. Error bars represent standard deviations. The vertical arrow indicates the point at which monolaurin was added.

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 ($\text{C}_{14:0}$), palmitic ($\text{C}_{16:0}$), palmitoleic ($\text{C}_{16:1}\Delta 9\text{cis}$), $\text{C}_{16:1}$ isomer, stearic ($\text{C}_{18:0}$), and vaccenic ($\text{C}_{18:1}$) 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 $\text{C}_{16:0}$ and $\text{C}_{18:1}$ 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 $\text{C}_{18:1}$ 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

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

Strain, condition	Total fatty acids ($\mu\text{g/g}$ [wet wt])	SFA/UFA ratio ^g	Fatty acid (%)						
			C _{12:0}	C _{14:0}	C _{16:0}	C _{16:1} isomer	C _{16:1} Δ 9cis	C _{18:0}	C _{18:1}
WT ^{a,b}	1,476 (13.3) ^e	1.26		4.7 (11.1)	43.1 (11.8)	1.6 (15.4)	4.5 (14.1)	7.9 (9.6)	38.0 (16.6)
WT, ML ^{c,d}	1,883 (9.4)		49.7 (11.8)	2.7 (6.7)	26.6 (6.9)	0.4 (5.5)	1.1 (4.5)	11.5 (8.7)	7.9 (5.2)
WT, ML	947 ^f	4.26		5.4	52.8	0.9	2.3	22.8	15.8
DGRM2 ^{a,b}	1,610 (14.3)	1.54		5.8 (15.4)	47.0 (16.3)	1.6 (14.4)	5.5 (17.0)	7.8 (20.7)	32.2 (9.7)
DGRM2, ML ^{c,d}	1,845 (0.8)		51.6 (0.9)	3.8 (1.1)	28.6 (0.4)	0.5 (4.7)	1.6 (1.4)	7.2 (1.4)	6.9 (2.5)
DGRM2, ML	893 ^f	4.43		7.8	59.0	1.0	3.3	14.8	14.2

^a WT, wild-type *E. faecalis* AR01/DGVS.

^b Average for two cultures, two extractions per culture, duplicate FAME analyses ($n = 8$).

^c ML, grown in the presence of 50 $\mu\text{g/ml}$ monolaurin.

^d Average for one culture, two extractions, duplicate FAME analyses ($n = 4$).

^e Values in parentheses are coefficients of variation (%).

^f Total fatty acids, excluding lauric acid.

^g Ratio of saturated fatty acids (SFA) to unsaturated fatty acids (UFA).

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 on *E. faecalis* AR01/DGVS growth, it was observed that monolaurin induced cell lysis immediately following its addition to actively growing cells. This observation was consistent with reports that monolaurin stimulates the autolytic peptidoglycan hydrolase of *Enterococcus faecium* ATCC 9790 through activation of a latent proteinase (56, 57).

In order to study the potential target of and resistance mechanisms to monolaurin in *E. faecalis*, a library of Tn917 *E. faecalis* AR01/DGVS mutants was generated and screened for

monolaurin resistance (MIC, >100 $\mu\text{g/ml}$). Three monolaurin-resistant mutants were isolated which were shown to have single Tn917 insertions in the same ORF. Sequence analysis of this ORF by BLAST revealed no similarity at the nucleotide level; however, the translated protein sequence showed 67% similarity to TraB of *E. faecalis* V583/pTEF1, a pheromone shutdown protein (11). TraB is a membrane-bound protein involved in reducing the amount of endogenous pheromone secreted by or associated with the surface of donor cells, and/or inducing secretion of the inhibitor peptide (9, 11). Analysis of the protein sequence revealed that it had a molecular mass of 26,847 Da and was composed of 238 amino acids of which 105 were hydrophobic. The Conserved Domain Database (NCBI) identified the presence of a conserved domain (COG1916) that includes three or four transmembrane loops (9). This domain, also found in other TraB (pAD1 and pPD1) proteins and PrgY-related proteins, can be found in a range of different organisms, including bacteria, archaea, plants, and mammals (9). Further sequencing of the 6.8-kb insert revealed the presence of *traA*, a key regulator of the pheromone response, and *res97*, an *E. faecalis* resolvase. Previous studies in our laboratory had demonstrated that both *traA* and *traB* were present on pJM01, an uncharacterized pheromone-responsive plasmid carried by *E. faecalis* AR01/DGVS which also harbors bacitracin and tetracycline resistance genes (36). Others have also reported *traB* and *traA* genes on pheromone-responsive plasmids in *E. faecalis* (2, 13, 39, 45, 60).

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-

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

Fatty acid	Fatty acid composition of cell membranes (%)			
	WT ^a	WT, ML ^b	DGRM2	DGRM2, ML
C _{12:0}	0.3	11.7	0.2	9.2
C _{14:0}	4.0	4.8	5.2	5.4
C _{16:0}	37.0	37.9	38.0	37.1
C _{16:1} isomer	1.8	0.9	1.9	1.5
C _{16:1} Δ 9cis	5.0	4.2	6.8	5.6
C _{18:0}	6.5	7.4	4.4	6.2
C _{18:1n9c}	3.5	7.6	2.0	4.2
C _{18:1}	41.6	25.0	41.0	30.4
C _{18:2}	0.3	0.5	0.5	0.4

^a WT, wild type.

^b ML, grown in the presence of 50 $\mu\text{g/ml}$ monolaurin.

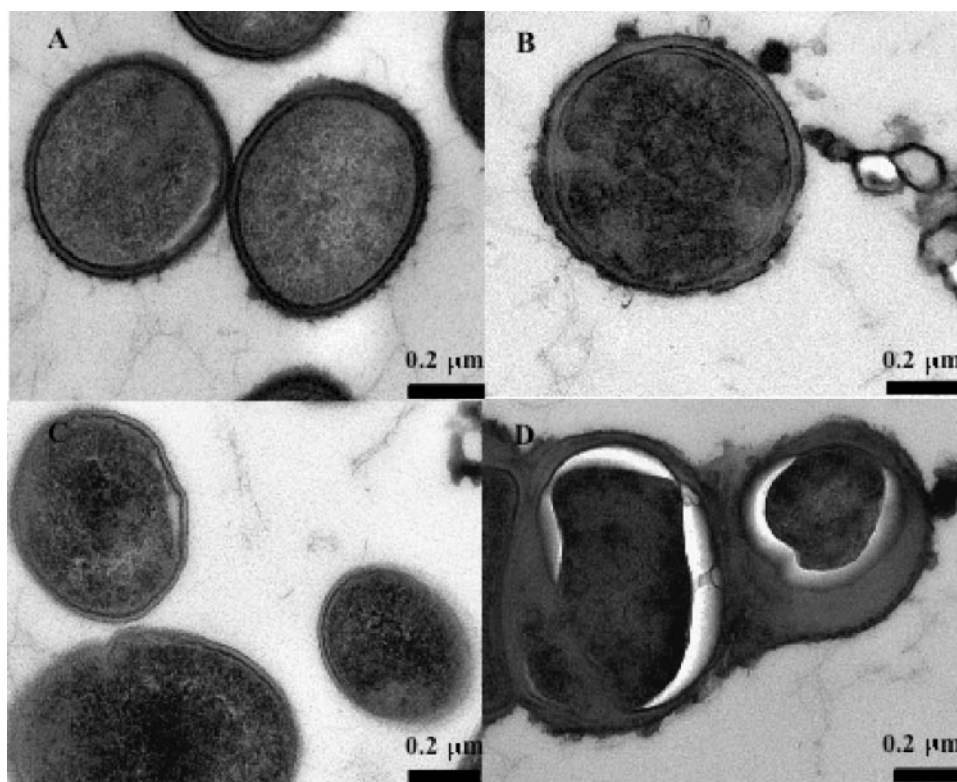


FIG. 4. TEM of *E. faecalis* AR01/DGVS (A and B) and DGRM2 (C and D) grown either in the absence (A and C) or in the presence (B and D) of monolaurin.

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 ($C_{14:0}$, $C_{16:0}$, and $C_{18:0}$) and the decrease in unsaturated fatty acid ($C_{18:1}$) 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 hydrophobicity. 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 hydrophobicity 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 hydrophobicity was observed in JH2-7349, a *traB* mutant (16). The lack of difference in cell surface hydrophobicity 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 hydrophobicity 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 a domain of the aggregation substance was binding directly to the lipoteichoic acid of the cell wall of *E. faecalis*. Thus, decreased lipoteichoic acid production in the presence of monolaurin (6) would result in lower levels of aggregation substance at the cell wall of AR01/DGVS, resulting 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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