Characterisation of Monolaurin Resistance in *Enterococcus faecalis*

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There is increasing concern regarding the presence of vancomycin-resistant enterococci (VRE) in domestically farmed animals, which may act as reservoirs and vehicle of transmission for drug-resistant enterococci to humans, resulting in serious infections. In order to assess the potential for 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 being resistant to monolaurin and were designated DGRM2, DGRM5, and DGRM12. The gene interrupted in all three mutants was identified as traB, an E. faecalis pheromone shutdown protein, whose complementation in trans restored monolaurin sensitivity in all three mutants. DGRM2 was selected for further characterisation. E. faecalis DGRM2 showed increased resistance to gentamicin and chloramphenicol (inhibitors of protein synthesis) while no difference in 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 is a food-grade glycerol monoester of lauric acid, reported to have the greatest antimicrobial activity among all 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 \textit{Staphylococcus aureus} (41), to block the induction of vancomycin resistance in \textit{E. 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 \textit{E. faecium} (40, 56, 57) and to inhibit glycerolipid and lipoteichoic acid biosynthesis in \textit{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 reports 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 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 foodborne illnesses and in spoilage of processed cooked meat, raw meat, milk, and
milk products (1, 14, 28). Enterococci are not important foodborne 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 VRE in nonhuman
reservoirs (1, 14, 18, 20, 52). In order to assess the utility of using monolaurin as a food preservative
against enterococci, it was important to determine its cellular target(s) and potential resistance
mechanisms to this compound.

In this communication, we report on the isolation and characterisation of monolaurin-
resistant E. faecalis mutants. We propose that monolaurin resistance in these mutants is mediated 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 chemicals were purchased from Sigma (Sigma
Chemical Co., St. Louis, Missouri, USA) and stock solutions were filter sterilized (0.2 µm Supor
Acrodisc, Gelman Sciences, Ann Arbor, Missouri, USA). Monolaurin and Lauricidin® (90% lauric
acid, 8% myristic acid, 2% capric acid; Med-Chem Labs, USA) 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 (LB) respectively. Strains in regular use were  
subcultured on either Luria agar (LA) or Brain Heart Infusion agar (BHIA) every two 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 OD$_{595nm}$ of 0.01 in BHI and 100 µl volumes dispensed  
into the wells of a flat bottom 96-well microtiter plate (Nalgene Nunc GmbH & Co. KG,  
Wiesbaden, Germany). Stock solution of monolaurin was diluted to attain final concentrations of:  
800, 400, 200, 100, 50, 25, 10, 5, and 1 µg/ml in BHI and 100 µl was added to each well when the  
bacterial cells reached an OD$_{595nm}$ 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 Microtiterplate Reader, LabSystems, Vantaa,  
Finland) with absorbance readings (595 nm) taken every 2 h. The minimum inhibitory concentration  
(MIC) was defined as the lowest concentration of monolaurin to show complete inhibition of  
bacterial growth (OD$_{595nm}$ < 0.1) after 24 h 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 using the following protocol.
A Lauricidin® stock solution was diluted to 500 µg/ml in BHI/Erm10 (BHI containing 10 µg/ml erythromycin) and 40 µl added to the wells of microtiter plates containing 100 µl of BHI/Erm10. Sixty µl volumes of an overnight culture of each mutant strain were added to each well and the optical density (595 nm) read at 0 h using the plate reader. Following 24 h incubation at 37°C, the absorbance was read again and the change in OD_{595nm} (ΔOD) calculated by subtracting the absorbance value at time 0 h from that at 24 h.

Genomic DNA extraction, transformation, and genetic techniques. E. faecalis chromosomal DNA was obtained using the method described previously (35). Transformation of E. faecalis using 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 to the manufacturer's instructions, using the PCR program described previously (31). Radiolabelled PCR products and plasmids were prepared by incorporation of [α-32P]dCTP-labeled deoxynucleotides using the Ready-to-go™ DNA labelling beads (Amersham, Buckinghamshire, England). Southern transfer and hybridisation was performed as previously described (35).

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

The site of Tn917 insertion in the selected monolaurin-resistant mutants was mapped using inverse PCR with Tn917-derived primers, ErmP2 5’-TACAAATTCCTCGTAGGC-3’ and HindIII
5’-GACATTATAAGCCGCTGTCG-3’. Total DNA from Tn917 mutants was digested with HindIII, self-ligated using T4 DNA ligase (Roche), and inverse PCR carried out using 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 using a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, UK.) and a model ABI377 automated DNA sequencer (Applied Biosystems). The nucleotide sequences were assembled using 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, N. Mex.), available via the internet.

**Cloning of 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 used for screening the transformants. The DNA insert of randomly selected transformants was sequenced to confirm the presence of the traB gene. To enable complementation, plasmid pAMCL6 was constructed by ligating pCL6 into the *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 confirmed by enzyme digests and PCR.

The traB::Tn917 mutants were originally selected on the basis of their increased resistance to monolaurin when 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 DNA
template for amplification of the traB gene using TraBF22 5’-CGGAGAGACACCGTCAGGG-3’
and TraBR1121 5’-CCTATAGCTCCTCCTAAATT-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 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 the plasmids
pPIT7349 and pPIT7013, respectively (provided by Dr Shuhei Fujimoto (16)) into E. faecalis JH2-
2.

Autolysis assay. Cell autolysis was assayed by modification of the method by 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
erthyromycin), respectively. One ml of overnight culture was used to inoculate THBG or
THBG/Erm10, which was incubated at 37°C until an OD_{600nm} of 0.3 was reached, at which point the
cells were chilled on ice for 15 minutes. The cells were washed twice in ice-cold MilliQ water and
resuspended in 50 mM Tris-HCl (pH 8.0) to an OD_{600nm} of 1.0. The cell suspensions were incubated
at 37°C for 6 h and autolysis determined by reading the OD_{600nm} at regular intervals. Two
concentrations of monolaurin were tested for their 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 acids analysis. The fatty acid composition of E. faecalis AR01/DGVS and DGRM2
cells and cell membranes was established when grown in the presence (50 µg/ml) or absence of
monolaurin. Overnight cultures of *E. faecalis* AR01/DGVS and *E. faecalis* DGRM2 were grown in BHI and BHI/Erm10, respectively.

For the preparation of the whole cell samples, bacterial cells were collected by centrifugation (7,000 x 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 MOPS buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.2 mg of lysozyme, and 2500 units of mutanolysin. Following 60 minutes 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 minutes at 37°C and then lysed by two passages through a French pressure cell at 20,000 psi. The crude lysate was centrifuged at 10,000 x g for 20 min at 4°C to remove unbroken cells. The supernatant was centrifuged for 1 h at 180,000 x 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 DTT, and 1 mM PMSF. 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 adjusted to 4.5 ml with MilliQ water. The internal standard (tridecanoic acid, Nu-Check Prep Inc., Elysian, MN, USA), 60 µl of 10 mg/ml in 1:1 (v/v) CHCl₃: MeOH (Merck, GR for analysis and LiChrosolv, 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 (v/v) CHCl₃:MeOH:water. The suspensions were shaken at room temperature (120 cycles/min, 30 min) and centrifuged (3000 x g, 10 min). The organic layer was transferred into another Kimax tube (series B tubes), which was centrifuged (1000 x 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 re-extracted in 2:1 (v/v) CHCl₃:MeOH. The organic layer was transferred to series B tubes and centrifuged as 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 was added 300 µl toluene and 1.4 ml 14% BF₃/MeOH. The tubes were heated at 100°C for 2 h 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 were shaken and then centrifuged (1000 x g, 10 min). An aliquot of the top layer was transferred to 2 ml autosampler vial containing a 250 µl insert for GC analysis.

Analysis of the fatty acid methyl esters was carried out on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 series autosampler (Agilent Technologies Inc, Wilmington, DE, USA). The injections were carried out in split mode (40:1) at 250°C. A flame ionisation detector (FID) at 260°C was used with gas flow rates of air 300 ml/min, hydrogen 30 ml/min and nitrogen at 30 ml/min. For quantitative analysis, separation was carried out on a BPX70 capillary column (50 m, 330 µm i.d., 0.25 µm film thickness; SGE Analytical Products, Melbourne, Australia) using hydrogen gas (2 ml min⁻¹, 33 cm sec⁻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, then increased to 230°C at 10°C min⁻¹. For confirmation of peak fatty acid methyl ester identities, separation was also carried out on a BPX5 capillary column (30 m, 250 µm i.d., 0.25 µm film thickness; SGE Analytical Products, Melbourne, Australia) using hydrogen gas (1.2 ml/min, 36 cm/sec, constant flow mode). The GC oven started at 60°C, then increased to 240°C at 2°C/min and held for 20 min. Data integration and computation were performed using HP Chemstation software (Hewlett Packard).
Identification of fatty acids was carried out using authentic standards and the retention times compared on two columns of differing polarity (see above). The authentic standards included a fatty acid methyl esters mixture (FAMEQ005), and a set of C18:1 isomers (6-cis-octadecenoic acid methyl ester, 6-trans-octadecenoic acid methyl ester, 11-cis-octadecenoic acid methyl ester, 11-trans-octadecenoic acid methyl ester (Nu-Check Prep Inc, Elysian, MN, USA).

**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 OD_{500nm} of 0.5. Four point eight ml 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 OD_{500nm} values of the aqueous phase were determined. The affinity of bacteria for the solvent was evaluated by: % Adherence = \((1 - A/A_0) \times 100\), where \(A_0\) is the optical density measured at 500 nm of the bacterial suspension before mixing and \(A\) is the optical density 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 (AR01/DGVS 50 µg/ml, DGRM2 100 µg/ml) or absence of monolaurin, 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 PBS (pH 7.0) and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) with 3 mg/ml of ruthenium red. After 2 h on a rotator (2 rpm) at room temperature, the samples were washed three times with the same buffer and post-fixed 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 embedded in 3% agarose. The samples were then loaded into a tissue processor, dehydrated using increasing concentration of ethanol and finally embedded in Quetol 651 resin. The samples were visualized using a Philips CM100 TEM (FEI/Philips, Eindhoven, Holland).

**Nucleotide sequence accession number.** The DNA sequence of traB from *E. faecalis* AR01/DGVS located on pJM01 has been deposited in GenBank under the 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 harbours two plasmids carrying antibiotic resistance genes, pJM01 (tetracycline<sup>R</sup>, bacitracin<sup>R</sup>) and pJM02 (vancomycin<sup>R</sup>, erythromycin<sup>R</sup>). *E. faecalis* AR01/DGVS was obtained by curing AR01/DG of its plasmid pJM02 (36). The plasmid pJM01 is 72 kb in size and has a frequency of conjugation of 7.28 x 10<sup>-3</sup> 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<sub>595nm</sub> 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 their ability to grow in BHI/Erm containing 100 μg/ml Lauricidin®. Lauricidin® was used in the initial screen because the cost of large amounts of monolaurin was prohibitive. A cell inoculum was added (30%, w/v) and Lauricidin® was present at the time of inoculation. The average increase in OD₅₉₅nm achieved after 24 h by the 8,000 mutants was 0.355 ± 0.001. Three mutants, designated DGRM2, DGRM5, and DGRM12, grew to OD₅₉₅nm of 0.713, 0.591, and 0.640 respectively. All three monolaurin-resistant mutants had a hybridisation 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 hybridisation 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 ORF was identified encoding a protein having 67% identity to TraB, a pheromone shutdown protein, from *E. faecalis* V583. Figure 2B indicates the site 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 μg/ml) demonstrating that Tn₉₁₇ insertion in the *traB* gene was responsible for the observed phenotype (data not shown).

**Characterisation of monolaurin-resistant mutants.** For the characterisation of our *E. faecalis* *traB* mutant strains, we decided to use for comparison an *E. faecalis* strain harbouring a pheromone-responsive plasmid carrying an inactivated (characterised) *traB* gene. For this purpose, we generated *E. faecalis* strain JH2-7349 carrying a pPD1 (pheromone-responsive plasmid) derivative, which had an inactivated *traB* due to 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 effects observed were due to the presence of *Tn917* on the plasmid pPD1 (16).

The MIC of monolaurin for strains DGRM2, DGRM5, DGRM12, and JH2-7349 was 300 µg/ml, while for AR01/DGVS, JH2-2 and JH2-7013 it was 100 µg/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 µg/ml monolaurin to actively growing cells resulted in an immediate decrease in optical density and inhibition of growth of *E. faecalis* AR01/DGVS, but had no effect on the growth of DGRM2 (Fig. 3). Based on 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 grown in the presence of monolaurin and 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).

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 two-fold 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 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 analysed when the bacterial cells were grown in the presence or absence of monolaurin. Myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1\(\Delta_9\)cis), C16:1 isomer, stearic (C18:0), and vaccenic (C18:1) acid 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 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 total fatty acid in both AR01/DGVS and DGRM2 (Table 3). The presence of monolaurin changed the cellular saturated to unsaturated fatty acid ratios from 1.4 (average of 1.26 and 1.54) to 4.35 (average 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 profile 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 in 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 4C). 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 VRE 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 µg/ml). Three monolaurin-resistant mutants were isolated which were shown to have single Tn917 insertions in the same ORF. Sequence analysis of this ORF using 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 weight of 26,847
Da, 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 3 or 4 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 species 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 uncharacterised pheromone-responsive plasmid carried by E. faecalis AR01/DGVS, which also harbours bacitracin and tetracycline resistance genes (36). Others have also reported traB and traA genes on pheromone-responsive plasmids in E. faecalis (2, 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 characterisation. 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) inhibited glycerolipid and lipoteichoic acid biosynthesis in S. mutans (6).

Fatty acids and their derivatives are 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 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, 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 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 would suggest that the incorporation of lauric acid in the cell membrane was responsible for the changes in fatty acid profile 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 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, the degree of predicted amino acids similarity, the presence of the conserved COG1916 domain, and the fact that both strains had an identical pattern of hydrophobicity and permeability to antibiotics, provide strong evidence that the function of these two proteins is similar.

It 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 the aggregation substance production (2). Waters et al. (59) have shown that a domain of the aggregation substance was binding directly to the lipoteichoic acid (LTA) of the cell wall of E. faecalis. Thus, decreased LTA 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 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 DGRM2 viewed by TEM when grown in 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 into the cytoplasmic membrane.

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

ACKNOWLEDGEMENTS

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Nucleic Acids Res. 18:15.

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adapted to benzalkonium chloride show resistance to other membrane-active agents but not


FIG. 1. Effect of monolaurin on the growth of *E. faecalis* AR01/DGVS. Monolaurin was added when the OD$_{595\text{nm}}$ reached 0.4 to final concentrations of: ($\lambda$) 0 $\mu$g/ml, (O) 1 $\mu$g/ml, (v) 5 $\mu$g/ml, (□) 10 $\mu$g/ml, (σ) 25 $\mu$g/ml, (Δ) 50 $\mu$g/ml, (◊) 100 $\mu$g/ml, (◊) 200 $\mu$g/ml, (×) 400 $\mu$g/ml, and (+) 800 $\mu$g/ml. Symbols represent the mean of triplicate samples. Error bars represent standard deviation. Vertical arrow indicate the point at which monolaurin was added.

FIG. 2. Sites of Tn917 insertions. Panel A: Autoradiogram from Southern blot of the restriction digests of total DNA from *E. faecalis* strains following hybridisation with $^{32}$P-labelled 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; and HindIII-digested *E. faecalis* mutants: lane 4, DGRM2; lane 5, DGRM5; and lane 6, DGRM12. Panel B: Triangles indicate the sites of Tn917 insertions for *E. faecalis* strains: 2, DGRM2; 5, DGRM5; 12, DGRM12; and 7349, JH2-7349. Tn917 insertions for strains DGRM2, DGRM5, and DGRM12 were located within the *traB* gene of pJM01 while Tn917 insertion for strain JH2-7349 was located within the *traB* of pPD1. The number of amino acids (A.A.) for each ORF is indicated in brackets.

FIG. 3. Growth curves of *E. faecalis* strains grown in the presence (■, □) or absence (λ, O) of monolaurin. Growth curves of *E. faecalis* strains AR01/DGVS (■, ●) and DGRM2 (□, O). Symbols represent the mean of triplicate samples. Error bars represent standard deviation. Vertical arrow indicate the point at which monolaurin was added.

FIG. 4. TEM of *E. faecalis* AR01/DGVS (Panels A and B) and DGRM2 (Panels C and D) grown either in the absence (Panels A and C) or presence of monolaurin (Panels B and D).
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>F-araD139 (ara-leu)7696 galE15 galK16 (lac)X74 rspL (strr)hsdR2 (r k-mk+) mcrA mcrB1 (7)</td>
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</tr>
<tr>
<td><em>E. faecalis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR01/DGVS</td>
<td>AR01/DG cured of pJM02 (VmR EmR); TcR BrCmR (36)</td>
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<tr>
<td>DGRM2</td>
<td>AR01/DGVS traB::Tn917; EmR TcR BrCmR MlR (36)</td>
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</tr>
<tr>
<td>DGRM5</td>
<td>AR01/DGVS traB::Tn917; EmR TcR BrCmR MlR (36)</td>
<td></td>
</tr>
<tr>
<td>DGRM12</td>
<td>AR01/DGVS traB::Tn917; EmR TcR BrCmR MlR (36)</td>
<td></td>
</tr>
<tr>
<td>AR01/DGVS/pAM401</td>
<td>AR01/DGVS harbouring pAM401; TcR BrCmR (This study)</td>
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<tr>
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<td>DGRM2 harbouring pAMCL6; EmR TcR BrCmR MlR (This study)</td>
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<td>DGRM12/pAMCL6</td>
<td>DGRM12 harbouring pAMCL6; EmR TcR BrCmR MlR (This study)</td>
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</tr>
<tr>
<td>JH2-2</td>
<td>Glycopeptide-susceptible strain commonly used for gene transfer experiments in <em>Enterococcus</em> (22)</td>
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<tr>
<td></td>
<td>JH2-7349 JH2-2 harbouring pPIT7349; EmR (This study)</td>
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<tr>
<td></td>
<td>JH2-7013 JH2-2 harbouring pPIT7013; EmR (This study)</td>
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<td><strong>Plasmid</strong></td>
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<tr>
<td>pTV1-OK</td>
<td>repA(Ts)-pWV01Ts aphA3 Tn917; KmR EmR (19)</td>
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<tr>
<td>pCL1921</td>
<td>Low copy number vector; StR SpR, 4.6 kb (33)</td>
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<tr>
<td>pCL6</td>
<td>pCL1921 harbouring 6.8 kb insert from AR01/DGVS containing traB; StR SpR (This study)</td>
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<tr>
<td>pAM401</td>
<td><em>E. coli</em>-<em>E. faecalis</em> shuttle vector; CmR TcR, 10.4 kb (61)</td>
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<td>pPIT7349</td>
<td>pPD1::Tn917 derivative with insertion in traB (16)</td>
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<td>pPIT7013</td>
<td>pPD1::Tn917 derivative with insertion in orfY (16)</td>
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</table>

R Resistant.  
S Sensitive.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Percent hydrophobicity</th>
<th>MIC (µg ml⁻¹) of strains for compound</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Monolaurin</td>
</tr>
<tr>
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<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>
<tr>
<td>Strain</td>
<td>Total fatty acids (µg/gww)</td>
<td>SFA/UFA ratio&lt;sup&gt;e&lt;/sup&gt;</td>
<td>C12:0</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>WT&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1476 (13.3)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.26</td>
<td>4.7 (11.1)</td>
</tr>
<tr>
<td>WT+ML&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1883 (9.4)</td>
<td>49.7 (11.8)</td>
<td>2.7 (6.7)</td>
</tr>
<tr>
<td>WT+ML</td>
<td>947&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.26</td>
<td>5.4</td>
</tr>
<tr>
<td>DGRM2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1610 (14.3)</td>
<td>1.54</td>
<td>5.8 (15.4)</td>
</tr>
<tr>
<td>DGRM2+ML&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1845 (0.8)</td>
<td>51.6 (0.9)</td>
<td>3.8 (1.1)</td>
</tr>
<tr>
<td>DGRM2+ML</td>
<td>893&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.43</td>
<td>7.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT: Wild-type *E. faecalis* AR01/DGVS.

<sup>b</sup> Average for 2 cultures, 2 extractions per culture, duplicate FAME analysis (n=8).

<sup>c</sup> ML: Grown in the presence of 50 µg/ml monolaurin.

<sup>d</sup> Average for 1 culture, 2 extractions, duplicate FAME analysis (n=4).

<sup>e</sup> Values in brackets give coefficient of variation (%).

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

<sup>g</sup> Ratio of saturated fatty acids (SFA) to unsaturated fatty acids (UFA).
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>C12:0</td>
<td>0.3</td>
</tr>
<tr>
<td>C14:0</td>
<td>4.0</td>
</tr>
<tr>
<td>C16:0</td>
<td>37.0</td>
</tr>
<tr>
<td>C16:1 isomer</td>
<td>1.8</td>
</tr>
<tr>
<td>C16:1Δ9cis</td>
<td>5.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>6.5</td>
</tr>
<tr>
<td>C18:1n9c</td>
<td>3.5</td>
</tr>
<tr>
<td>C18:1</td>
<td>41.6</td>
</tr>
<tr>
<td>C18:2</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.