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Alanine Esters of Enterococcal Lipoteichoic Acid Play a Role in Biofilm Formation and Resistance to Antimicrobial Peptides

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Enterococcus faecalis **is among the predominant causes of nosocomial infections. Surface molecules like D-alanine lipoteichoic acid (LTA) perform several functions in gram-positive bacteria, such as maintenance of cationic homeostasis and modulation of autolytic activities. The aim of the present study was to evaluate the effect of D-alanine esters of teichoic acids on biofilm production and adhesion, autolysis, antimicrobial peptide sensitivity, and opsonic killing. A deletion mutant of the *dltA* gene was created in a clinical *E. faecalis* isolate. The absence of D-alanine in the LTA of the *dltA* deletion mutant was confirmed by nuclear magnetic resonance spectroscopy. The wild-type strain and the deletion mutant did not show any significant differences in growth curve, morphology, or autolysis. However, the mutant produced significantly less biofilm when grown in the presence of 1% glucose (51.1% compared to that of the wild type); adhesion to eukaryotic cells was diminished. The mutant absorbed 71.1% of the opsonic antibodies, while absorption with the wild type resulted in a 93.2% reduction in killing. Sensitivity to several cationic antimicrobial peptides (polymyxin B, colistin, and nisin) was considerably increased in the mutant strain, confirming similar results from other studies of gram-positive bacteria. Our data suggest that the absence of D-alanine in LTA plays a role in environmental interactions, probably by modulating the net negative charge of the bacterial cell surface, and therefore it may be involved in the pathogenesis of this organism.**

Enterococci are commensals of the human and animal intestinal microflora. In recent years, they have emerged as one of the leading causes of nosocomial infections, with the majority of clinical isolates being *Enterococcus faecalis* (20). The increasing occurrence of enterococcal strains resistant to multiple antibiotics in recent decades represents a serious threat to therapy and underscores the necessity of a better understanding of the pathogenicity of these microorganisms.

The cell wall of gram-positive bacteria contains different types of anionic molecules. Lipoteichoic acid (LTA) is an amphiphilic polymer consisting of polyglycerolphosphate and a glycolipid anchor inserted into the cell membrane. The LTA polymer of most gram-positive bacteria is nonstoichiometrically replaced with D-alanine at C-2 of glycerol (29). In addition to LTA, enterococci, like many other gram-positive bacteria, synthesize a cell wall teichoic acid (WTA), usually consisting of a glycosylated and alanine-substituted alditol phosphate repeating unit covalently attached to the cell wall (29).

In the present study, we used targeted mutagenesis to inactivate the first enzyme involved in the teichoic acid D-alanylation pathway to investigate the contribution of teichoic acid alanylation to biofilm formation, autolysis, cationic antimicrobial peptide (CAMP) resistance, resistance to neutrophil killing, and adhesion to and invasion of epithelial cells.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Enterococci were grown at 37°C without agitation in tryptic soy broth (TSB; CASO broth; Merck), with the addition of 1% glucose as indicated (TSB-G), or on tryptic soy agar plates (TSA; CASO agar; Merck).

Escherichia coli DH5α and TOP10 (Invitrogen) were cultivated aerobically in Luria-Bertani broth at 37°C. Kanamycin was added for enterococci (2,000 μg/ml) and for *E. coli* (50 μg/ml); spectinomycin was added for cultivation of enterococci (500 μg/ml) and of *E. coli* (250 μg/ml); all the antibiotics were from Sigma Chemicals. For the antimicrobial peptide sensitivity assay, all the bacteria were grown in Mueller-Hinton broth (Merck); polymyxin B, colistin, and nisin were purchased from Sigma.

Molecular techniques. Chromosomal DNA from enterococci was prepared using the DNeasy Tissue kit (QIAGEN) according to the manufacturer's instructions. Plasmid DNA was prepared from enterococci or *E. coli* using the Wizard Plus SV Miniprep System or PureYield Plasmid Midiprep System (Promega). DNA was purified from PCRs or from agarose gels using the QIAquick PCR purification kit or the Gel Extraction kit (QIAGEN) according to the manufacturer's instructions. Restriction and modifying enzymes were obtained from Invitrogen and New England Biolabs. Custom primers were manufactured by Invitrogen. Electrocompetent enterococci were prepared according to the method of Fielder and Wirth (12), and electroporation was performed in a Bio-Rad Gene Pulser Xcell Electroporation System using the parameters given by Fiedler and Wirth (12). All the other methods (DNA ligations, electrophoresis, transformation of competent *E. coli*, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were performed using standard techniques (40).

Construction of a deletion mutant. The deletion of a portion of the gene *dltA* (EF2749 in the *E. faecalis* V583 genome; GenBank accession no. AAO82448.1) was created using the method described by Cieslewicz et al. (6) with the following modifications. Primers 1 and 2 (Table 2) were used to amplify a 598-bp fragment in the *dltA* gene, and primers 3 and 4 were used to amplify a 649-bp fragment in the *dltB* gene (EF2748 in the *E. faecalis* V583 genome; GenBank accession no. AAO82447.1) (Fig. 1). Primers 2 and 3 contain a 21-bp complementary sequence (underlined in Table 2). Overlap extension PCR was used to create a PCR product lacking a portion of *dltA*. The resulting fragment was cloned into the

TABLE 1. Enterococcal strains and plasmids used in this study

Strain or plasmid	Characterization	Reference or source
Strains		
E. faecalis V583	Reference strain, fully sequenced	32
E. faecalis ATCC 29212	Reference strain	
E. faecalis 12030	Clinical isolate, strong biofilm producer	18
E. faecalis 12030Δ*dltA*	*dltA* mutant	This study
E. faecalis 12030 R-wt	Wild-type revertant strain	This study
E. faecalis 12030Δ*dltA*/pEU*dltA*	Strain complemented with the *dltA* gene	This study
E. faecalis 12030Δ*dltA*/pEU*dltAB*	Strain complemented with the *dltA* and *dltB* genes	This study
E. coli DH5α	Gram-negative cloning host	
E. coli TOP10	Gram-negative cloning host	Invitrogen
Plasmids		
pCASPER	Gram-positive, temp-sensitive mutagenesis vector	4
pCRII-TOPO	Gram-negative cloning vector	Invitrogen
pEU327	Gram-positive expression vector	11
pCASPER/Δ*dltA*		This study
pEU*dltA*	Expression vector carrying the *dltA* gene	This study
pEU*dltAB*	Expression vector carrying the *dltA* and *dltB* genes	This study

gram-negative cloning vector pCRII-TOPO (Invitrogen) and cut with the restriction enzyme EcoRI; the fragment was inserted into the gram-positive vector pCASPER containing a temperature-sensitive origin of replication (4). The resulting plasmid, pCASPER/Δ*dltA*, was transformed into *E. faecalis* 12030 by electroporation, and integrants were selected at the nonpermissive temperature on TSA plates with kanamycin. A single colony was picked, and insertion of plasmid into the chromosome was confirmed by PCR. The integrant was passaged 10 times in liquid culture without antibiotics at the permissive temperature, and colonies were replica plated to screen for loss of kanamycin resistance. The excision of the plasmid either creates a reconstituted wild-type strain or leads to an allelic replacement with the deleted sequence in the chromosome. The deletion mutant created was designated *E. faecalis* 12030Δ*dltA*, and one wild-type revertant strain was designated *E. faecalis* 12030 R-wt; both strains were confirmed by PCR and automated sequencing.

Complementation of the deletion mutant. The expression vector pEU327 (11) contains a xylose promoter that has been shown to be expressed constitutively in *E. faecalis* (15). A 1,521-bp PCR product containing the entire *dltA* gene was amplified by use of primers 5 and 6 (Table 2), and a fragment containing the genes *dltA* and *dltB* was amplified using the primers 5 and 7 (Table 2). The resulting PCR products were digested with SalI (linkers underlined in Table 2) and ligated into pEU327, which had been digested with SalI and dephosphorylated. The chimeric plasmids pEU*dltA* and pEU*dltAB* were transformed in *E. coli* DH5α, and the correct inserts and orientations were confirmed by restriction analysis using HindIII. pEU327, pEU*dltA*, and pEU*dltAB* were transformed into *E. faecalis* 12030Δ*dltA* by electroporation, and transformants were selected on TSA plates containing spectinomycin. pEU327 also was electroporated in *E. faecalis* 12030 and used as a control. The presence of the plasmids was confirmed by plasmid preparations and subsequent restriction digestions.

TABLE 2. Primers used in this study

No.	Name	Sequence (5′–3′)[a]
1	dltA for	TTGATGAATGGGCACGTAAA
2	dltA OE L	ACTAGCGCGGCCGCTTGCTCCGC CAATGCGGGATAAATAGA
3	dltA OE R	GGAGCAAGCGGCCGCGCTAGTA GGTGAATGCCACATGATGA
4	dltA rev	GACACCACCAAGATAATGACTGA
5	dltA exp SalI F	CCCGTCGACAATGGAAAAAGTAA TTAATATGATTC
6	dltA exp SalI R	CCCGTCGACTCATGTGGCATTCA CCTCATT
7	dltB exp SalI R	CCCGTCGACTTATTTAAACCATA GTTTGTC

[a] Linkers are underlined.

Phenotypic analyses. The mutant and the wild-type strain were compared by means of a panel of standard biochemical reactions (Vitek Gram-Positive Identification Card; BioMerieux) and by agglutination with the group D antisera (Streptococcal Grouping kit; Oxoid).

Isolation and structural analysis of LTA. LTA from the wild type and the Δ*dltA* strain was extracted as described previously (28). Briefly, bacterial cells were grown in tryptic soy broth, harvested after 3 h, and resuspended in 0.1 M citrate buffer, pH 4.7. Bacteria were disrupted with glass beads using a Beadbeater (Glenn Mills, Clifton, NJ) and then stirred with an equal volume of n-butanol for 30 min. After phase separation by centrifugation, the aqueous layer was removed, dialyzed against 0.1 M ammonium acetate (pH 4.7), and lyophilized. The material was redissolved in chromatography starting buffer (15% n-propanol in 0.1 ammonium acetate, pH 4.7) and applied on octyl Sepharose for hydrophobic interaction chromatography. Bound material was eluted by a gradient of 15 to 80% n-propanol. Material eluting around 50% n-propanol was pooled and lyophilized repeatedly to remove residual ammonium acetate. Nuclear magnetic resonance (NMR) spectrometry was done after exchange with 99.9% ^2H_2O. All one-dimensional and two-dimensional spectra were recorded at 27°C with a Bruker DRX Avance 600-MHz spectrometer. NMR analysis of LTA was done as described previously (28).

Triton X-100-induced autolysis assays under nongrowing conditions. The Triton X-100-induced autolysis assay was performed as described previously (13, 25). In brief, bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 in TSB containing 1 M NaCl. The cells were pelleted by centrifugation, washed in the same volume of ice-cold sterile water, and resuspended in the same volume of 50 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100. The bacterial cells were then incubated at 30°C with shaking, and the OD_{600} was measured every 30 min. The results were normalized to the OD_{600} at time zero, i.e., percent lysis at time t = [(OD at time zero − OD at time t)/OD at time zero] × 100. All results shown are the averages of two independent experiments.

Biofilm plate assay. Enterococci were tested for production of biofilm using the protocol described by Baldassarri et al. (3). Briefly, bacteria were grown overnight at 37°C in TSB or TSB with spectinomycin (final concentration, 500 μg/ml) for strains carrying the expression vector. Polystyrene tissue culture plates (Brand, Wertheim, Germany) were filled with 180 μl of TSB or TSB-G, with spectinomycin (500 μg/ml) added when necessary, and 20 μl of overnight culture, and the plates were then incubated at 37°C for 18 h. The plates were read in an enzyme-linked immunosorbent assay reader (Bio-Rad microplate reader) at an optical density of 630 nm, the culture medium was then discarded, and the wells were washed three times with 200 μl of phosphate-buffered saline (PBS) without disturbing the biofilm on the bottom of the wells. The plates were dried at 60°C for 1 h and then stained with 2% Hucker's crystal violet for 2 min. Excess stain was removed by rinsing the plates under tap water, and the plates were then dried for 10 min at 60°C. The optical density at 630 nm was determined. Biofilm formation was normalized to growth with the biofilm index, which was calculated as OD of the biofilm × (0.5/OD of growth) (9). All experiments were repeated at least four times on different days.

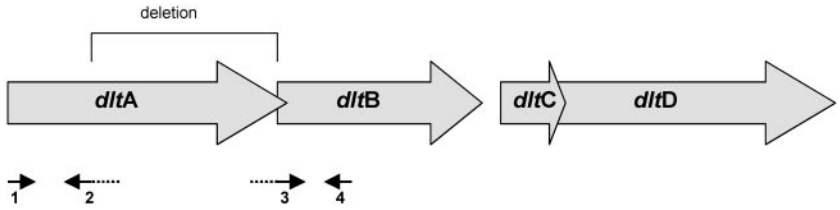

FIG. 1. Genetic organization of the *dlt* operon in *E. faecalis*.

Opsonophagocytic assay. The opsonophagocytic assay was done as described elsewhere (18) with some modifications. Serum raised against antibiotic-killed bacteria (*E. faecalis* 12030) was absorbed with either the wild type or the *dltA* mutant and tested for its opsonic killing activity against the wild-type strain. Polymorphonuclear neutrophils (PMN) were freshly prepared from human blood collected from healthy adult volunteers. About 20 to 30 ml was mixed with an equal volume of dextran/heparin buffer and incubated at 37°C for 45 min. The upper layer containing the PMNs was collected, and remaining erythrocytes were lysed by 1% NH_4Cl buffer and subsequent washing steps with RPMI supplemented with 15% fetal bovine serum (both from Gibco). Trypan blue exclusion was used to determine the number of viable cells, and the final PMN count was adjusted to 2×10^7 neutrophils/ml. Baby rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was used as the complement source (15% final concentration), and it was absorbed with the homologous *E. faecalis* strain at 4°C for 1 h before use.

For the final assay, the following components were mixed: 100 μl of PMNs; 100 μl of bacteria (2×10^7/ml, grown to mid-log phase in TSB and confirmed by viability counts); 100 μl of serum (serum dilution, 1:100); and 100 μl of absorbed baby rabbit complement. The mixture was incubated on a rotor rack at 37°C for 90 min, and samples were plated in duplicate at time zero and after 90 min. Percent killing was calculated by comparing the colony counts of a control not containing PMNs (PMN−) to the colony counts after a 90-min incubation at 37°C (t_{90}), using the following formula: {[(mean CFU PMN− at t_{90}) − (mean CFU at t_{90})]/(mean CFU PMN− at t_{90})} × 100.

Sensitivity to polymyxin B, nisin, and colistin. The MICs of polymyxin B, nisin, and colistin against enterococci was determined by a modified NCCLS broth dilution method (23). The experiments were performed in Mueller-Hinton broth, with spectinomycin added to a final concentration of 500 μg/ml when necessary, in 96-well microtiter tissue culture plates (Greiner). Wells were inoculated with 50-μl volumes of a suspension containing approximately 8×10^5 to 1×10^6 CFU/ml of the test organism, and samples of the bacterial suspensions were plated to confirm the proper range of concentrations.

The microtiter plates were incubated overnight at 37°C, and the MICs were defined as the lowest peptide concentration at which visible growth was inhibited.

Epithelial cell adherence. HEp-2 cells (human epithelioid carcinoma; purchased from ATCC, Manassas, VA) were cultivated in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% fetal calf serum (FCS) (all from Gibco). Cells were passaged at a ratio of 1:5 twice each week.

To evaluate the ability of *E. faecalis* 12030 and its isogenic mutant to adhere to and survive within epithelial cells, the antibiotic protection assay was performed as previously described (2) with slight modifications. Briefly, HEp-2 cells were prepared as described and seeded on 24-well plates at a concentration of 50,000 cells/ml. Bacterial cells grown overnight were washed and suspended into the cell medium to give a bacteria-to-cell ratio of 100:1. Epithelial cells were infected for 1 or 2 h at 37°C, washed to remove nonadherent bacteria, and further incubated for 3 h in medium supplemented with 1% FCS plus gentamicin (250 μg/ml; Sigma). Duplicate wells of infected cells were lysed with 0.1% Triton X-100 in PBS for 5 min both after adherence steps (1 h or 2 h) and after the invasion step (three additional hours in the presence of gentamicin). Dilutions of lysates were made in PBS and plated on TSA plates to quantitate viable bacteria. Throughout the experiment, viable cells were quantitated by trypan blue dye exclusion.

Transmission electron microscopy. Bacterial cells were first fixed for 20 min with 0.1 M cacodylate-buffered 2.5% glutaraldehyde containing 0.075% (wt/vol) ruthenium red (Glut-RR) (Merck) and 75 mM lysine at room temperature. They were then fixed with Glut-RR without lysine for 2 h and finally fixed with 1% OsO_4 plus ruthenium red for an additional hour. Fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30 to 100% ethanol), and embedded in Agar 100 (Agar Scientific Ltd., United Kingdom). Ultrathin sections obtained by an MT-2B Ultramicrotome (LKB-Pharmacia) were stained with uranyl acetate and lead citrate and examined by an EM 208 Philips electron microscope.

Statistical methods. Comparisons were made by a two-tailed Mann-Whitney or *t* test using the Prism4 software package (GraphPad, San Diego, CA). A *P* value of <0.05 was considered statistically significant.

RESULTS

The *dlt* operon in *E. faecalis* is responsible for D-alanylation of teichoic acids. The *dlt* operon consists of four genes, *dltA* to *dltD* (GenBank accession nos. AAO82448.1, AAO82447.1, AAO82446.1, and AAO82445.1, respectively, in the sequenced strain *E. faecalis* V583) (32) (Fig. 1). The gene *dltA* encodes a putative D-alanine-activating enzyme; inactivation of this gene in *E. faecalis* strain 12030 was achieved by targeted mutagenesis. The deletion mutant *E. faecalis* 12030Δ*dltA* lacks the 3′ region of the *dltA* gene (Fig. 1). Lipoteichoic acid (LTA) was purified from the wild type and the mutant strain, and the molecular composition was determined by NMR spectroscopy (Fig. 2). While D-alanine is a major substituent in the wild-type LTA, LTA from the mutant strain, while otherwise structurally intact, completely lacked D-alanine. The deletion in the mutant extends into the first base of the *dltB* ribosomal binding site, leading to the hypothesis that a polar effect on *dltB* translation may occur. Therefore, the mutant strain was complemented also with a plasmid containing both genes (i.e., *dltA* and *dltB*). Resistance to antimicrobial peptides was almost completely restored to wild-type levels (Table 3) when both genes (*dltA* and *dltB*) were expressed in *trans* on the expression vector, while biofilm formation also was restored efficiently by reconstitution with the *dltA* gene alone (data not shown). Comparison of the growth curves of the wild type, the revertant strain, and the mutant strain showed no significant difference (data not shown). All the strains showed identical reactions using standard biochemical tests (Vitek Gram-Positive Identification Card), and all agglutinated with group D antisera.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-protein extracts showed no differences in the electrophoretic profiles of the wild type and the mutant strain (data not shown).

Autolytic properties of the *dlt* mutant. Over a period of 4.5 h there was a decline in OD_{600} of about 20% in the wild type, the revertant, and the deletion mutant, and there was a decline of about 50% after overnight incubation, indicating that the *E. faecalis* strains tested in this study showed no difference in the Triton X-100-induced autolysis (data not shown).

D-alanylation of teichoic acid is involved in biofilm formation. The lack of D-alanine esters on the teichoic acid causes a stronger negative net charge on the bacterial cell surface, and

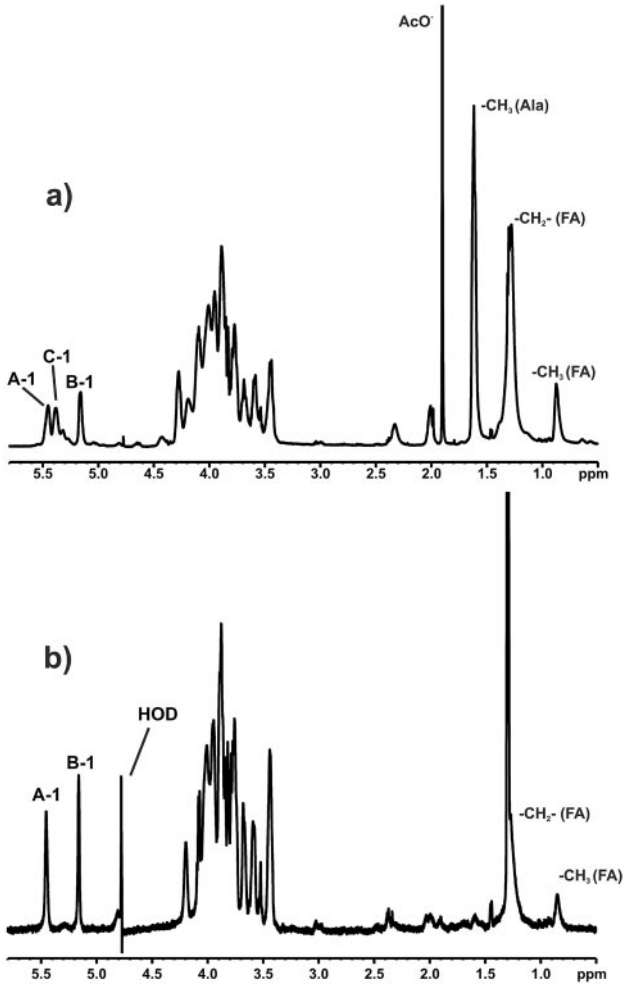

FIG. 2. ^{1}H NMR spectra of LTA isolated from *E. faecalis* strains 12030 (a) and 12030Δ*dltA* (b). Spectra were recorded at 27°C in $^{2}H_2O$. Residue A, →2)-α-D-glucopyranose-(1→; residue B, α-D-glucopyranose-(1→; residue C, →1)-Gro-(3→ P→, with Ala at C-2 (the Arabic numerals refer to the protons in the respective residues). The shifts at δ 1.629 and 5.388 are missing in the 12030 Δ*dltA* mutant, indicating the absence of bound D-alanine. The shift at δ ~1.9 represents a contamination from the chromatography buffer (NH_4OAc). (FA), fatty acid; HOD, deuterated water.

this can affect several bacterial properties. The two wild-type strains, *E. faecalis* 12030 and *E. faecalis* 12030 R-wt, were compared to the *dlt* mutant regarding formation of biofilm on polystyrene surfaces. In the presence of 1% glucose, the wild-type *E. faecalis* strain 12030 produced significantly more biofilm than the mutant strain 12030Δ*dltA* ($P = 0.008$, two-tailed *t* test; Fig. 3); no statistically significant differences were observed between the wild type, 12030 R-wt, and the complemented strains (data not shown).

TABLE 3. Activities of cationic antimicrobial peptides against the *E. faecalis* 12030 wild type, the deletion mutant, the complemented strains, and a reference strain

Strain	MIC		
	Colistin (μg/ml)	Polymyxin B (μg/ml)	Nisin (μM)
E. faecalis 12030	>1,024	256	>25
E. faecalis 12030Δ*dltA*	64	16	3.125
E. faecalis ATCC 29212	512	128	>25
E. faecalis 12030Δ*dltA*/pEU*dltA*	64	32	6.25
E. faecalis 12030Δ*dltA*/pEU*dltAB*	512	64	12.5

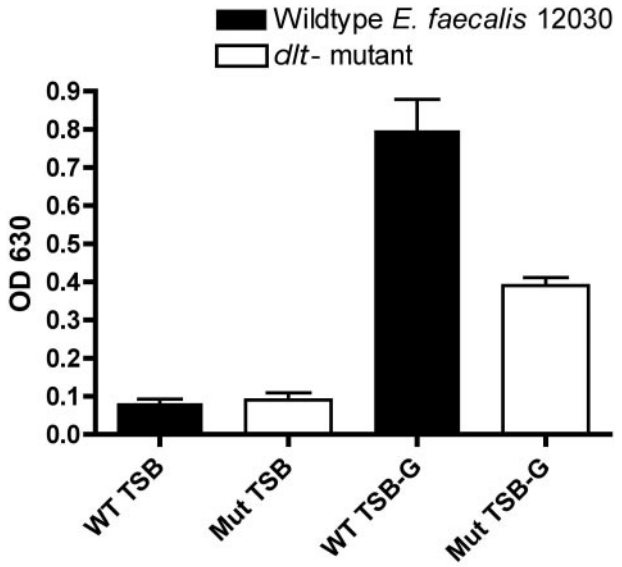

FIG. 3. The wild type (WT) and the *dltA* mutant (Mut) strain were tested for biofilm production in media without (TSB) and with 1% glucose (TSB-G). The amount of biofilm is expressed as the biofilm index (9). Error bars represent standard errors of the means ($n = 3$).

Opsonophagocytic assay. Absorption of serum raised against whole bacterial cells of the homologous strain with the *dlt* mutant reduced opsonophagocytic killing significantly less than that of serum absorption with the wild-type strain ($P = 0.028$, two-tailed Mann-Whitney test; Fig. 4).

LTA D-alanylation contributes to cationic antimicrobial peptide resistance. We tested whether the lack of D-alanylation

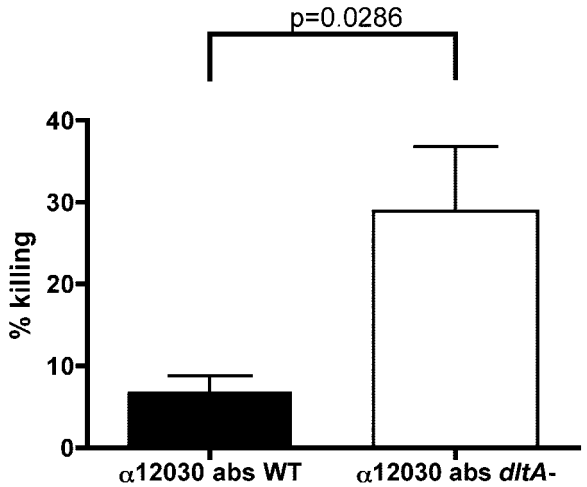

FIG. 4. Opsonic killing of the wild-type (WT) strain *E. faecalis* 12030 with serum raised against whole bacterial cells. The serum was absorbed (abs) with the homologous wild-type strain (black bar) or with the *dlt* mutant (white bar). Error bars represent standard errors of the means.

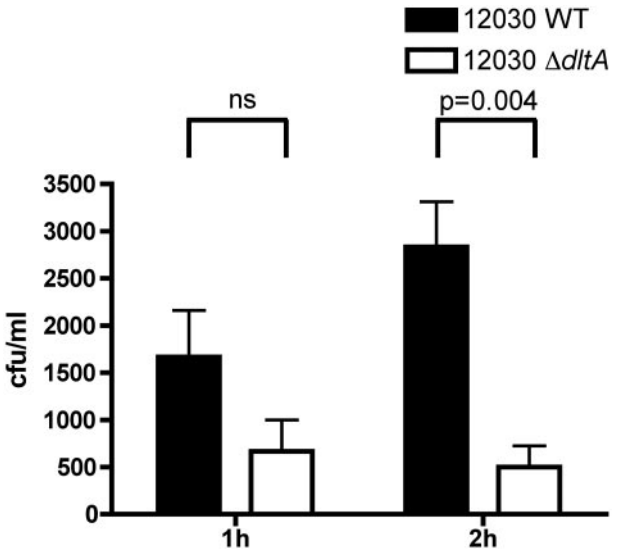

FIG. 5. *E. faecalis* 12030 (black bars) and the *dlt* mutant (white bars) were tested for their ability to adhere to and/or invade HEp-2 cells. While neither strain was able to survive within epithelial cells, there was a significant difference between the ability of the wild-type (WT) and that of the mutant to adhere to cells when the adherence step was prolonged from 1 to 2 h. Error bars represent standard errors of the means. ns, not significant.

and the subsequent increase in negative surface charge of the *E. faecalis* 12030Δ*dltA* mutant led to an enhanced susceptibility to cationic antimicrobial compounds. As shown in Table 3, compared to the wild-type strain, the *dlt* mutant exhibited decreased drug MICs in all the cationic antimicrobial peptides tested. No differences were seen between the wild-type strain and the R-wt strain (data not shown). The two complemented strains show a restored resistance to the CAMPs tested; however, the effect was more pronounced in the strain complemented with both genes (*dltA* and *dltB*; Table 3).

Adhesion. Neither *E. faecalis* 12030 nor its isogenic mutant was able to survive within epithelial cells, as indicated by the antibiotic protection assay. However, binding of the wild-type strain to epithelial cells significantly increased compared to that of the mutant when the adherence step was prolonged from 1 to 2 h (Fig. 5).

Electron microscopy. When the wild type and the mutant strain were examined by transmission electron microscopy, no obvious morphological differences could be observed. Both *E. faecalis* 12030 and the mutant strain exhibited extracellular polysaccharide material and a normal cell wall (Fig. 6). The filamentous protrusions seen on both strains are probably preparation artifacts caused by extracellular polymers, and the slightly reduced quantity of this material in the mutant strain may reflect the reduced biofilm formation by this strain.

DISCUSSION

The surface of gram-positive bacteria is exposed to different microenvironments in which the formation of D-Ala-LTA accomplishes several functions, such as maintenance of cationic homeostasis, modulation of autolytic activities, and assimilation of metal cations and other electromechanical properties of the bacterial envelope. Two different teichoic acids are present on the surface of gram-positive bacteria: lipoteichoic acid (LTA), noncovalently inserted into the cellular membrane, and a wall teichoic acid (WTA), covalently linked to the peptidoglycan of the cell wall. These two molecules are synthesized by separate biosynthetic pathways that have been well characterized in *Bacillus subtilis* (29).

Despite the separate biosynthetic pathways of LTA and

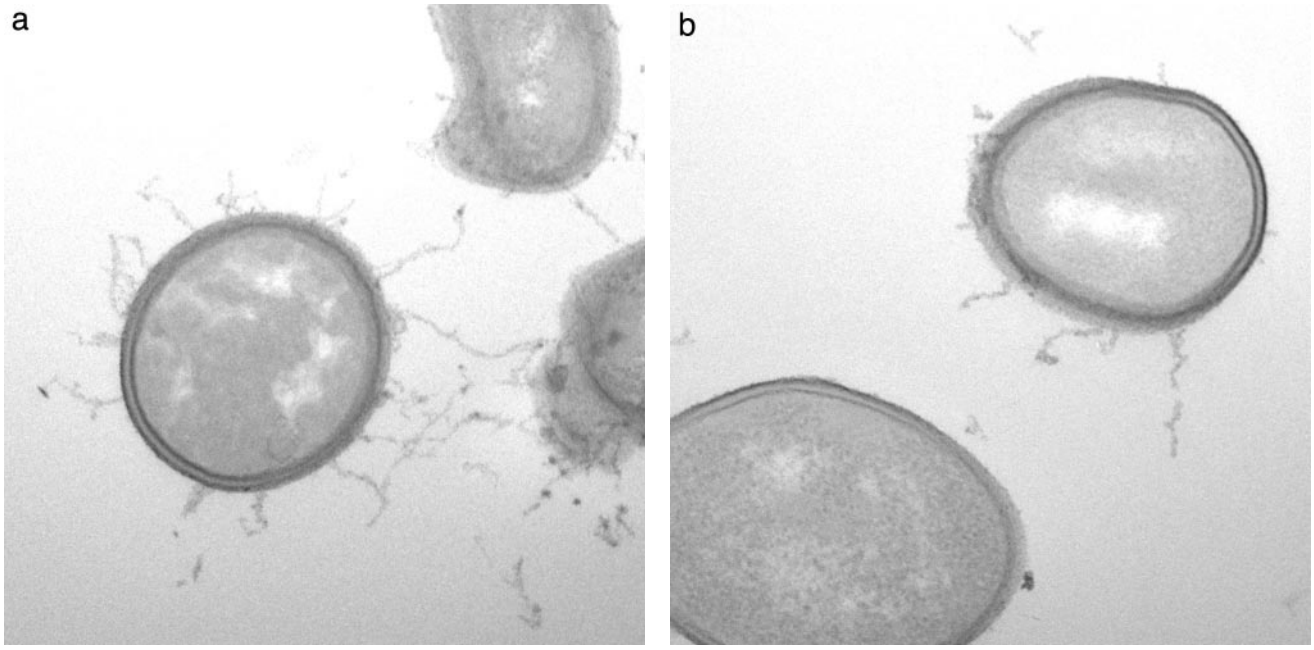

FIG. 6. Transmission electron microscopy of wild-type *E. faecalis* 12030 (a) and the *dlt* mutant (b).

WTA, D-alanyl-ester substituents of WTA originally derive from LTA and are later transferred to WTA by transacylation. Accordingly, there is a single *dlt* operon encoding the genes responsible for the D-alanine incorporation (23, 29, 33, 35, 38). The *dlt* operon of *B. subtilis* contains four genes, *dltA* to *dltD*, where *dltA* codes for a D-alanine-activating enzyme and *dltC* codes for a D-alanine carrier protein. *dltB* and *dltD* encode membrane proteins involved in the transport system (29). In *B. subtilis*, inactivation of the *dlt* operon enhanced the endogenous and β-lactam-induced cell lysis (33). The susceptibility to vancomycin has been shown to be affected in a *Staphylococcus aureus dlt* mutant (36). In addition, it affected the activity of autolysins (36), biofilm formation (14), and virulence in a mouse model (7, 47).

The *dlt* operon has been studied in several gram-positive bacteria, and its organization is almost identical in all of them (39). Inactivation of genes within this operon results in the complete absence or reduction of teichoic acid D-alanine esters. This is confirmed by our observation that *E. faecalis dlt* produced a lipoteichoic acid without alanyl-esters, as verified by NMR.

Gram-positive bacteria with mutations in the *dlt* operon showed a variety of phenotypic changes that could be attributed to the resulting charge modification of their cell surface. The lack of D-alanine esters resulted in a stronger negative net charge on the bacterial surface, because D-alanine esters introduce positively charged groups into the otherwise negatively charged teichoic acids. In *B. subtilis* (46) and in *S. aureus* (36), the absence of D-alanine has been shown to cause alterations in the activity of autolytic enzymes.

Wecke et al. found that in *B. subtilis*, autolysins bind to anionic sites on teichoic acids. A strain with a reduced amount of alanyl-esters binds more autolysins, leading to an increased rate of autolysis (46). An increased level of autolysis has recently been reported in a *Streptococcus pyogenes dlt* mutant (23) and in *Lactobacillus lactis dlt* (41) mutant strains.

In the present study, *E. faecalis* 12030 did not exhibit an increased autolysis in response to the deletion of the *dltA* gene. This may be related to the fact that the strain used for these experiments shows only very little autolysis, in contrast to other clinical isolates tested by us (data not shown).

Polycationic peptides with bactericidal activity are produced by microbes, plants, and the innate immune system of higher organisms. These compounds exert their effect by disruption of the bacterial cytoplasmic membrane, and resistance to cationic antimicrobial peptides (CAMPs) has been associated with the establishment of persistence and chronic infections (34). The susceptibility to CAMPs was increased in the *dlt* mutants of several gram-positive bacteria, such as *S. aureus* and *S. xylosus* (35), *Streptococcus agalactiae* (39), *S. pyogenes* (23), *Listeria monocytogenes* (1), and *Lactobacillus casei* (8). In *E. faecalis*, we found that the deletion of *dltA* also resulted in a significant decrease of the MICs of nisin, polymyxin B, and colistin. Resistance to other CAMPs with similar modes of action, such as β-defensins, may play a role in the colonization and persistence of enterococci in the gastrointestinal tract where several of these compounds are secreted (30), while resistance to cathelicidins or thrombocidins may be involved in other enterococcal infections, such as endocarditis or peritonitis (34, 48).

Production of biofilm is recognized as a virulence factor in a number of pathogens (31), and several authors have described the ability of enterococci to produce biofilm (3, 21, 22, 42, 44). Very little is known about the molecular mechanisms controlling biofilm formation and maintenance in enterococci (3, 31, 44). Testing a collection of clinical isolates, Baldassarri and colleagues found that 66% of 73 strains of *E. faecalis* tested were strong biofilm producers, that 14% were weak producers, and that 20% did not produce any biofilm at all (3). The formation of biofilm in these strains was strongly affected by the presence of additional monosaccharides in the growth medium. Bacteria producing biofilm were able to survive in peritoneal macrophages over extended periods (longer than 48 h), while slime-negative strains were killed in less than 24 h (3). The enterococcal surface protein Esp has been implicated in biofilm formation (43). However, current data on the role of Esp in biofilm formation are conflicting (19, 24). It is likely that *esp* represents one of a number of different mechanisms by which enterococci produce biofilm, and this interpretation is also supported by the findings of Toledo-Arana et al. that insertional mutagenesis in the *esp* gene abolished biofilm production in only two of three strains of *E. faecalis* (44).

Several recent papers discuss the role of *gelE* in the formation of biofilm by *E. faecalis* (5, 16, 24). These results were confirmed by Mohamed et al., who found that disruption of *gelE* resulted in fewer attached bacteria (26), and by Pillai and colleagues, who demonstrated that *fsr* mutants showed reduced biofilm formation on polystyrene plates (37). However, conflicting results have also been reported (27), and it seems clear that environmental factors (10), the genetic background of the strain, and possibly also an additional extracellular signaling molecule may play a role (5).

While a *dltA* mutant of a strong biofilm-producing *S. aureus* strain showed more than 50% less initial binding to polystyrene plates (14), there was no difference in biofilm formation in a *dltA* mutant of *E. faecalis* OG1RF compared to that of the wild type (26). The strain used in the present study (*E. faecalis* 12030) is a strong biofilm producer, and the *dlt* deletion mutant produced about 50% less biofilm than the wild type. Different mechanisms of biofilm formation are probably responsible for the observed phenotype in the specific strains used by different investigators. Alanylation of LTA affects the net surface charge of the bacteria, thereby modulating electrostatic interactions between cells and inanimate surfaces. This finding could have important implications for the design of new implantable devices and may also explain the enhanced adhesion of the wild-type strain to eukaryotic cells compared to that of the dealanylated mutant strain.

Our group has previously shown that a teichoic acid-like molecule is the target of opsonic antibodies directed against *E. faecalis* (17, 18, 45). While the structure reported by us did not contain alanine (45), we now believe that this teichoic acid is nonstoichiometrically alanylated (unpublished observations). The data presented here support this observation, since the wild-type strain resulted in 93.2% absorption while the dealanylated mutant was able to absorb only 71.1% of the killing activity. The difference in killing may be explained by a subpopulation of opsonic antibodies that are directed against alanylated LTA epitopes. Alternatively, alterations in chain length, amount of LTA inserted into the cell membrane, or

amount of LTA shed into the medium could be responsible for this effect.

In conclusion, the data presented here indicate that alanine esters of teichoic acids (lipoteichoic acid as well as wall teichoic acid) are involved in several aspects of the structural composition of the enterococcal cell wall. This feature may be responsible for the unspecific interaction of the bacteria with the environment. Therefore, this mechanism is probably also involved in pathogenesis of this organism and leads to increased biofilm formation, enhanced resistance to antimicrobial peptides, and increased attachment to eukaryotic cells.

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