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Inactivation of the *dlt* Operon in *Staphylococcus aureus* Confers Sensitivity to Defensins, Protegrins, and Other Antimicrobial Peptides*

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Positively charged antimicrobial peptides with membrane-damaging activity are produced by animals and humans as components of their innate immunity against bacterial infections and also by many bacteria to inhibit competing microorganisms. *Staphylococcus aureus* and *Staphylococcus xylosus*, which tolerate high concentrations of several antimicrobial peptides, were mutagenized to identify genes responsible for this insensitivity. Several mutants with increased sensitivity were obtained, which exhibited an altered structure of teichoic acids, major components of the Gram-positive cell wall. The mutant teichoic acids lacked D-alanine, as a result of which the cells carried an increased negative surface charge. The mutant cells bound fewer anionic, but more positively charged proteins. They were sensitive to human defensin HNP1–3, animal-derived protegrins, tachyplesins, and magainin II, and to the bacteria-derived peptides gallidermin and nisin. The mutated genes shared sequence similarity with the *dlt* genes involved in the transfer of D-alanine into teichoic acids from other Gram-positive bacteria. Wild-type strains bearing additional copies of the *dlt* operon produced teichoic acids with higher amounts of D-alanine esters, bound cationic proteins less effectively and were less sensitive to antimicrobial peptides. We propose a role of the D-alanine-esterified teichoic acids which occur in many pathogenic bacteria in the protection against human and animal defense systems.

Antimicrobial peptides play an important role in the defense of insects, vertebrates, and humans against pathogenic microorganisms (1) and have accordingly been designated "host defense peptides." Substances such as defensins from the granules of phagocytes, epithelial surfaces, and skin (2), protegrins from porcine leukocytes (3), tachyplesins from the hemocytes of the horseshoe crab (4), and magainins from amphibian skin (5) share an amphiphilic cationic structure and a membrane-damaging activity by forming pores or disintegrating the cytoplasmic membrane bilayer. Peptides with similar structure and activity are also produced by many Gram-positive bacteria and include the nonribosomally synthesized gramicidins and polymyxin B (6), ribosomally synthesized peptides, such as lactococcin A or pediocin PA-1 (7), and lantibiotics, which contain the posttranslationally formed thioether amino acid lanthionine (8). Because of their unique structural features and biotechnological importance, lantibiotics such as nisin, subtilin, Pep 5, epidermin, and gallidermin have been extensively investigated (8, 9).

Staphylococcus aureus, a major human pathogen, tolerates high concentrations of several host defense peptides and lantibiotics. Strains resistant to defensin-like platelet microbicidal proteins have been shown to be more virulent than sensitive ones (10). The mechanisms responsible for the resistance phenotype are unknown. The broad range of antimicrobial peptides tolerated distinguishes them from the highly specific self-protection systems of lantibiotic-producing staphylococcal strains (11, 12).

Gram-positive bacteria are not protected by an outer membrane. The *S. aureus* cell wall is instead formed by a thick peptidoglycan fabric and by polymers of alternating phosphate and alditol groups called teichoic acids. These polymer chains are either covalently connected to the peptidoglycan (wall teichoic acids, WTA)[1] (13) or to membrane glycolipids (lipoteichoic acids, LTA) (14). Teichoic acids of the various Gram-positive species are highly variable in the use of alditol groups (glycerol or ribitol) and in modifications of the alditol with glycosyl residues or D-alanine. The highly charged teichoic acids are essential for viability and seem to be involved in the control of cell shape, autolytic enzymes, and magnesium ion concentration within the cell envelope (13, 15).

In an attempt to understand the mechanisms by which staphylococci resist antimicrobial peptides, we isolated gallidermin-sensitive *Staphylococcus xylosus* and *Staphylococcus aureus* mutants, analysis of which revealed that the absence of D-alanine esters from teichoic acids leads to increased sensitivity toward cationic antimicrobial peptides.

EXPERIMENTAL PROCEDURES

Growth Conditions, Transposon Mutagenesis, and DNA Sequence Analysis—S. aureus Sa113 (16), *S. xylosus* C2a (17), and *Escherichia coli* DH5α (18) were grown in BM broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose) unless otherwise noted. Plasmid pTV1ts, used for transposon mutagenesis, is composed

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF032440 (S. xylosus C2a dlt operon) and AF101234 (S. aureus Sa113 dlt operon).

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[1] The abbreviations used are: WTA, wall teichoic acid(s); LTA, lipoteichoic acid(s); PCR, polymerase chain reaction; RP-HPLC, reversed-phase high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s).

of a temperature-sensitive replicon, a chloramphenicol resistance gene, and the transposon Tn*917,* which mediates erythromycin resistance (19). To generate a mutant library, *S. xylosus* (pTV1ts) was grown overnight at 30 °C in BM broth containing 5 μg of erythromycin/ml and 20 μg of chloramphenicol/ml. The culture was subsequently diluted 100-fold in BM broth containing 2.5 μg of erythromycin/ml and incubated for 14 h at 42 °C to select for transposon insertion mutants. This culture was diluted again in the same way and grown for another 14 h at 42 °C. Appropriate amounts of the bacterial suspension were spread on BM agar plates containing 2.5 μg of erythromycin/ml and incubated at 37 °C. Mutant clones (4000) were transferred to BM agar plates containing 3 μg of gallidermin/ml and monitored for impaired growth on gallidermin.

DNA was sequenced by cycle sequencing (18) on a DNA sequencer 4000 L (LI-COR Inc., Lincoln, NE) using the Thermo Sequenase fluorescent-labeled prime cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Tn*917*-specific primers were used to directly sequence genomic DNA upstream and downstream from the transposon insertion sites. Remaining sequence gaps were filled in by primer walking. To perform amino acid sequence similarity searches and to determine the degree of identity of two given sequences the program BLAST 2.0 with the nonredundant protein data base and program BLAST 2 Sequences of the National Center for Biotechnology Information (Bethesda, MD) were used, respectively. Multiple sequence alignments were accomplished using the Higgins-Sharp algorithm of the program MacDNASIS Pro (Hitachi Software Engineering, San Bruno, CA).

Construction of Plasmids and Homologous Recombination—In order to replace the *dltA* gene of *S. aureus* Sa113 by a spectinomycin resistance gene, DNA fragments of 1.5-kilobase pair flanking *dltA* were amplified by PCR, cloned into the *Sma*I site of pUC18 according to standard methods (18), and then sequenced. The upstream and downstream fragments were isolated from the resulting plasmids by cleavage with *Sph*I/*Sac*I and *Bam*HI/*Eco*RI, respectively, and inserted into the polylinker of the temperature-sensitive shuttle plasmid pBT2 (17) together with a 1277-bp *Sac*I-*Bam*HI fragment encoding the spectinomycin adenyltransferase gene (*spc*) from Tn*554* (20), as shown in Fig. 1. The resulting plasmid pBTΔdlt1 was transformed into *S. aureus* Sa113 by electroporation (21). By incubation at 42 °C and subsequent screening for spectinomycin-resistant clones without the plasmid-encoded chloramphenicol resistance, mutant AG1, which carries the *spc* gene instead of *dltA* in the chromosome, was identified. The recombination procedure has been described recently in detail (17). The proper integration of *spc* was verified by direct sequencing of the genomic DNA at the borders of the PCR-derived regions. A 1448-bp fragment comprising the *dltA* gene and additional 10 bp at the 5′ end was deleted; 18 bp of the *dltA* 3′ end bearing the ribosomal binding site of *dltB* were retained.

Plasmid pRBdlt1 was constructed by ligation of a 4655-bp PCR fragment bearing the *dltABCD* operon of *S. xylosus* C2a, together with 393 bp upstream of the *dltA* start codon with the putative promoter region and 185 bp downstream of the *dltD* stop codon with the terminator structure into the *Sma*I site of pUC18. After sequence analysis the fragment was isolated by *Bam*HI-*Eco*RI cleavage from the resulting plasmid, cloned into the multiple cloning site of the shuttle vector pRB473 (22), and transformed into *S. xylosus* C2a and *S. aureus* Sa113 by electroporation (21). Direct transformation of *S. aureus* and *S. xylosus dlt* mutants was unsuccessful, probably as a result of the altered surface charge. pRBdlt1 was therefore introduced into mutant protoplasts by fusion with wild-type protoplasts bearing the plasmid and subsequent selection for spectinomycin (*S. aureus*) or erythromycin (*S. xylosus*) resistance and the plasmid-encoded chloramphenicol resistance. Protoplast fusion was carried out as described previously (23). The resulting clones were verified by restriction fragment analysis of the plasmid and sequencing of the DNA flanking the chromosomal resistance determinant.

Isolation of WTA and LTA—Bacteria were grown overnight in 500 ml of BM broth containing 0.25% (*S. xylosus*) or 0.3% glucose (*S. aureus*), harvested by centrifugation, and washed in 100 ml of sodium acetate buffer (20 mM, pH 4.6). Cells were disrupted in the same buffer using glass beads and a Disintegrator S (Biomatik GmbH, Rodgau, Germany) as described previously (24).

In order to isolate WTA, 500-μl aliquots of the crude cell extracts were diluted 4-fold in sodium acetate buffer containing 2% SDS, sonicated for 15 min, and then vigorously shaken for 1 h at 60 °C. The cell walls were sedimented by centrifugation, subjected to repeated washings with sodium acetate buffer to remove the SDS, and finally resuspended in 1 ml of sodium acetate buffer. WTA was extracted by diluting 250 μl of purified cell walls 4-fold in 5% trichloroacetic acid and incubating at 60 °C for 4 h. The peptidoglycan was removed by centrifugation.

LTA was isolated from 250 μl of the crude cell extract after 2-fold dilution with sodium acetate buffer by extraction with 500 μl of aqueous phenol (vigorous shaking for 1 h at 60 °C) and subsequent hydrophobic interaction chromatography. The chromatographic purification was carried out essentially as described by Koch *et al.* (25). Briefly, the aqueous layer was further diluted by addition of 250 μl of sodium acetate buffer, mixed with 500 μl of octyl-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech), which had been equilibrated with sodium acetate buffer, and incubated for 15 min at room temperature. The suspension was applied to Ultrafree-MC centrifugal filter units with 0.45 μm pore size (Millipore, Bedford, MA), and the solvent was removed by centrifugation for 1 min at 340 × *g*. After three washing steps, each with 500 μl of 15% 1-propanol in sodium acetate buffer, the lipoteichoic acids were eluted by resuspending the gel in 500 μl of 50% 1-propanol in sodium acetate buffer and incubating for 15 min at room temperature. After collecting the eluate by centrifugation, the elution procedure was repeated, and eluates were combined.

Analysis of D-Alanine and Phosphorus—D-Alanine was analyzed according to an established method (26). WTA and LTA samples were adjusted to pH 9–10 with NaOH to a final volume of 100 μl and were incubated for 1 h at 37 °C to hydrolyze the D-alanine esters. Tris-HCl (200 μl, 0.2 M, pH 8.4) containing 2.5 mg of D-amino acid oxidase/ml (1.3 units/mg; Sigma) was added, and samples were incubated for 1 h at 37 °C. The reaction was stopped with 100 μl of 30% trichloroacetic acid, and the precipitated protein was removed by centrifugation. 2,4-Dinitrophenylhydrazine (100 μl of a 0.1% solution prepared in 2 M HCl) was added to the supernatant and incubated for 5 min. After addition of 200 μl of 2.5 M NaOH, the A_{525} was determined. The amount of phosphorus in WTA and LTA samples was determined according to Chen *et al.* (27).

Isolation of Defensin HNP1–3 from Human Neutrophils—Human neutrophil peptides HNP1–3 were isolated from human peripheral blood neutrophils as described previously (28). To improve the yield, blood from a patient who had been stimulated with granulocyte colony-stimulating factor was used (kindly provided by Dr. Handgretinger, Children's Hospital, University of Tübingen). The defensin peptides were highly enriched from the granules of the granulocytes by intensive extraction with 5% acetic acid and further purified by RP-HPLC yielding 5.1 mg of defensin peptides from 500 ml of blood. The lyophilized samples were stored at 4 °C and dissolved in 0.01% acetic acid at a concentration of 1 mg/ml. The product was composed of the three defensin variants HNP1, HNP2, and HNP3, which differ only in the first amino acid. The purity and quality was confirmed by ESI-MS yielding the expected masses.

Synthesis, Folding, Purification, and Characterization of Protegrins and Tachyplesins—Protegrins 3 and 5 (29) and tachyplesins 1 and 3 (4) were synthesized by standard methods for multiple parallel synthesis of peptides (30) using a Fmoc (9-fluorenylmethylcarbonyl protecting group) strategy. All peptides were synthesized as peptide-amides. After lyophilization, the crude, linear peptides were obtained in purities ranging from 75–82% as determined by RP-HPLC and ESI-MS. Folding of the raw peptides was achieved by the method of Aumelas *et al.* (29) after Tam *et al.* (31). Briefly, crude linear peptides were dissolved in 30% aqueous isopropyl alcohol (0.8 mg/ml) and then added slowly and with stirring to folding buffer (25% dimethyl sulfoxide, 10% isopropyl alcohol, 0.1 M Tris-HCl, final pH 6.8) at a ratio of 1.5 ml of peptide solution to 4 ml of folding buffer (final peptide concentration about 0.22 mg/ml). The reaction was allowed to proceed in capped glass vials with gentle stirring for 24 h and was followed by the periodic removal of 150 μl samples which were acidified by addition of 10 μl of trifluoroacetic acid and then characterized by RP-HPLC. No precipitation of the peptides was observed in any of the samples, and after 24 h disulfide bond formation appeared to have ceased. The purity of the crude, folded product was estimated to range from 56–69%, as judged by RP-HPLC. Crude, folded preparations were stored frozen at −20 °C prior to purification.

Peptides were purified by semipreparative RP-HPLC using appropriate acetonitrile gradients in 0.1% trifluoroacetic acid. After lyophilization, the folded peptides were characterized by RP-HPLC and ESI-MS; in each case purity was judged to exceed 95%, while masses showed a loss of 4 Da, suggesting oxidation of the peptides to form the respective disulfide bridges. Disulfide bond formation was also confirmed by a previously described method (32). Briefly, samples (5 μg) of each of the four peptides was alkylated with 4-vinylpyridine both in the presence and absence of a reducing agent (2-mercaptoethanol), purified by RP-HPLC, and then analyzed by ESI-MS. In no case were free thiol groups detected, suggesting that all of the cysteinyl thiol functions were oxidized and were involved in disulfide bonds.

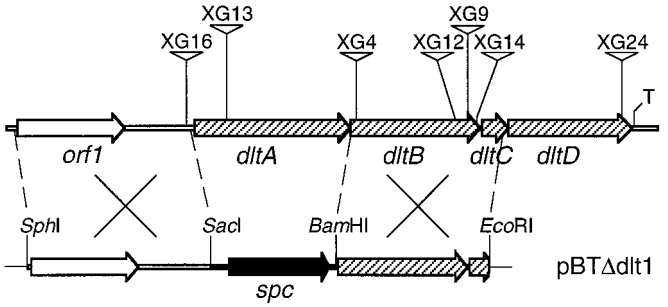

FIG. 1. **Organization of *dlt* genes in *S. xylosus* and *S. aureus* and disruption by transposon or resistance gene insertions.** Sites of Tn*917* integration in *S. xylosus* mutants are indicated by *triangles*. The *dlt* operon of *S. aureus* was disrupted by replacing the *dltA* gene with the spectinomycin resistance gene *spc* as shown in the *lower part* of the figure. The *spc* gene and PCR fragments flanking *dltA* were cloned using the restriction sites indicated to produce the integration vector pBTΔdlt1. The symbol *T* indicates a transcriptional terminator structure.

Analysis of Minimal Inhibitory Concentrations—Gallidermin was kindly provided by Dr. Karl Thomae GmbH, Biberach, Germany. Nisin, synthetic ([$A^{8,13,18}$]magainin II amide, gramicidin D, and polylysine (average molecular mass of 3.97 kDa) were purchased from Sigma. In a serial dilution test, LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing increasing concentrations of the antimicrobial peptides were inoculated with 1/100 volume of precultures. The cultures were incubated until the stationary phase was reached, and the turbidity of the cultures was monitored at 590 nm. Since the activity of human defensins is very sensitive to the salt concentration (28), LB broth without NaCl and with only half of the amount of tryptone and yeast extract was used. The tested strains all grew well in this medium.

Analysis of Green Fluorescent Protein, Cytochrome c, and Gallidermin Binding—Bacteria were grown to stationary phase in BM broth and harvested by centrifugation. In order to analyze the interaction with anionic and cationic proteins, cells were washed twice with sodium phosphate buffer (100 mM, pH 7) for incubation with green fluorescent protein (CLONTECH) and gallidermin. For incubation with cytochrome *c* (Merck, Darmstadt, Germany), MOPS buffer (20 mM, pH 7) was used. The cells were resuspended in the same buffers to a final A_{578} of 7 (green fluorescent protein and cytochrome *c*) or A_{578} of 4 (gallidermin), incubated for 10 min with 0.5 μg of protein/ml (green fluorescent protein and gallidermin) or 0.5 mg/ml (cytochrome *c*), and subsequently removed by centrifugation. The amount of green fluorescent protein in the supernatant was analyzed fluorometrically (excitation at 395 nm, emission monitored at 509 nm). Cytochrome *c* was quantitated photometrically at 530 nm, the absorption maximum of the prosthetic group. The amount of gallidermin in the supernatant was determined by RP-HPLC analysis using a linear gradient from 30 to 60% acetonitrile in 0.1% trifluoroacetic acid over 20 column volumes on a Spherisorb ODS2 column (Grom Analytik, Herrenberg, Germany).

RESULTS

Identification and Sequence Analysis of the S. aureus and S. xylosus dlt Operons—*S. aureus* Sa113 and the coagulase-negative *S. xylosus* C2a reveal high innate tolerances toward several antimicrobial peptides (see below). To investigate the resistance mechanism, *S. xylosus* C2a was mutagenized with Tn*917* as described under "Experimental Procedures," and the resulting transposon insertion mutants were analyzed for reduced growth on agar plates containing the antimicrobial peptide gallidermin. The nucleotide sequence upstream and downstream of the transposon from seven clones whose growth was specifically reduced in the presence of gallidermin was determined. In all seven mutants, the transposon had integrated into the same determinant of 4600 bp, which encodes four open reading frames, arranged in an operon-like structure, and followed by a typical terminator (Fig. 1). The open reading frames showed sequence similarity with the *Lactobacillus casei* and *Bacillus subtilis dltABCD* operons, which are responsible for esterification of teichoic acids with D-alanine (33–35). The transposon had integrated into the *S. xylosus dltA* (mutant XG13), *dltB* (XG4, XG12, XG9, XG14), or *dltD* genes (XG24) or into the putative promoter region (XG16) (Fig. 1). Growth rates in the absence of gallidermin and microscopic appearance of the mutants were indistinguishable from those of the wild-type strain.

The *S. xylosus dlt* operon showed similarity to a DNA sequence from *S. aureus* KAN96, which has recently been deposited to the GenBank™.[2] We sequenced and completed the respective DNA sequence from *S. aureus* Sa113 and found an identical organization to that in *S. xylosus* (Fig. 1). In both organisms, the *dltA* and *dltB* genes and the *dltC* and *dltD* genes overlap by 4 bp each, while the *dltB* and *dltC* genes are separated by 17 bp. The *dltA*, *dltB*, *dltC*, and *dltD* genes encode proteins of 485, 404, 78, and 391 amino acids (*S. aureus*) and 487, 404, 78, and 382 amino acids (*S. xylosus*), respectively. The protein sequences of the two organisms are 61–89% identical and 30–62% identical to the corresponding proteins of *B. subtilis* and *L. casei*. A further, less-pronounced sequence identity in the C-terminal 270 amino acids of the putative membrane proteins DltB was found to AlgI of *Pseudomonas aeruginosa* (28%), which is involved in substitution of the exopolysaccharide alginate with acetyl groups (36), and to a related gene product from *Helicobacter pylori* (24%). Three domains in the C-terminal portions of the proteins are particularly well conserved as shown in Fig. 2*A*. All four DltD proteins bear hydrophobic stretches at the N termini resembling a signal peptide. Putative signal peptidase I cleavage sites are, however, only weakly conserved (Fig. 2*B*).

The *dlt* operons are preceded in *S. aureus* and *S. xylosus* by *orf1*, which shows up to 44% identity to hydroxyacid dehydrogenases from various organisms. *orf1* and *dltA* are separated by noncoding regions of 573 bp (*S. aureus*) and 865 bp (*S. xylosus*), respectively.

Disruption of the S. aureus dlt Operon and Analysis of the D-Alanine Content in LTA and WTA—The *dltA* gene of *S. aureus* Sa113 was replaced by a spectinomycin resistance gene (*spc*) by homologous recombination producing the gallidermin-sensitive strain AG1. WTA and LTA of *S. aureus* and *S. xylosus* wild-type strains and mutants were isolated, and the molar ratios of D-alanine to phosphorus were determined as shown in Table I. In the wild-type strains, 75% (*S. aureus*) and 95% (*S. xylosus*) of the alditol phosphate residues in LTA were esterified with D-alanine, while only 51% (*S. aureus*) and 15% (*S. xylosus*) were esterified in WTA. In the *S. aureus dlt* mutant AG1 and in the *S. xylosus* mutants XG13, XG4, and XG24, bearing the transposon in the *dltA*, *dltB*, and *dltD* genes, respectively, no D-alanine was detected in LTA or WTA, indicating that the pathway for D-alanine incorporation was inactivated by the spectinomycin resistance gene and transposon insertions.

When the mutant strains *S. aureus* AG1 and *S. xylosus* XG13 were complemented with plasmid pRBdlt1 bearing the *dlt* operon, normal or slightly increased amounts of D-alanine were found in LTA and WTA. Transformation of the wild-type strains with pRBdlt1 resulted in an increase of D-alanine in LTA and WTA by 5–18% (Table I).

Sensitivity toward Antimicrobial Peptides—The minimal inhibitory concentrations of gallidermin and of several other membrane-damaging antimicrobial peptides, which were isolated or synthesized as described under "Experimental Procedures" were determined for the *S. aureus* and *S. xylosus* wild-type and mutant strains. The mutants were sensitive to a variety of antimicrobial peptides that bear a positive net charge

[2] The partial nucleotide sequence of the *S. aureus* KAN96 *dlt* operon has been deposited in the GenBank™ data base under GenBank™ accession number D86240.

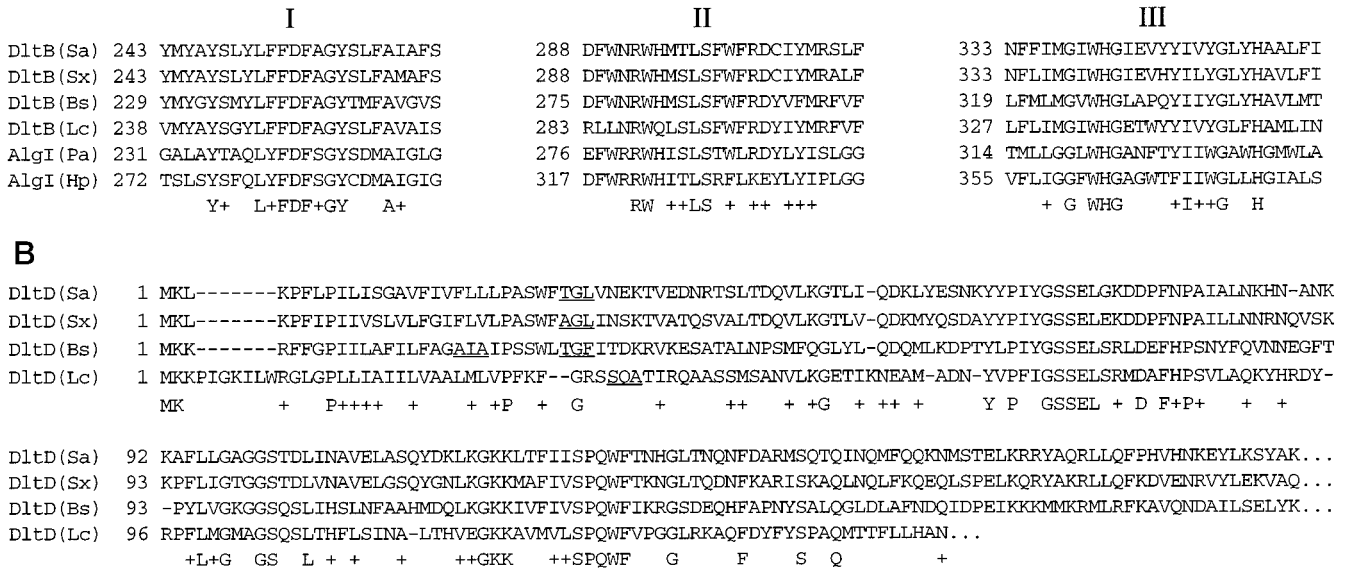

FIG. 2. **Sequence alignments of DltB and DltD.** *A*, three well conserved regions of the DltB proteins of *S. aureus* (*Sa*), *S. xylosus* (*Sx*), *B. subtilis* (*Bs*), and *L. casei* (*Lc*) are compared with the homologous AlgI proteins of *P. aeruginosa* (*Pa*) and *H. pylori* (*Hp*). *B*, alignment of the N-terminal portions of DltD proteins from *S. aureus* (*Sa*), *S. xylosus* (*Sx*), *B. subtilis* (*Bs*), and *L. casei* (*Lc*). Conserved amino acids are indicated below the sequence. Similar amino acids are indicated by +. Putative signal peptidase cleavage sites, as calculated according to von Heijne (40), are indicated by *underlining* the three preceding amino acids (the *B. subtilis* DltD contains two possible cleavage sites).

TABLE I
D-Alanine content of S. aureus and S. xylosus teichoic acids

Strain	Molar ratio of D-alanine to phosphorus in	
	LTA	WTA
S. aureus Sa113		
Wild-type	0.75	0.51
Wild-type (pRBdlt1)	0.82	0.69
dltA::*spc* (AG1)	0	0
dltA::*spc* (AG1) (pRBdlt1)	0.78	0.67
S. xylosus C2a		
Wild-type	0.95	0.15
Wild-type (pRBdlt1)	1.0	0.20
dltA::Tn*917* (XG13)	0	0
dltB::Tn*917* (XG4)	0	0
dltD::Tn*917* (XG24)	0	0
dltA::Tn*917* (XG13) (pRBdlt1)	0.96	0.15

(Table II). The sensitivity of the *S. aureus* mutant AG1 to defensin HNP1–3 from human neutrophils and to protegrins 3 and 5 from porcine leukocytes was at least 10–23-fold higher. Factors of 7–12 were determined with tachyplesin 1 and 3 from hemocytes of the horseshoe crab and to a variant of magainin II from clawed frog skin. The tolerance toward the lanthionine-containing peptides gallidermin from *Staphylococcus gallinarum* and nisin from *Lactococcus lactis* was 8–50-fold decreased; very similar results were obtained with the *S. xylosus* strains (Table II).

The increased sensitivity of *dlt* mutants seemed to be restricted to cationic peptides, since no considerable differences were observed in the inhibitory concentrations of the neutral peptide gramicidin D from *Bacillus brevis*. Furthermore, the mutants were not sensitive to cationic polylysine, indicating that cationic properties are not sufficient for activity of a peptide against *S. aureus* and *S. xylosus* strains lacking D-alanine esters in their teichoic acids.

The mutant strains AG1 and XG13 revealed normal sensitivities to gallidermin and nisin after complementation with the plasmid pRBdlt1. Wild-type strains bearing pRBdlt1 revealed increased tolerances against most tested cationic peptides, suggesting a direct correlation between the tolerance to cationic peptides and the D-alanine content of the teichoic acids.

Binding Studies with Anionic Green Fluorescent Protein and the Cationic Proteins Cytochrome c and Gallidermin—D-Alanine esters modulate the teichoic acid net charge by introducing positively charged amino groups to the negatively charged backbone (37). To determine whether the lack of D-alanine caused an alteration in the overall charge of the cell envelope, the capacity of the wild-type and *dlt* mutant cells to bind negatively or positively charged proteins was compared. The cells were incubated at pH 7 with either negatively charged green fluorescent protein (calculated pI 5.8), positively charged cytochrome *c* (calculated pI 10.0), or positively charged gallidermin. The bacteria were subsequently removed by centrifugation and the amounts of these substances remaining in the supernatants were determined (Fig. 3). The mutants bound lower amounts of anionic green fluorescent protein and higher amounts of cationic cytochrome *c* and gallidermin than the wild-type strains, while wild-type strains bearing pRBdlt1 revealed the opposite behavior (with the exception of green fluorescent protein binding by *S. aureus* Sa113 bearing pRBdlt1). These observations are in agreement with the proposed higher negative charge of the cell surface of the mutants and the lower negative charge of wild-type strains containing additional copies of the *dlt* operon.

DISCUSSION

The *dltABCD* genes of *S. aureus*, *S. xylosus*, *L. casei,* and *B. subtilis* are similar in sequence and organization. Studies in *L. casei* have demonstrated a role of DltA as a D-alanine-D-alanyl carrier protein ligase (Dcl), which activates D-alanine by hydrolysis of ATP and transfers it to the phosphopantetheine cofactor of a specific D-alanine carrier protein (Dcp), which is encoded by *dltC* (33, 34) (Fig. 4). The hydrophobic DltB is indispensable for D-alanine incorporation into teichoic acids and may be involved in the transfer of activated D-alanine across the cytoplasmic membrane (35) (Fig. 4). The essential role of DltD and the presence of a putative N-terminal signal peptide suggest an involvement in the transfer of D-alanine

TABLE II
Activity of antimicrobial peptides against wild-type strains S. aureus Sa113/S. xylosus C2a and dlt mutants S. aureus AG1/S. xylosus XG13

Peptide (origin)	Peptide, net charge	Minimal inhibitory concentration against			
		Wild-type	*dlt* mutant	Wild-type (pRBdlt1)	*dlt* mutant (pRBdlt1)
		μg/ml			
S. aureus Sa113:					
Defensin HNP1–3 (human neutrophils)	+2/+3	>100	10	>100	ND[a]
Protegrin 3 (porcine leukocytes)	+6	13	0.76	17.2	ND[a]
Protegrin 5 (porcine leukocytes)	+6	20	0.86	19	ND[a]
Tachyplesin 1 (horseshoe crab)	+7	11	1.0	ND[a]	ND[a]
Tachyplesin 3 (horseshoe crab)	+7	8.6	1.2	ND[a]	ND[a]
Magainin II (clawed frog skin)[b]	+4	45	3.8	46	ND[a]
Gallidermin (*S. gallinarum*)	+3	3.4	0.41	4.6	3.3
Nisin (*L. lactis*)	+3	22	0.45	35	19
Gramicidin D (*B. brevis*)	0	1.2	0.85	1.4	ND[a]
Polylysine[c]	Positive	>100	>100	ND[a]	ND[a]
S. xylosus C2a:					
Magainin II (clawed frog skin)[b]	+4	45	4.3	ND[a]	ND[a]
Gallidermin (*S. gallinarum*)	+3	3.3	0.23	3.8	4.2
Nisin (*L. lactis*)	+3	23	2.6	23	20
Gramicidin D (*B. brevis*)	0	2.3	2.1	2.7	ND[a]
Polylysine[c]	Positive	>100	>100	ND[a]	ND[a]

[a] ND, not determined.
[b] The synthetic peptide variant [$A^{8,13,18}$]magainin II amide with higher antimicrobial activity was used.
[c] Polylysine with an average molecular mass of 3.97 kDa was used.

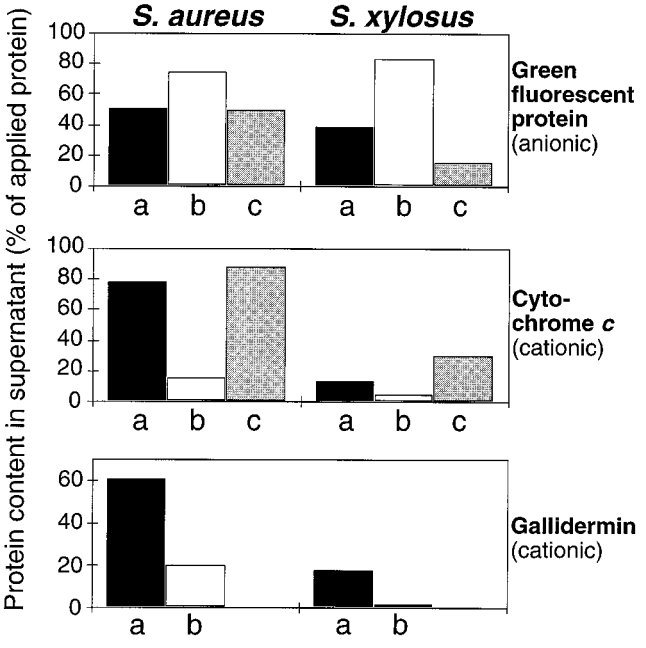

FIG. 3. **Interaction of *S. aureus* and *S. xylosus* strains with negatively and positively charged proteins.** *S. aureus* Sa113 and *S. xylosus* C2a wild-type strains (*black columns*, *a*), *dlt* mutants *S. aureus* AG1 and *S. xylosus* XG13 (*white columns*, *b*), and wild-type strains bearing additional copies of the *dlt* operon on the plasmid pRBdlt1 (*gray columns*, *c*) were incubated at neutral pH with anionic green fluorescent protein, cationic cytochrome *c*, or cationic gallidermin. The samples were then centrifuged, and the amounts of protein in the supernatants were determined (the capacities of wild-type strains bearing pRBdlt1 to bind gallidermin were not determined).

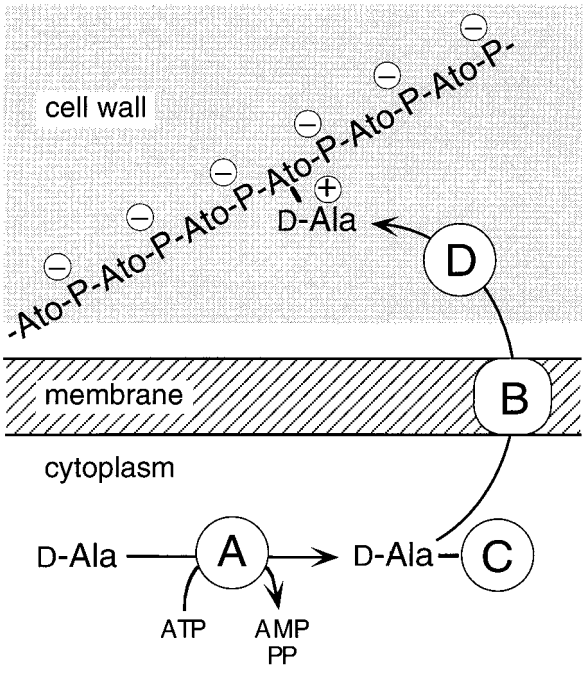

FIG. 4. **Schematic representation of the putative pathway of D-alanine transfer into teichoic acids.** A teichoic acid molecule is depicted as a chain of alternating alditol (*Ato*) and phosphate (*P*) residues. D-Alanine (D-Ala) is activated in the cytoplasm by DltA (*A*) via ATP hydrolysis and the release of pyrophosphate and is coupled to the phosphopantetheine prosthetic group of the D-alanine carrier protein DltC (*C*) (33, 34). The hydrophobic protein DltB (*B*) is likely to be involved in the transfer of D-alanine across the cytoplasmic membrane, and DltD (*D*), which bears a putative N-terminal signal peptide, is assumed to catalyze the esterification of teichoic acid alditol groups with D-alanine resulting in the introduction of positive charges into the otherwise negatively charged teichoic acids.

from the membrane carrier to teichoic acids (Fig. 4).

The increased sensitivity of *S. aureus* and *S. xylosus dlt* mutants toward a variety of membrane-active antimicrobial peptides provides evidence for a role of the D-alanine substituents in the protection of the bacteria against these substances. Inactivation of *dlt* genes caused considerably lower minimal inhibitory concentrations of (i) host defense peptides with β-sheet structure and disulfide bridges, such as human defensin HNP1–3, protegrins from porcine leukocytes, and tachyplesins from horseshoe crab hemocytes; (ii) the linear peptide magainin II from amphibian skin; and (iii) the bacteria-derived lantibiotics gallidermin and nisin, which contain thioether bridges. Since the common structural feature of these molecules is a positive net charge, and since the sensitivity toward neutral gramicidin D was the same in the wild-type and the *dlt* mutants, we propose that the basis for increased sensitivity is an altered electrostatic interaction of the peptides with the mutant cells. The teichoic acid backbone is highly charged by deprotonized phosphate groups, and esterification with D-ala-

nine causes a reduction of the net negative charge by introduction of basic amino groups (Fig. 4). Accordingly, the *S. aureus* and *S. xylosus dlt* mutants, whose teichoic acids are devoid of D-alanine, bound lower amounts of negatively charged green fluorescent protein but higher amounts of positively charged cytochrome *c* and gallidermin, while strains with increased D-alanine content showed the opposite behavior. Increased accumulation of antimicrobial peptides in the vicinity of the cytoplasmic membrane is therefore very likely to be the basis for the higher sensitivity of the *dlt* mutants.

Cationic properties are necessary for the initial interaction of membrane-damaging peptides with the negatively charged membrane surface (38). Reduction of the negative cell envelope charge by incorporation of D-alanine may thus be regarded as a multiple drug protection mechanism. A similar observation has been made with Gram-negative bacteria whose resistance to the cationic peptides polymyxin B and protamine was caused by a reduction of the anionic nature of the lipopolysaccharide (39). Bacteria have to cope with antimicrobial peptides in many environments. The production of bacteriocin-like molecules such as gallidermin or nisin is a prevalent strategy among microorganisms to inhibit the growth of competing strains (7, 8) and, in higher organisms, to support the innate immunity against bacterial infections (1). For bacteria such as staphylococci, which live in intimate contact with humans and animals, mechanisms protecting against host defense peptides are of particular benefit. Accordingly, a correlation between the capacity of *S. aureus,* coagulase-negative staphylococci, and streptococci to cause endocarditis, and the *in vitro* resistance to defensin-like platelet microbicidal proteins has been demonstrated (10). Understanding the mechanisms by which bacteria are able to circumvent the human defense systems has a great impact on the treatment of infections and the search for new antimicrobial agents. To our knowledge, the study presented here describes the first mechanism conferring resistance to host defense peptides in a Gram-positive organism. D-Alanine-esterified teichoic acids occur in many Gram-positive human pathogens including staphylococci, streptococci, enterococci, and listeria (14), and our study raises the question whether the D-alanine esters or further resistance systems against host defense peptides contribute to the virulence and persistence of these bacteria.

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