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ORIGINAL ARTICLE

Genetic Basis for In Vivo Daptomycin Resistance in Enterococci

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ABSTRACT

BACKGROUND

Daptomycin is a lipopeptide with bactericidal activity that acts on the cell membrane of enterococci and is often used off-label to treat patients infected with vancomycin-resistant enterococci. However, the emergence of resistance to daptomycin during therapy threatens its usefulness.

METHODS

We performed whole-genome sequencing and characterization of the cell envelope of a clinical pair of vancomycin-resistant *Enterococcus faecalis* isolates from the blood of a patient with fatal bacteremia; one isolate (S613) was from blood drawn before treatment and the other isolate (R712) was from blood drawn after treatment with daptomycin. The minimal inhibitory concentrations (MICs) of these two isolates were 1 and 12 μg per milliliter, respectively. Gene replacements were made to exchange the alleles found in isolate S613 with those in isolate R712.

RESULTS

Isolate R712 had in-frame deletions in three genes. Two genes encoded putative enzymes involved in phospholipid metabolism, GdpD (which denotes glycerophosphoryl diester phosphodiesterase) and Cls (which denotes cardiolipin synthetase), and one gene encoded a putative membrane protein, LiaF (which denotes lipid II cycle-interfering antibiotics protein but whose exact function is not known). LiaF is predicted to be a member of a three-component regulatory system (LiaFSR) involved in the stress-sensing response of the cell envelope to antibiotics. Replacement of the *liaF* allele of isolate S613 with the *liaF* allele from isolate R712 quadrupled the MIC of daptomycin, whereas replacement of the *gdpD* allele had no effect on MIC. Replacement of both the *liaF* and *gdpD* alleles of isolate S613 with the *liaF* and *gdpD* alleles of isolate R712 raised the daptomycin MIC for isolate S613 to 12 μg per milliliter. As compared with isolate S613, isolate R712 — the daptomycin-resistant isolate — had changes in the structure of the cell envelope and alterations in membrane permeability and membrane potential.

CONCLUSIONS

Mutations in genes encoding LiaF and a GdpD-family protein were necessary and sufficient for the development of resistance to daptomycin during the treatment of vancomycin-resistant enterococci. (Funded by the National Institute of Allergy and Infectious Diseases and the National Institutes of Health.)

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The treatment of enterococcal infections has become an enormous challenge for clinicians because these organisms frequently exhibit resistance to the standard drugs of choice — namely, ampicillin, vancomycin, and aminoglycosides (with high-level resistance to aminoglycosides). In addition, there has been a striking increase in the frequency of isolation and the spread of vancomycin-resistant enterococci in hospitals around the world, which has resulted in significant increases in mortality, length of hospital stay, and hospitalization costs.[1]

Enterococcus faecium is one of the so-called ESKAPE pathogens (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and enterobacter species) flagged by the Infectious Diseases Society of America as problem pathogens requiring new therapies.[2] The Food and Drug Administration (FDA) has approved only two compounds for the treatment of vancomycin-resistant enterococci infections: linezolid and quinupristin–dalfopristin. Both have important limitations when used for the treatment of severe vancomycin-resistant infection. Their use often results in clinical failure or recurrence of infection; they have an adverse toxicity profile, a limited spectrum of activity, and a bacteriostatic effect against vancomycin-resistant enterococci, and they are associated with increasing reports of resistance.

Daptomycin is a lipopeptide antibiotic with in vitro bactericidal activity against enterococci. Even though the FDA has not approved daptomycin for the treatment of infection with vancomycin-resistant enterococci, clinicians often use it off-label in patients with severe enterococcal infections because of the lack of other treatment options with established reliability.[3] However, a major drawback of the use of daptomycin for the treatment of infection with vancomycin-resistant enterococci is the development of resistance during therapy.[4-6] Although little is known about the mechanism of in vivo resistance, a critical step in the action of daptomycin is its interaction with the bacterial cell membrane in a calcium-dependent manner. In this study, comparative genomic sequencing and genetic manipulations of a clinical pair of daptomycin-susceptible and daptomycin-resistant *E. faecalis* strains, which were recovered from the bloodstream of a patient with fatal bacteremia, identified mutations in two genes not previously associated with antibiotic resistance in enterococci.

METHODS

BACTERIAL ISOLATES

The vancomycin-resistant clinical strain pair of daptomycin-susceptible and daptomycin-resistant *E. faecalis* has been described previously,[4] as has a similar strain pair of *E. faecium*[5] (for details, see the Supplementary Appendix, available with the full text of this article at NEJM.org). We also studied, with the use of pulsed-field gel electrophoresis, six unrelated clinical isolates of daptomycin-resistant enterococci (one *E. faecalis* isolate and five *E. faecium* isolates) recovered from different clinical sources in the United States. A daptomycin-resistant derivative of *E. faecalis* S613 was obtained after passing the bacterium through increasing subinhibitory concentrations of daptomycin twice daily for 17 days, starting with a concentration of 0.5 μg per milliliter. (See the Supplementary Appendix for the selection protocol.)

GENOMIC SEQUENCING AND ANALYSIS

Paired-end sequence reads of the *E. faecalis* strain pair (S613 and R712) were generated with the Illumina Genome Analyzer IIx at the Washington University Genome Institute, producing 100 base-paired end reads that were assembled with the use of Velvet software.[7] Genomic analysis and comparisons were performed by applying standard methods (see the Supplementary Appendix for details). All synonymous and nonsynonymous mutations were confirmed by means of polymerase-chain-reaction (PCR) sequencing of both strands in accordance with the Sanger dideoxy-terminator method of sequencing, with target genes resequenced in their entirety. The homologues of the genes encoding the LiaFSR regulatory system (*liaF*, *liaS*, and *liaR* [*lia* denotes lipid II cycle-interfering antibiotics protein]),[8] the glycerophosphoryl-diester-phosphodiesterase (*gdpD*)–family protein, and cardiolipin synthetase (*cls*) were sequenced from all isolates and derivatives of S613.

ALLELIC REPLACEMENTS

To establish a direct link between the presence of specific gene mutations and the development of the daptomycin-resistant phenotype, we replaced the native genes encoding the LiaF and GdpD proteins of the *E. faecalis* S613 isolate with those of the *E. faecalis* R712 isolate. The replacements were performed independently for each gene and then in combination, with the use of the *p*-chlo-

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rophenylalanine sensitivity counterselection system, as described previously,[9,10] except for the fact that plasmid pHOU3 was constructed and used (Fig. S1 in the Supplementary Appendix). The mutants were characterized with the use of pulsed-field gel electrophoresis, and the open reading frames of the three genes (*liaF*, *cls*, and *gdpD*) were sequenced. To detect small differences in the susceptibility to daptomycin, minimal inhibitory concentrations (MICs) were determined with the use of Etest (AB Biodisk).[11]

ULTRASTRUCTURAL CHARACTERISTICS OF THE CELL ENVELOPE

Transmission electron microscopy was used to assess the ultrastructural characteristics of *E. faecalis* strain pair S613 and R712 in accordance with standard methods.[12] The number of cell-division events (cells with a septum) in 100 cells chosen randomly in two blinded experiments was also determined with the use of transmission electron microscopy. The thickness of the cell walls was measured from the outer border of the cell membrane to the outer edge of the cell wall (on the basis of 100 observations of each isolate with a minimum of 50 cells, in cells from different fields, at a magnification of 190,000). The mean (±SD) cell-wall thickness was determined for each strain, and mean differences were compared with the use of Student's t-test.

Cell-surface charge, daptomycin-mediated cell-membrane permeability, and the effect of daptomycin on cell-membrane potential were measured with the use of a modified cytochrome *c* assay,[13] a highly sensitive bacterial viability kit (LIVE/DEAD BacLight, Invitrogen),[14] and the cell-membrane potential-sensitive 3,3-dipentyoxacarbocyanine assay,[15] respectively (see the Supplementary Appendix for details).

RESULTS

MUTATIONS CONFERRING DAPTOMYCIN RESISTANCE

A total of 3082 open reading frames were found in isolate S613 (2,727,367 base pairs were matched in both genomes), and comparative analysis and resequencing of genes of the clinical strain pair revealed changes in four predicted proteins in the daptomycin-resistant isolate, R712, as compared with the daptomycin-susceptible isolate, S613. Three of the four mutations consisted of in-frame deletions in a stretch encoding a repeated amino acid (Table 1). The proteins encoded included two enzymes (GdpD and Cls) predicted to be involved in phospholipid metabolism and likely to participate in cell-membrane homeostasis and a putative transmembrane protein that is a homologue of LiaF (48% similarity) from *Bacillus subtilis*[8] (Table 1). The regions of these predicted proteins are highly conserved among all *E. faecalis* isolates whose genomes have been sequenced, and these deletions are unique to the daptomycin-resistant isolate as compared with all *E. faecalis* homologues analyzed (www.ncbi.nlm.nih.gov//genomes/geblast.cgi?gi=7128#SearchSet). The fourth mutated gene encodes a putative LacI (lactose-operon-repressor)-family transcriptional repressor that is probably involved in carbohydrate metabolism. However, the substitution (Gly→Val) in position 2 is also present in other enterococcal homologues of the putative protein. It is of interest that none of the enterococcal homologues of staphylococcal genes associated with daptomycin resistance in previous studies (*mprF*, *yycG*, *yycH*, *dltABCD*, *rpoB*, *rpoC*, *vraSR*, and *graSR*)[20-24] exhibited any change when their gene sequences were compared with those of the vancomycin-resistant *E. faecalis* clinical strain pair.

To confirm the association of the genetic changes (Table 1) with the development of daptomycin-resistance, we initially exposed the daptomycin-susceptible *E. faecalis* S613 isolate to increasing concentrations of daptomycin in vitro. A daptomycin-resistant derivative was readily obtained after 17 days of exposure, with a daptomycin MIC similar to that of R712 (isolate S613R) (Table 2). On pulsed-field gel electrophoresis, the S613R isolate had a pattern that was identical to that of isolate S613, and we identified the same mutations found in the genes encoding the LiaF protein and the GdpD enzyme (but not in *cls*), suggesting that *gdpD* and *liaF* play a predominant role in the development of resistance to daptomycin in this in vitro–selected mutant.

To establish a direct link between the mutations in these two genes and the development of resistance to daptomycin, we replaced the *liaF* and *gdpD* alleles of the S613 isolate with those derived from the R712 isolate, both individually and in combination (with *liaF* replaced first, followed by *gdpD*). The correct allelic replacements were confirmed by sequencing the corresponding entire open reading frames in all constructs (including the *cls* gene that was not manipulated and re-

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Table 1. Genetic Changes Identified in the Vancomycin-Resistant *Enterococcus faecalis* Isolate That Was Resistant to Daptomycin (Strain R712) as Compared with the Daptomycin-Susceptible Isolate (Strain S613).*

Predicted Gene Product†	Nucleotide Change in Strain R712	Predicted Amino Acid Change in Strain R712	Comments
LiaF	Deletion of ATT	Deletion of Ile at position 177 in a stretch of four Ile residues	Part of a conserved gram-positive, three-component regulatory system that orchestrates the cell-envelope response to antimicrobial peptides and antibiotics that target the cell membrane[16-19]; the *Bacillus subtillis* LiaF negatively interferes with histidine kinase (LiaS) autophosphorylation through direct interactions, thereby suppressing activation of the cognate response regulator (LiaR)[8]
GdpD	Deletion of ATT	Deletion of Ile at position 170 in a stretch of four Ile residues	A transmembrane protein predicted to be involved in phospholipid metabolism; participates in glycerol metabolism in other organisms through hydrolysis of several glycerophosphodiesters; the mutation occurs within a predicted transmembrane domain
Cls‡	Deletion of AAA	Deletion of Lys at position 61 in a stretch of three Lys residues	A transmembrane protein predicted to be involved in phospholipid metabolism; the putative enzyme contains two conserved phospholipase D domains; the mutation is located nine amino acids away from the C-terminal of the second predicted transmembrane domain outside the phospholipase D domains

* Cls denotes cardiolipin synthetase, GdpD glycerophosphoryl diester phosphodiesterase, and LiaF lipid II cycle-interfering-antibiotics protein.[8]
† The genome accession numbers, locus tags, and gene homologues in *E. faecalis* V583 are specified in the Supplementary Appendix.
‡ In some *E. faecalis* genomes, the gene has been annotated as coding for a phospholipase D–transphosphatidylase enzyme.

mained unchanged in all constructs). Table 2 shows that the introduction of the *liaF* allele of the R712 isolate quadrupled the daptomycin MIC of the S613 isolate but not to the level of the R712 isolate; replacement of the *gdpD* allele alone had no effect on the susceptibility of isolate S613 to daptomycin. However, when the *gdpD* allelic replacement was introduced into the S613 derivative harboring the deletion in *liaF*, the daptomycin MIC was increased to 12 μg per milliliter — a concentration that was above the clinical breakpoint and identical to that for isolate R712, confirming our in vitro observation that mutations in these two genes are necessary and sufficient to confer clinical resistance to daptomycin in *E. faecalis* isolate S613.

ULTRASTRUCTURAL CHANGES AND RESISTANCE

Transmission electron microscopy revealed important differences in the cell morphology of the two isolates; R712 cells tended to clump and formed aggregates with longer chains, as compared with S613 cells. At higher magnifications, the presence of multiple septal structures before complete cell separation was evident in R712 (Fig. 1). In addition, the R712 cell envelopes appeared to be markedly altered as compared with those of the S613 isolate (Fig. 1). The number of cells with a septum was consistently higher in the R712 isolate than in the S613 isolate (P<0.001). The cell-wall thickness of R712 was also greater than that of S613 (average thickness, 18.12±2.23 nm vs. 10.43±1.34 nm; P<0.001). Collectively, our results suggest that the development of daptomycin resistance in vivo in *E. faecalis* is associated with profound ultrastructural changes in the cell envelope, septal apparatus, and cell wall. The development of daptomycin resistance in R712 was also associated with a cell surface with a greater positive charge, striking reductions in daptomycin-induced permeabilization of the cell membrane, and alterations in the ability of daptomycin to depolarize the membrane of the target cell, as compared with isolate S613 (Fig. S2 in the Supplementary Appendix).

MUTATIONS IDENTIFIED IN OTHER ENTEROCOCCI

We sought to determine whether the genetic changes identified in the daptomycin-resistant strain of *E. faecalis* (Table 1) could also be found in other clinical isolates of daptomycin-resistant enterococci, particularly *E. faecium*, since infection with this species is much more difficult to treat than infection with *E. faecalis*. Indeed, in a clinical strain pair of *E. faecium* recovered from a patient before and after daptomycin therapy, we found an Arg218→Gln substitution in the Cls enzyme but not in the LiaFSR or GdpD proteins (Table 3). The Arg218→Gln substitution in Cls was also found in an unrelated daptomycin-resistant clinical isolate

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Table 2. Amino Acid Changes in Daptomycin-Susceptible and Daptomycin-Resistant Clinical and Laboratory-Derived Isolates of Vancomycin-Resistant *Enterococcus faecalis*.*

Isolate	MIC of Daptomycin†	Predicted Amino Acid Change		
		Cls	LiaF	GpdD
	μg/ml			
S613‡	1	None	None	None
R712‡	12	Deletion of Lys at position 61	Deletion of Ile at position 177	Deletion of Ile at position 170
S613R§	12	None	Deletion of Ile at position 177	Deletion of Ile at position 170
S613*liaF*$_{\Delta Ile177}$¶	4	None	Deletion of Ile at position 177	None
S613*gdpD*$_{\Delta Ile170}$¶	1	None	None	Deletion of Ile at position 170
S613*liaF*$_{\Delta Ile177}$*gdpD*$_{\Delta Ile170}$¶	12	None	Deletion of Ile at position 177	Deletion of Ile at position 170

* Cls denotes cardiolipin synthetase, GdpD glycerophosphoryl diester phosphodiesterase, and LiaF lipid II cycle-interfering-antibiotics protein.
† The minimal inhibitory concentration (MIC) of daptomycin was determined with the use of Etest (AB Biodisk)[11] on brain–heart infusion agar.
‡ The clinical strain pair of *E. faecalis* isolates (S613 and R712) was recovered from a single patient, with S613 recovered before the administration of daptomycin and R712 recovered after the administration of daptomycin.[4]
§ S613R is an in vitro derivative of *E. faecalis* S613 that was obtained by serial passage through increasing concentrations of daptomycin.
¶ In this isolate, the native allele of S613 was replaced with the allele belonging to the R712 isolate.

of *E. faecium* (isolate R501) (Table 3), suggesting that this switch in amino acids (within the phospholipase D domain of the enzyme) may play an important role in the development of daptomycin resistance in *E. faecium*. Furthermore, we invariably found changes in *cls*, *liaF*, *liaS*, or *liaR* in other daptomycin-resistant clinical isolates of enterococci (Table 3).

DISCUSSION

The off-label use of daptomycin occurs often in the treatment of severe enterococcal infections, including infections with vancomycin-resistant enterococci or those species exhibiting high-level resistance to aminoglycosides. However, a major drawback for the successful use of this antibiotic is the emergence of resistance during therapy. In addition, in vitro, enterococci are less susceptible to daptomycin than *S. aureus*, with a clinical threshold for sensitivity that is four times as high (≤4 μg per milliliter, vs. ≤1 μg per milliliter for *S. aureus*).[25]

An essential event for the activity of daptomycin is calcium-mediated interaction with the cell membrane, a property that this antibiotic shares with related cationic antimicrobial peptides that are part of the human host defense against microbes. The change in the bacterial surface also appears to play an important role in the interaction of daptomycin with the cell membrane, and it has been postulated that a more positively charged cell envelope "repels" the cationic daptomycin from the cell membrane, contributing to the development of resistance.[15] A major factor in the cell-envelope charge is the phospholipid composition of the inner and outer cell-membrane leaflets, such as the negatively charged phospholipid cardiolipin and the positively charged amino derivatives of phosphatidylglycerol. In some *S. aureus* isolates, reduced susceptibility to daptomycin has been attributed to a decrease in the negative surface charge of the cell membrane as a result of modifications in phospholipid content, mainly through increased synthesis and translocation ("flipping") of the positively charged lysyl-phosphatidylglycerol from the inner to the outer leaflet of the cell membrane.[15,26] It has also been shown that lysyl-phosphatidylglycerol attenuates membrane perturbations caused by cationic antimicrobial peptides.[27]

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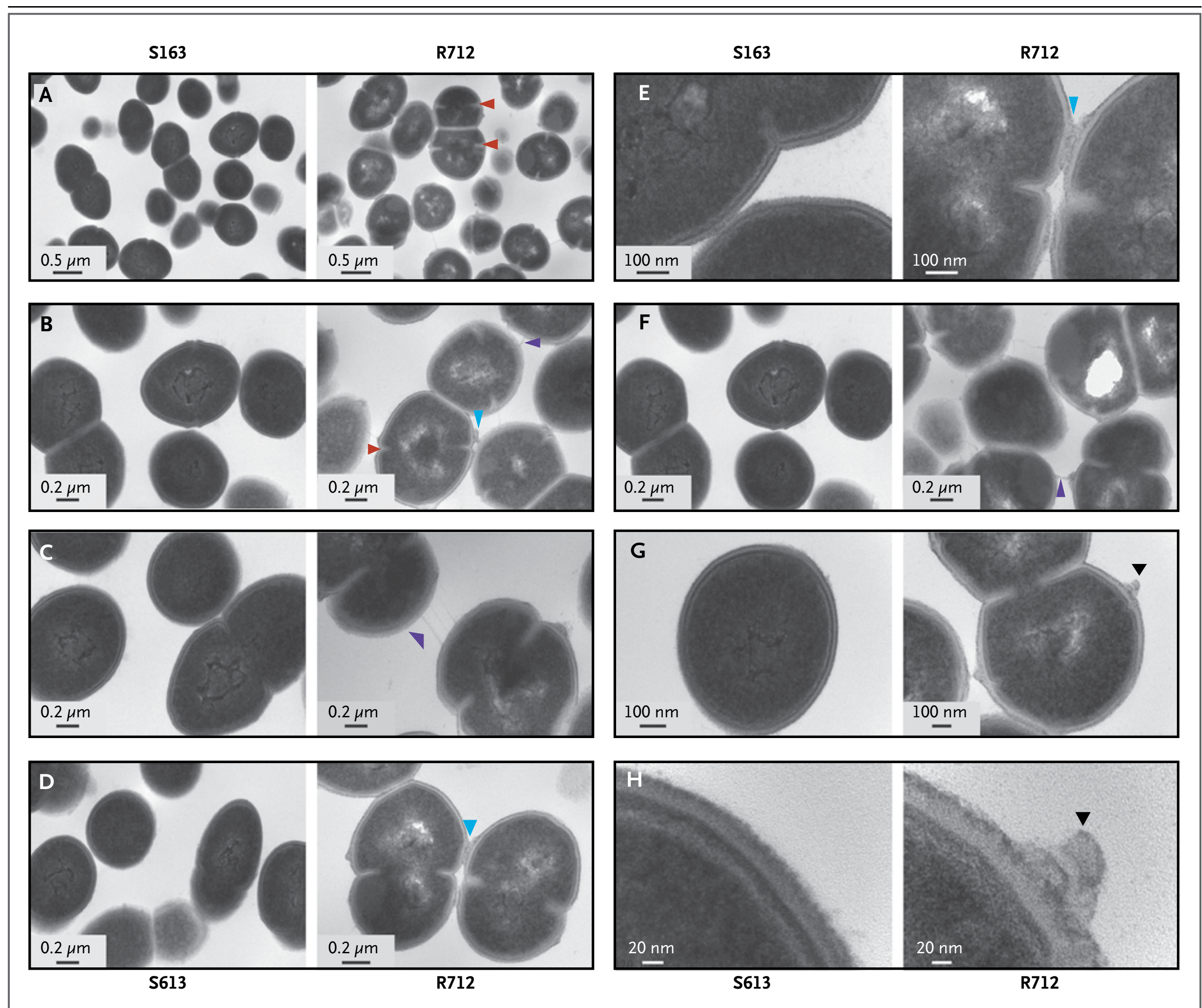

Figure 1. Transmission Electron Microscopy of the Cell Envelope of *Enterococcus faecalis* Isolates, One Susceptible to Daptomycin (S613) and the Other Resistant (R712).

At high magnification, cells in the S613 isolate have symmetric septa, with only a single septum observed between two cells, and cell separation is easily detected, with single cells visible (Panels A, B, and G). In contrast, multiple septal structures can be seen before complete cell separation in the R712 isolate (Panels A and B, red arrowheads). There are also prominent distortions in the cell envelopes of R712 cells. First, the envelopes appear to be altered at the point of cell contact (which is likely to be the point at which the cells separate) (Panels B, D, and E; blue arrowheads), and the surfaces of the R712 cells lack the smooth appearance of the S613 cells, even when they are in close proximity (a feature observed in all R712 cells analyzed) (Panel E). Second, several structures appear to be connecting the R712 cells (Panels B, C, and F; purple arrowheads); these connecting projections, which were not observed in S613 cells, appear to originate in the cell envelope and maintain the contact between adjacent cells even after the cells have separated. Third, localized protrusions of the cell envelope are a common feature of R712 cells (Panels G and H, black arrowheads). These protrusions were usually observed in proximity to a septal stricture that appeared to originate from the cell envelope. On the other hand, the surface of S613 cells is smooth and symmetric, without any obvious projections or protrusions (more than 100 cells analyzed) (Panels G and H).

Our findings in this study indicate that the development of resistance to daptomycin in the vancomycin-resistant *E. faecalis* isolate R712, like the development of resistance in *S. aureus*, is associated with alterations in the cell envelope and biophysical properties of the cell membrane. However, the genes linked to these changes in enterococci appear to be different from those described in *S. aureus*. Indeed, in the R712 isolate, none of the genes associated with the emergence of re-

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Table 3. Changes in Genes Encoding Cls or Members of the LiaFSR System in Clinical Isolates of Enterococci.*

Isolate	MIC†		Predicted Amino Acid Change	
	Daptomycin	Vancomycin	Cls	LiaFSR‡
	μg/ml			
Enterococcus faecalis				
R508	6	2	None	Ala180→Thr of LiaS
E. faecium				
S447§	2	256	None	None
R446§	32	16	Arg218→Gln	None
R501	48	256	Arg218→Gln	None
R494	64	1	None	Leu39→Phe of LiaF
				Trp73→Cys of LiaR
R496	32	1	Asn13→Ile	Ile144→Thr of LiaF
R497	24	1	Insertion of Met-Pro-Leu at position 110	Thr120→Ala of LiaS
R499	48	256	His215→Arg	Trp73→Cys of LiaR

* Cls denotes cardiolipin synthetase. LiaFSR is a lipid II cycle-interfering-antibiotics system.
† The minimal inhibitory concentration (MIC) of daptomycin was determined with the use of Etest (AB Biodisk)[11] on brain–heart infusion agar, and the MIC of vancomycin with the use of the agar dilution method.[25]
‡ LiaFSR proteins are homologous to YvqF, VraS, and VraR, which were previously described in *Staphylococcus aureus*.
§ The clinical strain pair of *E. faecium* (S447 and R446) was recovered from a single patient, with S447 recovered before the administration of daptomycin and R446 recovered after the administration of daptomycin.[5]

sistance to daptomycin in *S. aureus*[20-24] differed from those in the daptomycin-susceptible parental isolate, S613. Instead, our data provide direct evidence that changes in two genes — namely, *liaF* and *gdpD* — are sufficient for the development of resistance to daptomycin in the *E. faecalis* clinical strain pair.

The alteration of the LiaFSR system is probably a pivotal initial event in the development of resistance, since replacement of only the *liaF* allele in the S613 isolate with that from the R712 isolate decreased the susceptibility of the S613 isolate to daptomycin.

LiaF is part of the three-component LiaFSR regulatory system, which is known to orchestrate the response of the cell envelope to antibiotics and antimicrobial peptides in some gram-positive bacteria. The LiaFSR system has been well characterized in *B. subtilis*,[8,16] *Streptococcus mutans*,[17] and pneumococci.[18] In *B. subtilis* and *S. mutans*, the LiaFSR system is usually activated by the presence of antibiotics that disrupt cell-membrane and peptidoglycan synthesis through alterations of lipid-II metabolism (i.e., bacitracin, daptomycin, ramoplanin, nisin, and vancomycin).[16,19] In *B. subtilis*, LiaF is a membrane-anchored, negative regulator of LiaS (which is the sensor protein of the system and also functions as a histidine kinase that phosphorylates the cognate-response regulator, LiaR).[8] Therefore, it is predicted that mutations in *liaF* may release the inhibitory effect of LiaS, resulting in activation of this system.

Nonetheless, our genetic experiments indicated that mutations in *liaF* are not sufficient for full expression of the resistant phenotype. Indeed, the subsequent introduction of a mutation in *gdpD* was sufficient to increase the MIC to a level similar to that in the daptomycin-resistant R712 isolate, indicating that both genes are needed for the full expression of the resistant phenotype. Thus, it appears that resistance to daptomycin in enterococci requires two major steps. First, an initial activation of the LiaFSR system occurs through mutations in *liaF* or other components of the LiaFSR system (which might be selected by means of exposure to antibiotics that alter lipid-II metabolism); activation of the system may influence cell-envelope homeostasis by affecting the transcrip-

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tion of several genes that can help mitigate the damage caused by the antibiotic. Second, a subsequent alteration in the cell membrane occurs through changes in enzymes involved in phospholipid metabolism (e.g., GdpD or Cls), leading to critical and compensatory changes in the composition or distribution of phospholipids in the cell membrane. Indeed, the bacterial GdpD has been shown to be important in glycerol metabolism, hydrolyzing several cell-membrane glycerophosphodiesters[28] that affect phospholipid metabolism. Similarly, cardiolipin has been found to play several key roles in cell-membrane physiology, such as in bacterial cell division,[29,30] transporter localization (in *Escherichia coli*),[31] and the triggering of compensatory changes in the phospholipid composition of the cell membrane, which affect bacterial adaptive responses.[32]

In support of our hypothesis that the changes discussed above are also important in other daptomycin-resistant isolates of enterococci, we found changes in genes encoding the LiaFSR system and Cls in three additional clinical isolates of daptomycin-resistant enterococci. In four other daptomycin-resistant clinical isolates, only one of these genes appeared to be altered, suggesting that additional loci involved in cell-wall homeostasis or phospholipid metabolism may be important in these enterococcal isolates. Indeed, unlike the *S. aureus* cell membrane, the enterococcal cell membrane has several amino acid–containing phospholipids, apart from lysyl-phosphatidylglycerol (including arginyl-phosphatidylglycerol and alanyl-phosphatidylglycerol[33]). In addition, there are other two-component systems present in enterococci that can potentially modulate the response to the antimicrobial challenge.

It is also of interest that the amino acid changes in the LiaF and GdpD proteins of the R712 isolate occurred in a region that harbors repeats of Ile; this suggests that these in-frame changes may have originated from recombination between adjacent repetitive nucleotide sequences. Mutations that occur by means of this mechanism were observed to alter the function of LiaF in *B. subtilis*[8] and the histidine kinase $VanS_B$ involved in *E. faecium* resistance to vancomycin,[34] and these mutations suggest the presence of an underlying genetic mechanism for the development of resistance to daptomycin in *E. faecalis*.

In summary, our data indicate that the emergence of resistance to daptomycin is the result of concomitant alterations in genes (*liaF* and *gdpD*) encoding proteins that are probably involved in regulating the stress response to antimicrobial agents acting on the cell envelope and enzymes that are responsible for phospholipid metabolism in the cell membrane.

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