

Whole-Genome Analyses of *Enterococcus faecium* Isolates with Diverse Daptomycin MICs

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Daptomycin (DAP) is a lipopeptide antibiotic frequently used as a “last-resort” antibiotic against vancomycin-resistant *Enterococcus faecium* (VRE). However, an important limitation for DAP therapy against VRE is the emergence of resistance during therapy. Mutations in regulatory systems involved in cell envelope homeostasis are postulated to be important mediators of DAP resistance in *E. faecium*. Thus, in order to gain insights into the genetic bases of DAP resistance in *E. faecium*, we investigated the presence of changes in 43 predicted proteins previously associated with DAP resistance in enterococci and staphylococci using the genomes of 19 *E. faecium* with different DAP MICs (range, 3 to 48 $\mu\text{g/ml}$). Bodipy-DAP (BDP-DAP) binding to the cell membrane assays and time-kill curves (DAP alone and with ampicillin) were performed. Genetic changes involving two major pathways were identified: (i) LiaFSR, a regulatory system associated with the cell envelope stress response, and (ii) YycFGHIJ, a system involved in the regulation of cell wall homeostasis. Thr120→Ala and Trp73→Cys substitutions in LiaS and LiaR, respectively, were the most common changes identified. DAP bactericidal activity was abolished in the presence of *liaFSR* or *yycFGHIJ* mutations regardless of the DAP MIC and was restored in the presence of ampicillin, but only in representatives of the LiaFSR pathway. Reduced binding of BDP-DAP to the cell surface was the predominant finding correlating with resistance in isolates with DAP MICs above the susceptibility breakpoint. Our findings suggest that genotypic information may be crucial to predict response to DAP plus β -lactam combinations and continue to question the DAP breakpoint of 4 $\mu\text{g/ml}$.

The surge of *Enterococcus faecium* as an important nosocomial pathogen has been associated with an expanding pandemic caused by a hospital-associated (HA) genetic clade (1, 2). Indeed, isolates belonging to this genetic lineage are frequently multidrug resistant (MDR) with high MICs of ampicillin and vancomycin (3). Daptomycin (DAP) is a cell membrane (CM)-targeting lipopeptide that has *in vitro* bactericidal activity against MDR *E. faecium* and, due to the paucity of other bactericidal options, is often used as first-line therapy despite lacking U.S. Food and Drug Administration approval for these organisms. However, one of the major problems when using DAP against enterococci is the emergence of resistance during therapy (4–6).

The mechanisms of DAP resistance in enterococci are not fully understood, but recent evidence suggests that there are several genetic pathways involved and that resistance results from a sequential and ordered mutational pathway (7–9). In *Enterococcus faecalis*, we have previously shown that emergence of resistance during therapy involves substitutions in three proteins: (i) LiaF, a member of the three-component regulatory system LiaFSR which, in *Bacillus subtilis* and other Gram-positive bacteria (10), has been shown to orchestrate the cell envelope response to stress; (ii) GdpD, a glycerophosphoryl-diester phosphodiesterase, involved in phospholipid metabolism; and (iii) Cls, a cardiolipin synthase (11). The mechanism of DAP resistance in Gram-positive bacteria was initially postulated to involve repulsion of the calcium-decorated DAP from the cell surface (12). However, we recently provided evidence that DAP resistance in an *E. faecalis* strain was due to diversion of DAP from the division septum associated with redistribution of CM cardiolipin (CL) microdomains, without repulsion of DAP from the cell surface (8). Of note, deletion of Ile at

position 177 of LiaF was sufficient for membrane remodeling and also abolished DAP *in vitro* bactericidal activity (8, 13).

Genomic analyses in *E. faecium*, using clinical-strain pairs of DAP-susceptible and DAP-resistant isolates recovered during therapy, have identified many genetic changes associated with DAP resistance (14, 15), but the specific role of each of these mutations remains to be established. Interestingly, in a collection of clinical *E. faecium* isolates recovered from bacteremia, ca. 75% of DAP-“susceptible” isolates with MICs close to the established breakpoint (4 $\mu\text{g/ml}$) harbored changes in LiaFSR (16). Furthermore, recent data suggest that the combination of DAP with certain β -lactams (e.g., ampicillin and ceftaroline) restores DAP activity *in vitro* and *in vivo* by increasing the ability of the antibiotic to bind to its CM target (17–20, 41), but the mechanism or genetic basis for such synergism is unknown. Therefore, in an attempt to dissect the genetic determinants implicated in DAP resistance in

Received 6 March 2014 Returned for modification 18 April 2014

Accepted 19 May 2014

Published ahead of print 27 May 2014

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02686-14>.

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doi:10.1128/AAC.02686-14

TABLE 1 *E. faecium* strains included in this study^a

Strain	MIC ($\mu\text{g/ml}$) ^b				<i>van</i> gene	MLST	BioProject accession numbers	Source or reference
	VAN	TEI	DAP	AMP				
503	>256	32	3	>64	<i>vanA</i>	280	PRJNA181868	This study
504	1	0.12	4	>64		649	PRJNA181867	This study
505	1	0.12	6	>64		SLV ^c ST39	PRJNA181866	This study
506	>256	64	6	>64	<i>vanA</i>	18	PRJNA181865	This study
509	256	0.25	4	>64	<i>vanB</i>	17	PRJNA181864	This study
510	1	0.12	4	>64		18	PRJNA181863	This study
511	1	0.25	8	>64		17	PRJNA181862	This study
513	1	0.25	4	>64		736	PRJNA181861	This study
514	1	0.25	8	>64		17	PRJNA181860	This study
515	0.5	0.5	3	>64		549	PRJNA181859	This study
S447	256	16	3	>64	<i>vanA</i>	203	PRJNA181832	11, 15
R446	16	4	16	>64	<i>vanA</i>	203	PRJNA181838	11, 15
R501	256	8	32	>64	<i>vanA</i>	17	PRJNA181833	11
R494	1	0.12	48	>64		664	PRJNA181837	11
R496	1	0.25	32	>64		412	PRJNA181836	11
R497	1	0.25	16	>64		752	PRJNA181835	11
R499	256	8	48	>64	<i>vanA</i>	412	PRJNA181834	11
V689	256	64	4	>64	<i>vanA</i>	736	PRJNA181831	21
P1190	256	128	3	64	<i>vanA</i>	125	PRJNA181840	21

^a All strains (except S447 and R446, which were isolated from the same patient) had no epidemiological relationship between them and were recovered at different time points and geographical areas in the United States. Isolates V689 and P1190 were recovered in Venezuela and Peru, respectively, before DAP was introduced in these countries. The pulsed-field gel electrophoresis patterns of all strains were different and categorized as unrelated (data not shown).

^b VAN, vancomycin; TEI, teicoplanin; DAP, daptomycin; AMP, ampicillin.

^c SLV, single locus variant.

E. faecium and the interaction with β -lactams, we used whole-genome sequencing of a collection of 19 unrelated clinical isolates of *E. faecium* with a diverse range of DAP MICs and investigated the presence of genetic changes in 43 predicted proteins previously associated with DAP resistance. Note that, although the accepted term is “DAP nonsusceptibility,” we use the term “DAP resistance” throughout for ease of presentation.

(Parts of the results of this study were presented at the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy, September 10 to 13, 2013, Denver, Colorado, USA.)

MATERIALS AND METHODS

Bacterial isolates, molecular typing, and MIC determinations. A total of 19 clinical strains of *E. faecium* were included in the present study (DAP MICs, 3 to 48 $\mu\text{g/ml}$) (Table 1). We included 17 U.S. isolates from different patients and submitted to reference laboratories from diverse geographical areas. In addition, there were two *E. faecium* strains recovered in South America (21) before the introduction of DAP to the region (Table 1). Typing of isolates was performed using *in silico* multilocus sequence typing (MLST) analysis derived from the genomic sequence. Determination of DAP MICs was performed by Etest (bioMérieux, Marcy l’Etoile, France) on Mueller–Hinton agar according to the manufacturer’s instructions. Etest was used for DAP since our goal was to detect small variations in DAP MICs. The MICs for each strain were determined in triplicate. Two independent observers read the results, and a third investigator was consulted whenever a disagreement was identified. MICs of other antibiotics were determined by an agar dilution method (22).

Genome sequencing and mutational analysis. Whole-genome analysis, genomic assemblies, and annotation were performed as described previously (15). A total of 43 predicted proteins (Fig. 1; see also Table S1 in the supplemental material) were included in the analysis representing genes previously associated with DAP resistance in enterococci (11, 14, 15, 23) and *E. faecium* homologues of six genes associated with DAP resistance in *S. aureus* (*mprF*, *pgsA*, and the *dlt* cluster; see Tables S2 and S3 in

the supplemental material) (24–27). A relevant change was defined as a nucleotide change that resulted in an amino acid substitution that was not present at the same position on the predicted protein sequences of other *E. faecium* genomes publicly available (independent of the DAP MIC). Sequence comparisons were performed using the multiple sequence alignment program ClustalW2 and *E. faecium* DO (TX16), a DAP-susceptible (MIC = 2 $\mu\text{g/ml}$) clinical strain (whose genome is sequenced and closed) (28, 29), as the template to refine comparisons. All accession numbers are shown in Table 1.

Time-kill assays and evaluation of synergism between ampicillin (AMP) and DAP. To assess the bactericidal activity of DAP against *E. faecium*, we used time-kill assays with two representative isolates of each of the two most common genetic pathways identified displaying/or with DAP MICs below and above the susceptibility breakpoint (4 $\mu\text{g/ml}$), respectively, as follows: (i) 503 and R497 (LiaFSR pathway representatives) with MICs of 3 and 16 $\mu\text{g/ml}$, respectively (11), and (ii) 515 and R446 (15) (YycFGHIJ pathway) with MICs of 3 and 16 $\mu\text{g/ml}$, respectively. *E. faecium* DO (MIC 2 $\mu\text{g/ml}$) was used as a control for these experiments. Time-kill assays were performed with an initial bacterial inoculum of 10^7 CFU/ml in Mueller–Hinton broth (MHB) supplemented with calcium (50 mg/liter). DAP was added at concentrations of $5\times$ the MIC of each strain, and bacterial counts were performed at 0, 6, and 24 h. Antibiotic carryover was controlled by centrifugation (bacterial cell suspensions [1-ml samples] were centrifuged, and the pelleted bacteria were suspended in the same volume of 0.9% saline solution before plating) as described earlier (13, 30, 31). Bactericidal activity was defined as a ≥ 3 \log_{10} reduction in CFU/ml at 24 h in comparison to the initial inoculum. The limit of detection was 200 CFU/ml, assuming maximum plating efficiency. We also tested the ability of AMP (64 $\mu\text{g/ml}$) to achieve synergistic activity when combined with DAP against the same strains. Synergism was defined as a decrease of ≥ 2 \log_{10} CFU/ml in bacterial counts at 24 h compared to the most active single agent alone. All assays were performed in triplicate.

BODIPY-labeled daptomycin (BDP-DAP) assays. We used BDP-DAP to assess the interaction of DAP with the bacterial CM, as described

Strain	DAP MIC (µg/ml)	Protein																																				
		LiaFSR	Cls	GdpD	Cfa	PTS EIIA	YycFGHJ	HD domain protein	SulP	XpaC	RrmA	Beta Fructuronidase	PTS IIA	Bacteriophage minor structural protein	PspC	LepB	Hypothetical Protein	Aad	NrdR	PTS IID	EzrA	Alfa Manosidase	Outer surface protein	Hypothetical protein	Hypothetical protein	RpeS-5S	BrnQ	RpoN	Hypothetical protein	Hypothetical protein	Hypothetical protein	TelA	MprF	PgsA	DiABCDC			
S447	3																																					
503	3	■																																				
515	3																																					
P1190	3	■																																				
504	4	■																																				
509	4																																					
510	4																																					
513	4	■																																				
V689	4	■																																				
505	6	■																																				
506	6	■																																				
511	8																																					
514	8																																					
R497	16	■																																				
R446	16																																					
R496	32	■																																				
R501	32																																					
R499	48																																					
R494	48	■																																				
Total of strains with changes		10	7	1	2	7	5	1	1	3	0	0	2	1	1	0	2	1	1	1	2	0	2	2	0	1	3	1	0	0	3	2	0	1				

FIG 1 Amino acid changes in 43 predicted proteins associated with DAP resistance in *E. faecium* isolates. Black squares indicate the presence of amino acid changes. Cls, cardiolipin synthase; GdpD, glycerophosphodiester phosphodiesterase; Cfa, cyclopropane-fatty-acyl-phospholipid synthase; PTS-EIIA, phosphotransferase system, phosphoenolpyruvate-dependent sugar EIIA 2; SulP, sulfate transporter; XpaC, 5-bromo-4-chloroindolyl phosphate hydrolysis protein; RrmA, rRNA (guanine-N1-)-methyltransferase A; PTS-IIA, PTS system fructose IIA component; PspC, phage shock protein C; LepB, signal peptidase I; Aad, aldehyde-alcohol dehydrogenase; NrdR, nicotinamide mononucleotide transporter; PTS IID, mannose/fructose/sorbose transporter subunit IID; EzrA, septation ring formation regulator; RpeS-5S, ribosomal protein S5; BrnQ, branched-chain amino acid transport protein; RpoN, RNA polymerase sigma factor 54; TelA, telurite resistance protein; MprF, lysylphosphatidylglycerol synthetase; PgsA, CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase.

before (8). The assays were performed using isolates 503, R497, R446, and 515 (representatives of the most common mutational pathways [see above]). The protocol for BDP-DAP staining followed techniques previously described (7, 8, 32, 33). Briefly, isolates were grown in Luria-Bertani (LB) broth at 37°C and incubated with BDP-DAP at two concentrations (4 and 64 µg/ml in LB broth supplemented with Ca²⁺ at 50 mg/liter) for 10 min in darkness. Fluorescence was assessed using a standard fluorescein isothiocyanate filter set (excitation at 490 nm and emission at 528 nm). A minimum of two independent experiments was performed for each strain on different days. In order to estimate the amount of BDP-DAP bound to *E. faecium* strains, the fluorescence intensity was quantitated and normalized to protein concentration of the samples, as described previously (8).

RESULTS

Changes in genes involved in cell envelope homeostasis. We sought to identify substitutions in 43 predicted proteins previously associated with DAP resistance in enterococci and staphylococci within unrelated *E. faecium* isolates exhibiting a diverse range of MICs (3 to 48 µg/ml). The “control” strain for our genomic comparisons was *E. faecium* DO (28, 29), a DAP-susceptible clinical isolate (DAP MIC 2 µg/ml) whose genome has been

sequenced and closed. The changes are shown in Fig. 1 and are summarized according to their frequency in Table S3 in the supplemental material. The most common genes affected were those encoding regulatory systems involved in cell envelope homeostasis, *liaFSR* and/or *ycyFG* (including the accessory genes *ycyHII*). Indeed, 10 isolates with DAP MICs ≥ 3 µg/ml harbored changes in *LiaFSR*, with the most common substitutions found in *LiaS* (the putative histidine kinase of the system [*n* = 9]), followed by the response regulator *LiaR* (*n* = 7) (Fig. 1; see also Table S3 in the supplemental material). A T120A substitution was often identified in *LiaS* (*n* = 7), and W73C was found in all isolates (*n* = 7) with changes in *LiaR*. Moreover, *LiaS*^{T120A} was always present in isolates harboring *LiaR*^{W73C}, suggesting that these substitutions might have coevolved. Of note, mutations in *liaF*, which was previously associated with DAP resistance in *E. faecalis*, were found in four isolates, and each predicted change was unique (Fig. 1; see Table S3 in the supplemental material). Three of these isolates (DAP MICs of 4, 6, and 48 µg/ml) also harbored changes in *LiaSR*, and the remaining isolate (MIC of 32 µg/ml) harbored an I142T substitution in *LiaF* without concomitant changes in *LiaRS*. Overall, alterations in *LiaFSR* were identified in 50% (5 of 10 isolates)

with MICs between 3 and 4 $\mu\text{g/ml}$ and in 50% (5 of 10) with MICs $> 4 \mu\text{g/ml}$ (Fig. 1; see Table S3 in the supplemental material). Among seven isolates harboring mutations in the YycFGHIJ system, the most commonly involved proteins were YycH, a putative signal transduction protein ($n = 3$), followed by YycG (sensor histidine kinase of the system [$n = 2$]) and YycI, a putative regulatory protein ($n = 2$) (15, 34, 35). Three isolates harbored mutations in both LiaFSR and YycFGHIJ systems concomitantly, suggesting that these two systems may interact to develop DAP resistance in some isolates.

Substitutions in phospholipid metabolism enzymes. We had previously postulated that changes in enzymes involved in phospholipid metabolism were likely to appear at latest stages of the DAP resistance pathway in *E. faecalis* (8, 9). Interestingly, after genes predicted to function in cell envelope homeostasis, the most common gene affected was *cls* ($n = 7$), encoding a CL synthase involved in the last committed step of synthesis of CL from the precursor phosphatidylglycerol. The predicted amino acid changes were located in the phospholipase D domains (PLD1 and PLD2) and in the linker region joining the two putative transmembrane helices, as previously described (36). Of note, Cls substitutions were found mostly in isolates with high DAP MIC ($>4 \mu\text{g/ml}$ [$n = 6$]) and only in one isolate with an MIC of 4 $\mu\text{g/ml}$ (Fig. 1; see Table S3 in the supplemental material). Moreover, the changes in Cls were always observed in isolates that also had changes in one of the above-mentioned regulatory systems (LiaFSR or YycFG), supporting our previous hypothesis that Cls substitutions follow initial changes in cell envelope homeostasis and “enhance” the resistance phenotype (9). Other less frequent changes found in enzymes involved in phospholipid metabolism were (i) in the homolog of MprF (a lysylphosphatidylglycerol synthetase [$n = 2$]) (25, 37); (ii) Cfa, a putative cyclopropane fatty acid synthase ($n = 2$) (38); and (iii) GdpD a glycerophosphoryl-diester phosphodiesterase ($n = 2$) (11) (Fig. 1; see Table S3 in the supplemental material).

Other, less frequent mutations. A total of five isolates with MICs between 4 and 48 $\mu\text{g/ml}$ exhibited amino acid changes in a protein of unknown function that harbors an HD domain, which designates a superfamily of enzymes that possess phosphohydrolase activity and may be involved in nucleic acid metabolism and signal transduction (39). Four strains (MICs = 4 and 32 $\mu\text{g/ml}$) showed changes in the 23S rRNA methyltransferase, RrmA (40). In addition, three isolates exhibited substitutions in TelA, a putative tellurite resistance protein (Fig. 1; see also Table S3 in the supplemental material), previously associated with DAP resistance in *E. faecalis* (23). Changes in TelA, RrmA, and in the HD domain protein were always identified in conjunction with substitutions in LiaFSR or YycFGHIJ. Other genes altered less commonly are shown in Fig. 1 and in Table S3 in the supplemental material. Of note, we were unable to identify any changes in predicted proteins associated with DAP resistance in two DAP-resistant isolates (both with MICs of 8 $\mu\text{g/ml}$) (Fig. 1), indicating that additional genetic pathways leading to DAP resistance in *E. faecium* remain to be identified.

Mutational pathways influence *in vitro* bactericidal activity of DAP. Our previous work (8, 9, 11, 13, 15, 16) and the current genomic analysis indicate that LiaFSR and/or YycFGHIJ are the two most common genetic pathways resulting in DAP resistance. Our previous studies in *E. faecalis* indicated that a single *liaF* mutation abolished the *in vitro* bactericidal activity of DAP (DAP-

tolerant phenotype) (13) but did not increase the MIC above the breakpoint. Thus, we examined the bactericidal activity of DAP against *E. faecium* 503 (Table 1), a DAP-susceptible isolate (MIC 3 $\mu\text{g/ml}$) that harbors LiaR^{W73C} and LiaS^{T120A}. Figure 2A shows that DAP (5 \times the MIC) lacked bactericidal activity against *E. faecium* 503 with reductions of $<1 \log_{10}$ CFU/ml at 24 h. A similar effect was observed against *E. faecium* R497 (Fig. 2B), a DAP-resistant (16 $\mu\text{g/ml}$) isolate also carrying LiaR^{W73C} and LiaS^{T120A} but no changes in YycFGHIJ. Interestingly, for both 503 and R497, DAP bactericidal activity was restored by adding AMP (64 $\mu\text{g/ml}$), an observation consistent with previous reports (18, 41). Next, we sought to test the *in vitro* bactericidal activity of DAP in two representative isolates altered in the YycFG pathway (515 and R446) with MICs of 3 and 16 $\mu\text{g/ml}$, respectively. As expected, DAP did not have any killing effect against R446 (Fig. 2C) but also lacked bactericidal activity against 515 which has an MIC within the susceptible range (Fig. 2D). However, in contrast to representatives with LiaFSR changes, addition of AMP (64 $\mu\text{g/ml}$) to DAP had no synergistic effect, suggesting that an altered YycFGHIJ system is not affected by the combination of DAP and β -lactams. For the DAP-susceptible strain TX16/DO, both DAP and AMP exhibited bactericidal activity (Fig. 2E).

Lack of DAP binding to the cell surface is the predominant mechanism of DAP resistance in *E. faecium*. Two main mechanisms of DAP resistance have been postulated in enterococci: (i) electrostatic “repulsion” of calcium-decorated DAP (positively charged) from the cell surface due to a more positively charged cell envelope (23) and (ii) “diversion” of DAP from the division septum (the main DAP cell target) (only described in *E. faecalis*) (8). In order to gain insights into the mechanism of DAP resistance and the genetic background, we used fluorescent BDP-DAP to study the interactions of the antibiotic with the CM in representative strains of the LiaFSR or YycFGHIJ pathways, as described earlier (8). We used DAP-susceptible *E. faecium* DO (MIC = 2 $\mu\text{g/ml}$) as the control and performed the assays with two BDP-DAP concentrations (4 and 64 $\mu\text{g/ml}$) since DAP binding to the cell membrane target is concentration dependent (7, 8). Figure 3 shows that binding of BDP-DAP was significantly decreased with the DAP-resistant strains R497 (LiaFSR pathway) and R446 (YycFGHIJ pathway) at low and high concentrations compared to the control (*E. faecium* DO), suggesting that antibiotic “repulsion” may play a prominent role in resistance. In contrast, the pattern of BDP-DAP binding at low concentrations was similar to that of DO for DAP-susceptible (MIC = 3 $\mu\text{g/ml}$) 503 (with LiaFSR changes) and 515 (altered YycFGHIJ) (Fig. 3). However, at high BDP-DAP concentrations (64 $\mu\text{g/ml}$), 515 had significantly less binding to the cell membrane than DO, whereas no statistically significant difference in BDP-DAP binding was observed between 503 (LiaFSR pathway) versus DO. Our results suggest that diversion (rather than repulsion) may be the mechanism for decreased DAP killing with this isolate; however, further analyses are required to corroborate this hypothesis.

DISCUSSION

Using genomic analyses of clinical isolates with a wide variety of DAP MICs, we investigated the genetic basis of DAP resistance in *E. faecium*. Our findings suggest that two regulatory systems are likely to be involved in development of DAP resistance in *E. faecium*: (i) LiaFSR, which has been associated with the cell envelope response to cell wall acting antibiotics and antimicrobial peptides

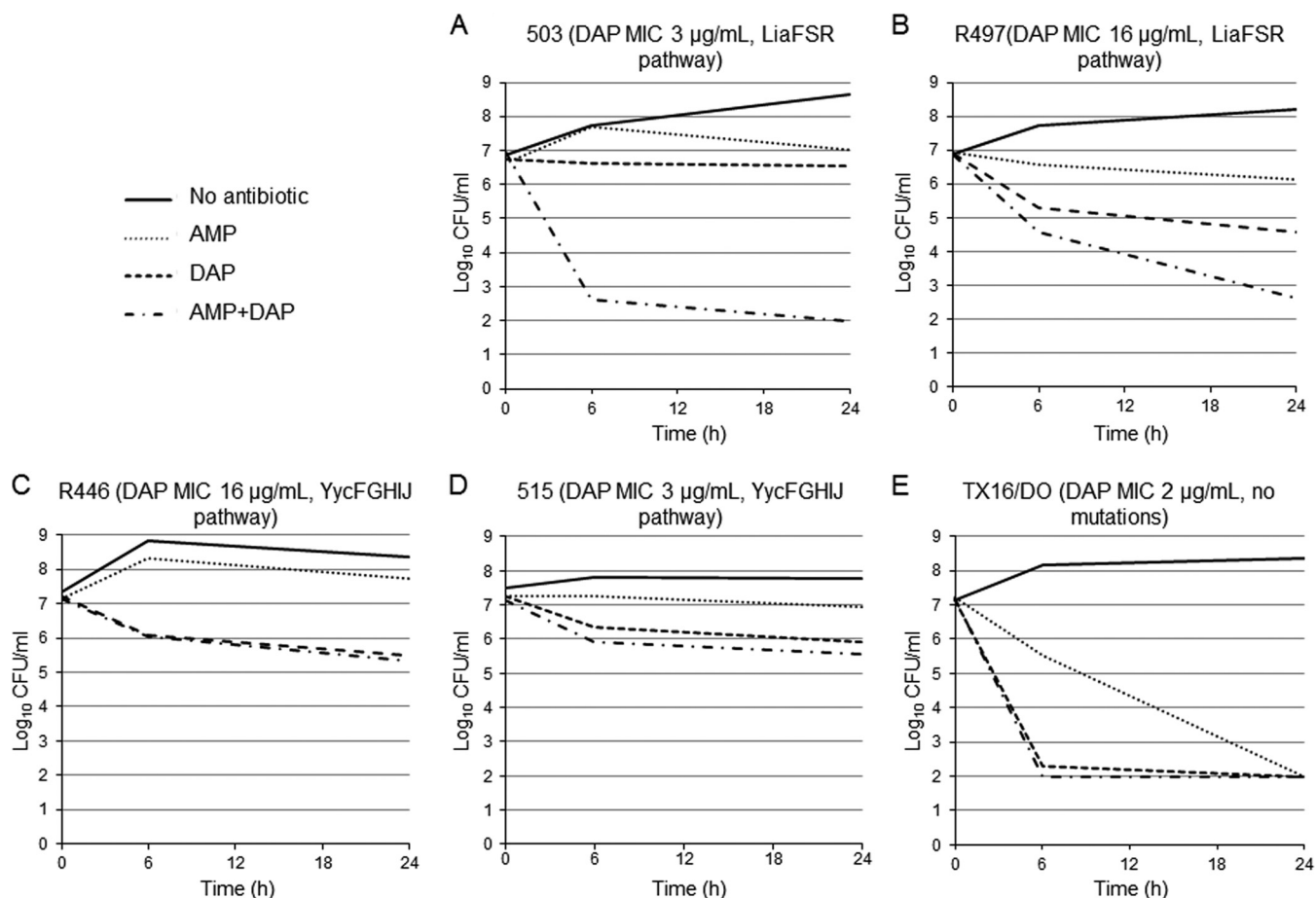


FIG 2 Time-kill assays with DAP (5 \times the MIC) with or without AMP (64 $\mu\text{g/ml}$). Bacteria were grown in Mueller-Hinton broth (MHB) supplemented with calcium (50 mg/liter). The *E. faecium* strains are indicated at the top of each panel. AMP, ampicillin; DAP, daptomycin. The limit of detection was 200 CFU/ml.

(10, 42), and (ii) YycFGHIJ, an essential two-component regulatory system characterized in several Gram-positive organisms (including *E. faecalis*) and shown to be involved in cell wall homeostasis and cell division (34, 43, 44, 47).

Our previous work indicated that development of DAP resistance in *E. faecalis* is a stepwise and ordered process (9, 11, 13). One distinct pathway involves initial mutations occurring in genes encoding the three-component regulatory system LiaFSR followed by changes in genes encoding enzymes involved in phospholipid metabolism (such as CL synthase) (9). Furthermore, we previously found that a single mutation in *liaF* of *E. faecalis* was sufficient to produce DAP tolerance and that substitutions in LiaRS were commonly found in *E. faecium* bloodstream isolates with MICs of ≥ 3 $\mu\text{g/ml}$ but absent in isolates with MICs of ≤ 2 $\mu\text{g/ml}$ (16). In the present study, we expand these observations and provide several additional lines of evidence to support the pivotal role of the LiaFSR three-component regulatory system in the pathway leading to DAP resistance in *E. faecium*. First, two substitutions (LiaR^{W73C} and LiaS^{T120A}) were commonly found in unrelated clinical isolates of *E. faecium* with DAP MICs of ≥ 3 $\mu\text{g/ml}$. Moreover, identical changes in these two predicted proteins were found in two isolates (V689 and P1190) with DAP MICs of 3 $\mu\text{g/ml}$ which were recovered in countries where DAP had not been introduced in clinical practice, suggesting that these muta-

tions can be selected even without DAP exposure, as previously reported (45). Second, the presence of the same LiaRS substitutions was associated with the DAP-tolerant phenotype, as previously shown for *E. faecalis*. Indeed, *E. faecium* 503, which only harbors LiaR^{W73C} and LiaS^{T120A} and no other changes in predicted proteins associated with DAP resistance, behaved as a DAP-resistant isolate in time-kill assays, despite of the fact that its DAP MIC is within the "susceptible" range. Our findings continue to question the stated CLSI DAP breakpoint of 4 $\mu\text{g/ml}$ and suggest that 2 $\mu\text{g/ml}$ would likely be a better cutoff value if considering DAP for *E. faecium* causing deep-seated infections.

Our genomic studies also suggest that a second pathway for DAP resistance involves changes in the YycFGHIJ system, as previously observed (15). After LiaFSR, substitutions in this system were the second most common changes observed in *E. faecium* with an MIC of ≥ 3 $\mu\text{g/ml}$. YycFGHIJ has been implicated in development of DAP nonsusceptibility in *E. faecium* and also in vancomycin and DAP nonsusceptibility in *Staphylococcus aureus* (15, 26, 46). Therefore, it is tempting to speculate that YycFGHIJ is another important regulatory network that contributes to the response to the cell membrane attack by DAP (and possibly other cell membrane-acting antimicrobials) and may be more relevant in isolates whose DAP MICs are above the breakpoint. Although our findings support a role of the YycFGHIJ in DAP nonsuscep-

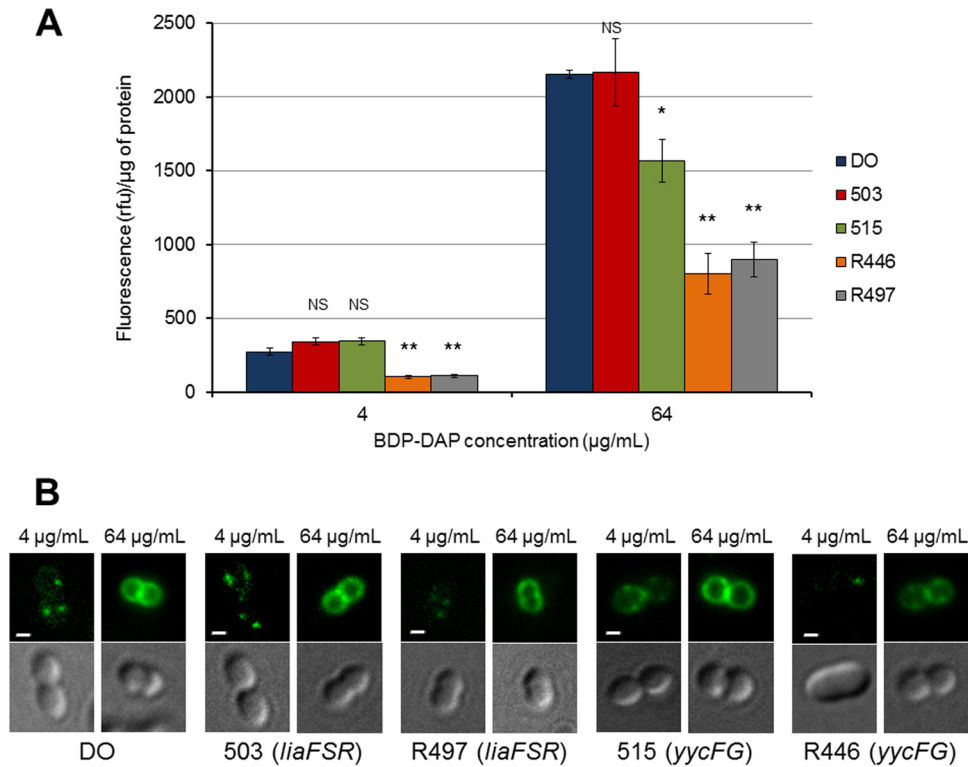


FIG 3 BODIPY-labeled DAP (BDP-DAP) staining of *E. faecium* strains. (A) Fluorescence intensities of representative *E. faecium* strains. Cells were treated with BDP-DAP and fluorescence was normalized to cell protein content. Intensities were compared to *E. faecium* DO. Rfu, relative fluorescence units; *, $P < 0.05$; **, $P < 0.001$; NS, not significant. (B) BDP-DAP staining of representative *E. faecium* cells at concentrations of 4 $\mu\text{g/ml}$ and 64 $\mu\text{g/ml}$. The top images capture bacterial cells under fluorescence microscopy (bars, 1 μm). The bottom images are the same bacterial cell in phase contrast. The mutated pathway is indicated in parentheses.

tibility, direct evidence of such contribution is still lacking and is the object of our future studies. Further evidence that the LiaFSR and YycFGHII systems may lead to distinct pathways in the development of DAP resistance comes from our time-kill curve assays with selected isolates representing each pathway. Recent data suggest that the addition of AMP to DAP may restore the bactericidal activity of DAP by mechanisms that are unclear (18, 41). Here, we show that the synergistic effect of the AMP plus DAP combination was seen only in isolates representing the LiaFSR pathway (503 and 497) regardless of the MIC, but it was absent with R446 or 515 (DAP-resistant and -susceptible, representing the YycFGHII pathway). These findings suggest that the synergistic effect is dependent on the genetic pathway and that it is not a universal phenomenon seen in all DAP-nonsusceptible *E. faecium*, a finding with important therapeutic implications.

Our data using the fluorescent derivative BDP-DAP also provide evidence that in DAP-nonsusceptible isolates (MICs above the *E. faecalis* breakpoint), reduced binding of the antibiotic molecule from the surface appears to be the main mediator of resistance regardless of the mutational pathway. However, specific genetic changes also seem to affect the interaction of DAP with the cell membrane in isolates with MICs below the breakpoint that exhibit DAP tolerance. Indeed, in *E. faecium* 503 (DAP MIC = 3 $\mu\text{g/ml}$) binding of BDP-DAP did not differ from that of DO (control) even at high antibiotic concentrations, similar to what has been shown previously in *E. faecalis* (8). Thus, it is tempting to speculate that in *E. faecium* 503, LiaFSR-mediated “diversion” of

DAP from lethal target sites (i.e., septum) is pivotal for the DAP tolerance phenotype. It is important to note that changes in these systems may not be mutually exclusive since two strains with mutations in both YycFGHII and LiaFSR were identified, adding complexity to the genetic changes associated with DAP resistance.

Finally, our findings also support our previous hypothesis that changes in phospholipid enzymes are common among DAP-resistant *E. faecium* and are likely to be associated with later stages of development of DAP resistance. In *E. faecium*, changes in CL synthase seem to be the prevailing “route” in isolates with high DAP MIC although changes in this enzyme were not found in all isolates. We had previously shown that mutations in the predicted CLs active site appear to increase the catalytic activity of the enzyme (36), which may be sufficient to optimize the DAP resistance phenotype. Although changes in many other predicted proteins were found, we speculate that these changes may play a less important role in the mutational sequence and may be minor contributors since they were found in small number of isolates. The role of these proteins in DAP resistance remains to be elucidated.

In summary, we provide genomic evidence that two major pathways (LiaFSR and YycFGHII) appear to be the most important changes associated with development of DAP resistance in *E. faecium* clinical isolates. Although the sequence of mutations appear to be complex, initial changes in regulatory systems that control the cell envelope response to stress may be of paramount importance to fully develop DAP resistance. Our findings also open the possibility of using genotypic information to identify

isolates that are likely to fail DAP therapy *in vivo* and those in which the combination of DAP plus β -lactams may be effective.

ACKNOWLEDGMENTS

We thank Silvia Munoz-Price, James H. Jorgensen, Helio Sader, Ronald Jones, Chris Pillar, Daniel Sahm, Manuel Guzman, and Carlos Carrillo for providing the enterococcal isolates. We also thank Isabel Reyes for technical support and Jared A. Silverman and Aileen Rubio for providing BODIPY FL-labeled daptomycin.

Support for this study was provided by the Instituto Colombiano para el Desarrollo de la Ciencia y Tecnología, Francisco José de Caldas, COLCIENCIAS (graduate scholarship to L.D.), the American Society of Microbiology (Latin American Fellowship for Epidemiology to L.D.), and the Universidad El Bosque (graduate fellowships to L.D. and J.R.). C.A.A. was supported by National Institutes of Health (NIH-NIAID) grant R01 AI093749. Y.S. was supported by NIH-NIAID grant R01 AI080714, and B.E.M. was supported by NIH-NIAID grant R01 AI047923. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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