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Genotypic and Phenotypic Evaluation of the Evolution of High-Level Daptomycin Nonsusceptibility in Vancomycin-Resistant *Enterococcus faecium*

Romney M. Humphries,[a] Theodoros Kelesidis,[b] Ryan Tewhey,[c] Warren E. Rose,[d] Nicholas Schork,[c,e] Victor Nizet,[f] and George Sakoulas[f]

Department of Pathology & Laboratory Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, USA[a]; Department of Medicine, Division of Infectious Diseases, David Geffen School of Medicine at UCLA, Los Angeles, California, USA[b]; Scripps Genomic Medicine, Scripps Translational Science Institute, La Jolla, California, USA[c]; University of Wisconsin—Madison School of Pharmacy, Madison, Wisconsin, USA[d]; Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA[e]; and Division of Pediatric Pharmacology & Drug Discovery, University of California San Diego School of Medicine, La Jolla, California, USA[f]

Whole-genome sequencing and cell membrane studies of three clonal *Enterococcus faecium* strains with daptomycin MICs of 4, 32, and 192 μg/ml were performed, revealing nonsynonymous single nucleotide variants in eight open reading frames, including those predicted to encode a phosphoenolpyruvate-dependent, mannose-specific phosphotransferase system, cardiolipin synthetase, and EzrA. Membrane studies revealed a higher net surface charge among the daptomycin-nonsusceptible isolates and increased septum formation in the isolate with a daptomycin MIC of 192 μg/ml.

Daptomycin-nonsusceptible (NS) *Enterococcus faecium* strains are emerging yet remain highly uncharacterized (11–13). This study reports whole-genome sequencing (WGS), cell surface charge, membrane fluidity, and cell wall thickness data for a daptomycin-susceptible strain and two isogenic daptomycin-NS *E. faecium* strains.

The isolates studied here are listed in Table 1. Daptomycin MICs were determined by broth microdilution (6) at the time of isolation from the blood of a patient with recurrent vancomycin-resistant *E. faecium* bacteremia secondary to decubitus ulcers and a colonic fistula. Isolate 5938 (daptomycin MIC, 192 μg/ml) was isolated from the patient following 26 days of daptomycin treatment (6 mg/kg every 48 h). Four and 8 months thereafter, *E. faecium* isolates 8019 and 5994 (NS; daptomycin MIC, 32 μg/ml) were recovered from blood cultures, respectively. These isolates were confirmed as >99% identical to isolate 5938 by repetitive-element PCR analysis as described elsewhere (11).

WGS was performed with an Illumina HiSeq 2000 sequencer by using standard protocols (8). Assemblies were produced with Edena v3 (9) and consisted of 138 to 155 contigs with an N50 of 54.7 to 58.4 kb and a total assembly size of 2.97 to 3.02 Mb. Read alignment and variant calling were performed with Stampy/Burrows-Wheeler Aligner (BWA) and SAMtools using the *de novo* assembly of isolate 8019 as a reference (16–18). Larger insertion and deletion events were evaluated by mapping Edena contigs to each other using BLAT (14). Gene calling was performed by rapid annotation using subset technology servers (RAST) (3), and mutational effects were assessed by custom Perl scripts. Mutations identified between 8019 and 5938 or 5994 were confirmed by amplification and Sanger sequencing on an ABI 3130xl using standard protocols.

WGS revealed 19 single nucleotide variants (SNVs) and four insertion/deletion events among the three *E. faecium* strains. SNVs included 6 synonymous substitutions (not shown), 11 nonsynonymous substitutions, and 1 nonsense substitution in coding regions of the genome and three SNVs in noncoding regions of the genome (Table 2). Consistent with prior data (2, 19), SNVs were identified at distinct loci in the cardiolipin synthetase gene of both daptomycin-NS isolates (Table 2).

Both strains 5938 and 5994 harbored the nonconserved substitution L238F in the membrane-bound IID domain of the mannose-specific phosphotransferase system (PTS). PTS mutation has been associated with resistance to class IIa bacteriocins (7, 15), which are cationic antimicrobial peptides produced by Gram-positive bacteria. Interaction between the bacteriocins and their target cells is both electrostatic and receptor mediated, wherein the IIC and IID domains of the PTS act as receptors (7). Although mutation of PTS has not been described in daptomycin-NS *E. faecalis* (2, 19), the PTSs of *E. faecalis* and *E. faecium* are phylogenetically distinct (5). Thus, it is possible that both altered cell surface charge and modification of PTS as a putative receptor may mediate daptomycin nonsusceptibility in *E. faecium*, although this concept remains to be confirmed by mutagenesis studies.

Four additional nonsynonymous SNVs were identified in both strains 5938 and 5994, in open reading frames (ORFs) predicted to encode alpha-mannosidase, alcohol dehydrogenase, EzrA, and a hypothetical protein (Table 2). In addition, three and four contigs absent from strain 8019 were identified in isolates 5938 and 5994, respectively, by *de novo* assembly of the genomes. This insertion contained >70 ORFs and amounted to approximately 50 kb of sequence, all of which are present in the *E. faecium* DO reference strain. Homology mapping of this region revealed that the contigs likely span a single continuous mobile genetic element. While no genes with an obvious direct impact on daptomycin susceptibility

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Address correspondence to Romney M Humphries, rhumphries@mednet.ucla.edu.

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TABLE 1 *E. faecium* isolates investigated in this study and results of phenotypic cell membrane studies

Isolate	Time point of isolation from blood	Daptomycin MIC (μg/ml)	Mean cytochrome *c* binding[c] ± SD	Mean polarization index[d] ± SD	Mean cell wall thickness[e] ± SD	% of cells with septum formation	Mean % survival in presence of LL37 ± SD
5938	Initial	192	0.378 ± 0.001	0.232 ± 0.011	33.6 ± 4.6	57.8[a]	75.3 ± 4.6[a]
8019	+4 mo	4	0.363 ± 0.009	0.232 ± 0.018	33.6 ± 4.5	46.6	50.3 ± 6.0
5994	+8 mo	32	0.413 ± 0.003	0.224 ± 0.008	37.3 ± 5.5[a]	50.4	108 ± 9.4[b]

[a] Significantly different ($P < 0.05$) from strain 8019.
[b] Significantly different ($P < 0.05$) from both strains 8019 and 5938.
[c] Cytochrome *c* binding results are expressed as units of optical density at 530 nm of unbound cytochrome *c* in supernatant.
[d] The polarization index is expressed as degree of fluorescence polarization (4).
[e] Cell wall thickness is expressed in nm.

were detected, multiple transcriptional regulators, putative membrane-associated proteins, stress response regulators, and additional genes from the PTS family are present on this genetic element (not shown).

Cell surface charge (21, 22) was assessed by cytochrome *c* binding (22), membrane fluidity through fluorescence polarization (4, 10), and cell wall thickness and septum formation by transmission electron microscopy (TEM) (20). Isolate 8019 bound the most cytochrome *c* (Table 1, $P = 0.007$, Kruskal-Wallis analysis of variance) and had the highest polarization index, indicative of a lower net surface charge (22), and lower membrane fluidity than the daptomycin-NS isolates. Neither the degree of cytochrome *c* binding nor the polarization indices were correlated with the daptomycin MIC (Table 1). Differences in the polarization indices among the three isolates studied did not achieve statistical significance ($P > 0.2$, Mann-Whitney U test). Isolate 5994 had a significantly thicker cell wall than isolates 5938 and 8019 (Table 1, $P < 0.001$). In contrast, a higher proportion of cells with septa visible by TEM was found to correlate with a higher daptomycin MIC (Table 1, $P < 0.05$). Although strain 5938 harbored three mutations relative to strain 8019 not found in strain 5994 (Table 2), septum formation was the only phenotype observed in these studies to correlate with an increased daptomycin MIC. Both strains 5938 and 5994 harbored a mutation in *ezrA* (Table 2), which encodes a transmembrane protein that is a negative regulator of the septation ring formation protein FtsZ (1) and may in part be responsible for increased septum formation among daptomycin-NS isolates. Daptomycin-NS *E. faecalis* also demonstrated a greater proportion of cells with septa than did susceptible isolates (2), but to date, no genotypic correlate to this phenotype has been identified (2). This finding may signal a morphological link to the daptomycin–β-lactam "seesaw effect" (21) as areas of septal formation have enhanced binding to the penicillins.

Finally, daptomycin-NS isolates were tested for susceptibility to the human cathelicidin LL37 as previously described (21). Strain 8019 was more susceptible to LL37 killing, which was reflected by 50% survival in the assay following 90 min of exposure to 4 μM LL37, than were daptomycin-NS isolates 5938 and 5994, which demonstrated 75% and 108% survival, respectively (Table 1). The difference between the survival of the daptomycin-NS isolates and that of strain 8019 was statistically significant ($P < 0.05$), and again, the degree of resistance to LL37 killing was not proportional to the daptomycin MIC.

These data are the first to provide WGS and phenotypic characterization of *in vivo*-selected daptomycin nonsusceptibility among clinical isolates of *E. faecium*. Further studies are needed to

TABLE 2 Nonsynonymous nucleotide mutations identified between strain 8019 (daptomycin susceptible) and strains 5938 and 5994 (daptomycin NS) by WGS

Nucleotide mutation (predicted amino acid mutation)		Mutation	*E. faecium* DO		
5938	5994	class	Locus	Locus tag	Predicted gene product
Deletion of 227 bp (fs)[a]	WT[b]	Indel[c]		HMPREF0351_11791 HMPREF0351_11792	Hypothetical protein PTS system, mannose-specific IIA component
Insertion of T at 2508 (fs)	Insertion of T at 2508 (fs)	Indel		No match	NtrC family transcriptional regulator
WT	Deletion of T at 1839 (fs)	Indel		HMPREF0351_10870	Hypothetical protein
WT	Insertion of A at 229 (fs)	Indel	*pspC*	HMPREF0351_12014	Conserved hypothetical protein
WT	Insertion of T at 704 (fs)	Indel		No match	ABC transporter
G187A (R63-)	WT	Nonsense	*lepB*	HMPREF0351_11130	Signal peptidase I
C236T (G79E)	C236T (G79E)	SNV		HMPREF0351_11658	Hypothetical protein
G1253A (C418Y)	G1253A (C418Y)	SNV	*aad*	HMPREF0351_10204	Alcohol dehydrogenase
G303T (L101F)	WT	SNV		HMPREF0351_10668	NrdR-regulated deoxyribonucleotide transporter
G712A (L238F)	G712A (L238F)	SNV		HMPREF0351_12883	PTS system, mannose-specific IID component
G979A (A327T)	G979A (A327T)	SNV	*ezrA*	HMPREF0351_12190	EzrA
T38C (N12S)	T644C (H215R)	SNV	*cls*	HMPREF0351_11068	Cardiolipin synthetase
T874A (S292T)	T874A (S292T)	SNV		HMPREF0351_12498	Alpha-mannosidase
WT	A749T (V250E)	SNV		HMPREF0351_11298	Outer surface protein of unknown function
WT	G35A (G12E)	SNV		HMPREF0351_10875	Hypothetical protein
WT	T98C (V33A)	SNV	*sdhC*	HMPREF0351_10830	Hypothetical protein

[a] fs, frameshift.
[b] WT, wild type (i.e., same as strain 8019).
[c] Indel, insertion or deletion of a nucleotide(s).

examine daptomycin-NS *E. faecium* from other patients and hospitals, as well as to perform genetic mutagenesis and complementation studies to confirm the putative roles of the mutations identified in this study in daptomycin resistance in *E. faecium*.

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