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Daptomycin resistant *Enterococcus faecalis* has a mutation in *liaX*, which encodes a surface protein that inhibits the LiaFSR systems and cell membrane remodeling

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ABSTRACT

The emergence of daptomycin (DAP) resistant *Enterococcus* species has increased worldwide, but the mechanisms for DAP resistance are not fully understood. We report a case of DAP resistant *Enterococcus faecalis,* from a clinical sample of a patient with diabetic ulcers, after DAP therapy. Whole-genome sequencing analysis revealed that the isolate had a loss-of-function point mutation within *liaX* encoding DAP-sensing surface protein, which inhibits the LiaFSR systems and cell membrane remodeling. This is the first case report of a clinical DAP resistant *E. faecalis* with a mutation in *liaX*.

Keywords:

daptomycin, LiaX, *Enterococcus faecalis*, whole-genome sequencing

Abbreviations:

DAP: daptomycin

MIC: minimum inhibitory concentration.

MRSA: methicillin-resistant *Staphylococcus aureus*

TAZ/PIPC: tazobactam/piperacillin

Introduction

Daptomycin (DAP) is a lipopeptide antibiotic that is active against Gram-positive microbes. DAP was approved for the treatment for bacteremia, right-sided endocarditis, and skin and skin-structure infections such as necrotizing fasciitis due to methicillin-resistant *Staphylococcus aureus* (MRSA) in Japan [1]. *Enterococcus* has increasingly become a major nosocomial pathogen worldwide because these organisms may develop resistance to many antimicrobials, and DAP is an alternative option for the treatment of enterococcal infections. However, DAP resistant *Enterococcus* isolates are an increasing problem and several reports have described the emergence of resistance during DAP therapy [2-4]. Recently, gene mutations that alter the susceptibility to DAP have demonstrated that they directly affect the cell membrane composition [5]. In *Enterococcus faecalis*, it has been proposed that substitutions in three proteins, namely LiaF (lipid-Ⅱ cycle interfering antibiotics protein), GdpD (glycerophosphoryl diester phosphodiesterase), and Cls (cardiolipin synthetase), are mainly involved in resistance to DAP [5-8]. However, the genetic background that might contribute to the DAP resistance is poorly understood in the species.

In this study, we report a case of a DAP resistant *E. faecalis* isolate, obtained after DAP therapy. Also, the susceptibility profile and molecular characteristics of the isolate are described. This isolate harbored a mutation in *liaX*, which is recently characterized as the main regulator of the cell membrane response to antimicrobials.

Case report

A 49-year-old man was admitted to our hospital for treatment of left lower extremity ulcers. He had a medical history of diabetes mellitus, diabetic retinopathy, nephropathy, and myocardial infarction. He had undergone below-knee amputation of his right lower extremity. He subsequently complained of fever and left leg pain. A physical examination revealed necrosis and ulceration spread to his left foot. Laboratory studies showed elevated serum levels of inflammation markers (C-reactive protein, 15.99 mg/dL; procalcitonin, 1.70 ng/mL; Table 1). Therefore, we performed an amputation of his necrotic left toes and debridement around his ulcers for infection control and treatment after admission. In addition, we treated the patient with 350 mg of daptomycin (DAP) every other day for 20 days and 4.5 g/day of tazobactam/piperacillin (TAZ/PIPC) for 12 days after amputation (Figure 1). After improvement of systemic inflammation, antibiotic treatment was stopped. However, he had osteomyelitis of the left metatarsal bone and underwent Chopart amputation on day 29 and below-knee amputation of his left leg on day 43 after admission. In addition, clinical samples of wound bacterial culture were collected on admission, and on day 33 after DAP therapy and Chopart amputation. *E. faecalis*, MRSA, *Staphylococcus lugdunensis*, and *Serratia marcescens* were detected on admission. The antibiograms of *E. faecalis*, MRSA, and *S. lugdunensis* isolates were susceptible to DAP. On the other hand, DAP resistant *E. faecalis*, *S. marcescens*, and *Raoultella ornithinolytica* were detected after DAP therapy and Chopart amputation on day 33 (Table 2). We continued to treat *E. faecalis*, which was susceptible to piperacillin, with TAZ/PIPC and improved his systemic inflammation.

The bacteria were identified using the Microflex LT system and MALDI Biotyper Compass software 4.1.100 (Bruker, Billerica, MA, USA). The minimum inhibitory concentrations (MICs) for clinical *E. faecalis* isolates were determined using the 96-well

MicroScan panels and the MicroScan WalkAway® 96 Plus system (Beckman Coulter, Brea, CA, USA), or using the ETEST strip (bioMérieux, Marcy I′Etoile, France) (Table 2). The "susceptible", "intermediate", or "resistance" categories were based on the CLSI document M100-S30 [9]. The MIC of DAP using ETEST strip was 12 μg/mL for *E. faecalis* isolated after DAP therapy and Chopart amputation. Whole-genome sequencing was performed using the MiSeq system (Illumina, San Diego, CA, USA) for DAP resistant *E. faecalis* to identify mutations associated with DAP resistance. DNA extraction and purification were performed using the NucleoSpin® Microbial DNA kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The library preparations and sequencing were conducted by the Eurofin Sequencing Service (Eurofins Genomics, Tokyo, Japan). The raw reads were assembled *de novo* using the A5-Miseq assembly pipeline. The sequence type of DAP resistant *E. faecalis* was defined as ST179 by using MLST 2.0 available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). *E. faecalis* ATCC 29212 was obtained from American Type Culture Collection (Manassas, VA, USA) and used as a drug-susceptible control strain (MIC of DAP was 1.0 μg/mL). To identify amino acid substitutions, we investigated the following genes potentially related to DAP resistance in *E. faecalis* based on previous reports: *liaF*, *liaX*, *cls*, and *gdpD* [6-8,10]. Compared to antibiotic-susceptible *E. faecalis* ATCC 29212 reference sequence (GenBank accession no. NZ_CP008816), the DAP resistant *E. faecalis* had a nonsense point mutation (C to T) at position 454 of *liaX*, and there was no point mutation in *liaF*, *cls*, or *gdpD*. This result indicated that the product of a truncated *liaX* was only 151 amino acids long, and the C-terminal domain of the DAP-sensing surface protein was missing. Next, we performed quantitative real-time PCR to determine the expression levels of *liaS* and *liaR* in DAP resistant *E. faecalis* with a mutation in *liaX* compared to antibiotic-susceptible *E. faecalis* ATCC 29212 reference strain which has wildtype *liaX* gene (GenBank accession no.

NZ_CP008816). RNA extraction, DNase treatment, and cDNA generation were performed in exponential phase growth strain using RNeasy Mini Kit (Qiagen, Hilden, Germany), RQ1 RNase-Free DNase (Promega, Madison, WI, USA), and ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan), respectively. Evaluation of expression levels was conducted using Power SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MS, USA) and previously reported primers [10] in Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). Relative expression ratio was calculated by normalizing to the housekeeping genes *gdhA*. The expression levels of *liaS* and *liaR* in DAP resistant *E. faecalis*, which has a mutation in *liaX*, highly elevated compared to the *E. faecalis* ATCC 29212 reference strain.

The study was approved by the institutional review board of Hamamatsu University School of Medicine (HUSM 17-318). The patient provided written informed consent.

Discussion

In this report, we describe the emergence of a DAP resistant *E. faecalis* isolate after DAP therapy for the treatment of wound infection. A whole-genome sequencing analysis indicated that the DAP resistant *E. faecalis* isolate contained a mutation in *liaX* which inhibits the LiaFSR system and cell membrane remodeling. To our knowledge, this is the first report of a loss-of-function point mutation in *liaX* in a clinical DAP resistant *E. faecalis*.

DAP is commonly used to treat enterococcal infections in clinical practice. However, the clinical use of DAP against *Enterococcus* isolates has been threatened by the emergence of resistance, but the mechanisms of DAP resistance have not been fully understood in the species [2-4]. Recently, some hypotheses involving DAP resistance have been proposed in *E. faecalis* [5]. First, the amino acid changes in the proteins associated with the metabolism of cell membrane phospholipids were shown to be responsible for the development of DAP resistance. Among them, Cls, which synthesize cardiolipin, and GdpD, which are involved in glycerol turnover for phospholipid biosynthesis, are the two prominent enzymes associated with the DAP resistant phenotype [5-8]. However, we did not identify mutations in these proteins in our isolate by whole-genome analysis. Next, the development of DAP resistance has also been shown to be associated with mutations in genes encoding regulatory systems that orchestrate cell envelope stress response. In particular, the DAP-sensing surface protein LiaX is a mediator of the cell membrane adaptive response to antimicrobials and is the main inhibitor of the LiaFSR system [10]. The LiaFSR system, which encodes a three-component regulatory system controlling cell envelope integrity to counteract membrane damage caused by external agents, such as antibiotics, is well conserved in Gram-positive bacteria [5,11]. The protein LiaF acts as a specific inhibitor of LiaS and LiaR, which are the histidine kinases and response regulators of the LiaFSR system, respectively [5]. Indeed, Jordan *et al.* reported that

the presence of DAP strongly activated the LiaSR regulon, leading to cell membrane protection [11]. The study showed that a nonpolar *liaF* deletion led to constitutive activation of LiaR-dependent promoters [11]. In addition, Kahn *et al.* demonstrated that the C-terminal domain of LiaX acted as a negative regulator of the LiaFSR system, and the LiaFSR system without this domain led to constitutive activation and cell membrane remodeling [10]. Consequently, the loss of function of the negative regulator of the LiaFSR system causes cell membrane remodeling and results in a DAP resistant phenotype. In this case, the whole-genome sequencing analysis of *liaX* showed a point mutation leading to the nonsense mutation at Arg152 to stop codon resulting in the C-terminal deletion. Furthermore, expression levels of *liaS* and *liaR*, which are regulated negatively by *liaX*, highly elevated in DAP resistant *E. faecalis* with a mutation in *liaX* compared to the reference strain. Therefore, we concluded that the development of DAP resistance in our isolate may have been caused by cell membrane remodeling through activation of the LiaFSR system due to nonsense mutation in *liaX* (Figure 2). However, the limitation of the present study is only one case with whole genome sequence data and molecular data.

In summary, we isolated a strain of DAP resistant *E. faecalis* from a clinical sample of the patient after DAP treatment. Whole-genome sequencing detected a nonsense mutation within *liaX* that may be responsible for DAP resistance in *E. faecalis*.

Conflict of Interest: None of the authors has any financial relationships with commercial entities that have an interest in the subject of this manuscript.

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Figure Legends

Figure 1. Clinical course. The abbreviations used are: DAP; daptomycin; TAZ/PIPC; tazobactam/piperacillin; WBC, white blood cell; CRP, C-reactive protein.

Figure 2. Gene expression levels of *liaS* and *liaR* in daptomycin resistant *Enterococcus faecalis* (Gray bar) compared with *E. faecalis* ATCC 29212 reference strain (Black bar). Data are the mean ± SEM of three independent experiments.

Figure 3. A hypothetical mechanism of daptomycin resistance in our isolate. The LiaFSR system regulates cell membrane remodeling. The LiaX inhibits the LiaFSR system in *Enterococcus faecalis* wild-type isolate (A). *E. faecalis* with LiaX nonsense mutation leads to cell membrane remodeling, resulting in daptomycin resistance (B).

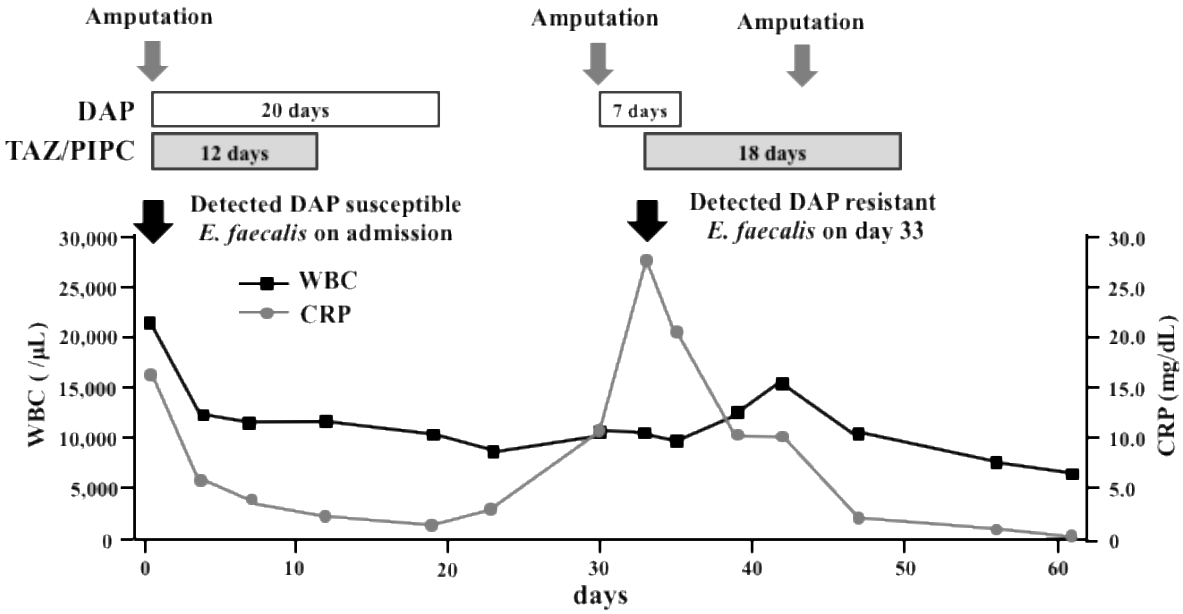
Amputation
Amputation
Amputation
DAP
20 days
7 days
TAZ/PIPC
12 days
18 days
Detected DAP susceptible
E. faecalis on admission
Detected DAP resistant
E. faecalis on day 33
WBC
CRP
WBC (/μL)
CRP (mg/dL)
30,000
25,000
20,000
15,000
10,000
5,000
0
30.0
25.0
20.0
15.0
10.0
5.0
0
0
10
20
30
40
50
60
days

Figure 1.

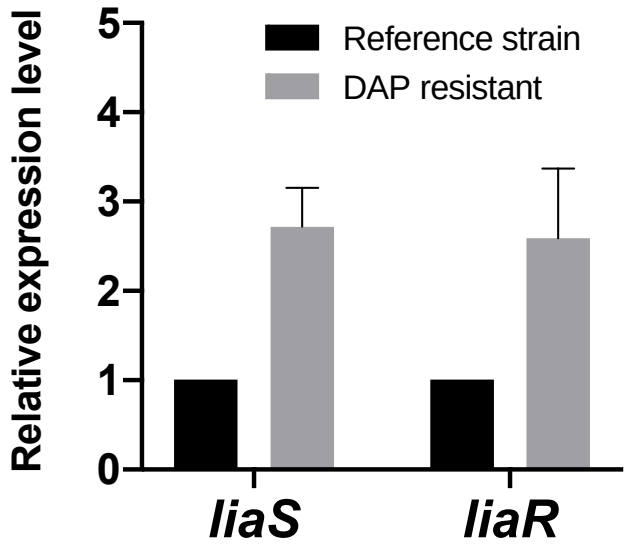
Relative expression level
5
4
3
2
1
0
Reference strain
DAP resistant
liaS
liaR

Figure 2.

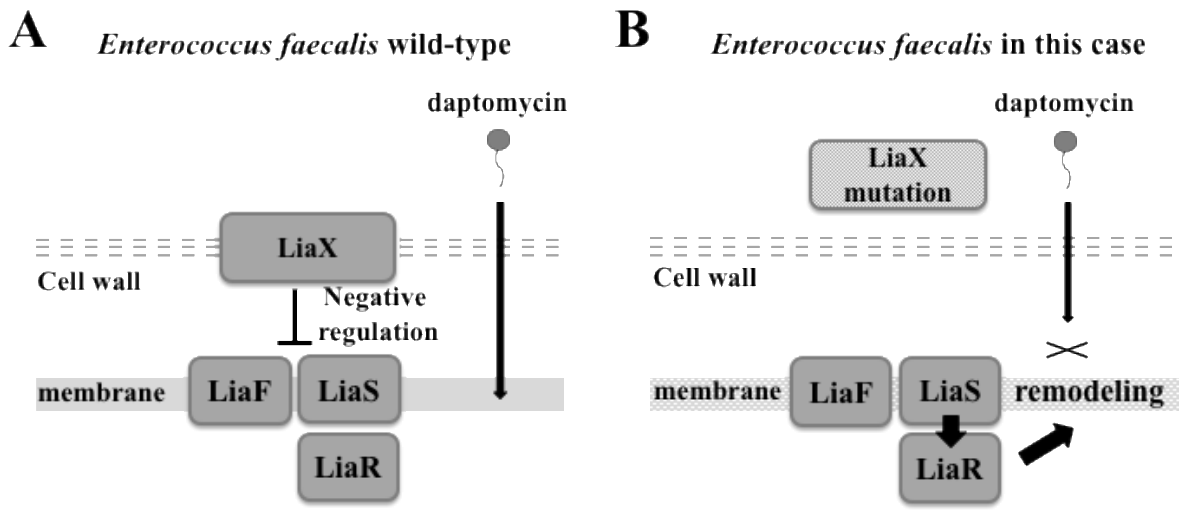
A
Enterococcus faecalis wild-type
daptomycin
LiaX
Cell wall
Negative
regulation
membrane
LiaF
LiaS
LiaR
B
Enterococcus faecalis in this case
daptomycin
LiaX
mutation
Cell wall
membrane
LiaF
LiaS
remodeling
LiaR

Figure 3.

Table 1. Laboratory findings on admission.

WBC	21,690	/μL	Na	133	mmol/L	PT	12.5	sec
Seg	83.0	%	K	6.1	mmol/L	APTT	41.0	sec
Band	7.0	%	Cl	91	mmol/L	Fibrino-gen	927	mg/dL
Eos	0	%	LD	195	U/L	D-dimer	1.6	μg/mL
Baso	0	%	AST	17	U/L			
Lym	4.0	%	ALT	9	U/L			
Mono	6.0	%	ALP	395	U/L			
RBC	5.42	$\times 10^6$/μL	TP	7.5	g/dL			
Hgb	16.1	g/dL	Alb	3.2	g/dL			
Hct	52.1	%	BUN	61.0	mg/dL			
Plt	30.7	$\times 10^4$/μL	Cre	8.39	mg/dL			
			CRP	15.99	mg/dL			
			PCT	1.70	ng/mL			
			FBS	188	mg/dL			
			HbA1c	7.4	%			

WBC: white blood cells; Seg: segmented neutrophils; Band: band neutrophils; Eos: eosinophils; Baso: basophils; Lym: lymphocytes; Mono: monocytes; RBC: red blood cells; Hgb: hemoglobin; Hct: hematocrit; Plt: platelets; LD: lactate dehydrogenase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TP: total protein; Alb: albumin; BUN: blood urea nitrogen; Cre: creatinine; CRP: C-reactive protein; PCT: procalcitonin; FBS: fasting blood sugar; HbA1c: hemoglobin A1c; PT: prothrombin time; and APTT: activated partial thromboplastin time

1 ***Table 2. Antibiograms of Enterococcus faecalis* isolates.**

Antibiotics	MIC (μg/mL)			
	Enterococcus faecalis on admission		*Enterococcus faecalis* on day 33	
Penicillin G	2	S	1	S
Ampicillin	≦2	S	≦2	S
Ampicillin/Sulbactam	≦8		≦8	
Amoxicillin/Clavulanic acid	≦2		≦2	
Oxacillin	>2		>2	
Cefazolin	>16		16	
Ceftriaxone	>16		>16	
Cefepime	>16		>16	
Cefmetazole	>32		>32	
Imipenem	≦1		≦1	
Meropenem	4		2	
Levofloxacin	1	S	1	S
Gentamicin	>8		>8	
Vancomycin	1	S	1	S
Teicoplanin	≦1	S	≦1	S
Linezolid	2	S	2	S
Daptomycin	0.5	S	12	R
Erythromycin	>4	R	>4	R
Clindamycin	>2		>2	
Minocycline	>8	R	8	I
Sulfamethoxazole/Trimethoprim	≦1		≦1	
Fosfomycin	16		16	
Rifampicin	>2	R	2	I

2 MIC: minimum inhibitory concentration