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Roles of pyruvate dehydrogenase and branched-chain α-keto acid dehydrogenase in branched-chain membrane fatty acid levels and associated functions in *Staphylococcus aureus*

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Abstract

Purpose. Membrane fluidity to a large extent is governed by the presence of branched-chain fatty acids (BCFAs). Branched-chain α-keto acid dehydrogenase (BKD) is the key enzyme in BCFA synthesis. A *Staphylococcus aureus* BKD-deficient strain still produced substantial levels of BCFAs. Pyruvate dehydrogenase (PDH) with structural similarity to BKD has been speculated to contribute to BCFAs in *S. aureus*.

Methodology. This study was carried out using BKD-, PDH- and BKD : PDH-deficient derivatives of methicillin-resistant *S. aureus* strain JE2. Differences in growth kinetics were evaluated spectrophotometrically, membrane BCFAs using gas chromatography and membrane fluidity by fluorescence polarization. Carotenoid levels were estimated by measuring A_{465} of methanol extracts from 48 h cultures. MIC values were determined by broth microdilution.

Results/Key findings. BCFAs made up 50 % of membrane fatty acids in wild-type but only 31 % in the BKD-deficient mutant. BCFA level was ~80 % in the PDH-deficient strain and 38 % in the BKD : PDH-deficient strain. BKD-deficient mutant showed decreased membrane fluidity, the PDH-deficient mutant showed increased membrane fluidity. The BKD- and PDH-deficient strains grew slower and the BKD : PDH-deficient strain grew slowest at 37 °C. However at 20 °C, the BKD- and BKD : PDH-deficient strains grew only a little followed by autolysis of these cells. The BKD-deficient strain produced higher levels of staphyloxanthin. The PDH-deficient and BKD : PDH-deficient strains produced very little staphyloxanthin. The BKD-deficient strain showed increased susceptibility to daptomycin.

Conclusion. The BCFA composition of the cell membrane in *S. aureus* seems to significantly impact cell growth, membrane fluidity and resistance to daptomycin.

INTRODUCTION

Staphylococcus aureus is a significant human pathogen with the ability to cause minor to severe life-threatening illnesses [1–5]. Clinical management of these infections is complicated due to widespread emergence of methicillin-resistant *S. aureus* (MRSA) strains. Some of these MRSA strains are now resistant to more than 20 different antimicrobial agents [6–9].

S. aureus encounters a variety of environmental conditions to which it must adapt in order to survive, including changing temperature [10, 11], fluctuations in pH [12], osmotic pressure [13, 14], nutrient availability and antibiotic stress [13, 15]. Specifically, membrane fluidity is a critical determinant in its adaptation [16–18]. *S. aureus,* like other species, maintains membrane fluidity by altering its fatty acid composition [17]. Branched-chain fatty acids (BCFAs) occupy significantly larger cross-sectional areas than saturated straight-chain fatty acids (SCFAs), disrupt the close packing of membrane fatty acids and allow the membranes to remain fluid at lower temperatures [19–21].

Recent studies have revealed that the membrane lipid composition in *S. aureus* is remarkably plastic, and this is largely determined by the nutritional environment [22]. When grown in artificial media, *S. aureus* fatty acids are a mixture of BCFAs and SCFAs [22, 23]. The percentage of BCFAs typically exceeds that of SCFAs in standard media such as brain–heart infusion and tryptic soy broth, and the proportion of BCFAs is very high in cells grown in Mueller–Hinton

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Abbreviations: BCFA, branched-chain fatty acid; BKD, branched-chain α-keto acid dehydrogenase; PDH, Pyruvate dehydrogenase; SCFA, straight-chain fatty acid; SCUFA, straight-chain unsaturated fatty acid.

broth and defined medium [22, 24]. However, when grown in serum [22] or fed individual fatty acids, *S. aureus* can incorporate significant quantities of SCFAs and straight-chain unsaturated fatty acids (SCUFAs) [25, 26]. Additionally, the triterpenoid carotenoid 'staphyloxanthin' is a characteristic of *S. aureus* cells and its level fluctuates widely in different strains of this bacterium and under different growth conditions [22, 24].

Given the importance of BCFAs in membrane structure and function, a BCFA-deficient mutant was created by insertional inactivation of the *lpdA* gene of *S. aureus* strain SH1000, the first gene of the cluster that codes for the four polypeptides of the branched-chain α-keto acid dehydrogenase complex (BKD) [23]. BCFAs were reduced to 35.4 % from 63.5 % in the mutant compared to the wild-type. The mutant had a less fluid membrane, was more susceptible to cold, alkaline and oxidative stress and diminished survival in mice [23]. BCFAs are biosynthesized from the branched-chain amino acids isoleucine, leucine and valine via branched-chain amino acid transaminase and BKD. The biosynthetic route to the substantial percentage of BCFAs present in the BCFA-deficient *S. aureus* mutant is not clear.

BKD is a multi-subunit complex composed of four polypeptides: a dehydrogenase (E1α), a decarboxylase (E1β), a dihydrolipoamide acyltransferase (E2) and a dihydrolipoamide dehydrogenase (E3) [27, 28]. It is anticipated that the residual levels of BCFAs [23] in the BKD-deficient *S. aureus* are produced by another enzyme complex sharing structural and/or functional homology to BKD. In this context, pyruvate dehydrogenase (PDH) is also a multi-enzyme complex similar to BKD. In *S. aureus*, the chromosomal locus possesses four genes that encode the PDH polypeptides: pyruvate dehydrogenase E1 component (alpha subunit), pyruvate dehydrogenase E1 component (beta subunit), branched-chain alpha-keto acid dehydrogenase (E2 subunit) and dihydrolipoamide dehydrogenase (LpdA). The PDH enzyme complex indeed shares significant sequence homology with the BKD complex [29, 30], and was predicted to be a prime candidate in contributing to BCFA metabolism [30–32], as activity of PDH with some branched-chain α-keto acids has been demonstrated [33, 34].

To investigate the roles of these complexes in BCFA production, BKD- and PDH-deficient *S. aureus* strains were studied. Surprisingly, lack of PDH activity led to a very significant increase in BCFAs, opposite to the effect that was seen in BKD-deficient *S. aureus*. These changes in BCFAs also impacted physiological functions in *S. aureus*.

METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *S. aureus* cultures were grown in tryptic soy broth or agar (TSB or TSA; Becton Dickinson). When needed, erythromycin (10 μg ml^{-1}), kanamycin (100 μg ml^{-1}) and chloramphenicol (10 μg ml^{-1}) were added to the growth medium. Mueller–Hinton broth (MHB, Becton Dickinson) was used for measuring minimum inhibitory concentrations (MICs) of antibiotics. TSB was used to culture *S. aureus* for the analysis of fatty acid composition. Cloning and plasmid DNA preparations were made by culturing appropriate *Escherichia coli* cells in Luria–Bertani broth or agar in the presence of ampicillin at 100 μg ml^{-1}.

Construction of *lpdA* (BKD-deficient) and *pdhA* (PDH-deficient) mutants

The previously reported mutation in the *lpdA* gene of strain SH1000 [23] was transduced in *S. aureus* strain JE2 using a phage transduction procedure as described previously [23]. A PDH-deficient derivative of *S. aureus* strain JE2 (NE1724 of the Nebraska Transposon mutant library) [35] was

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Bacteria		
S. aureus JE2	Most common CA-MRSA isolate, resistant to methicillin/oxacillin	[57]
lpdA	JE2 with mutation in the *lpdA* (Kan^r)	This study
lpdA+pCU-*bkd*	JE2 : *lpdA* complemented with pCU-*bkd* plasmid (Kan^r, Cam^r)	This study
NE1724	JE2 with a transposon insertion in the *pdhA* gene (Erm^r)	[35]
pdhA+pCU-*pdh*	JE2 : *pdhA* complemented with pCU-*pdh* plasmid (Erm^r, Cam^r)	This study
lpdA : *pdhA*	JE2 with mutation in the *lpdA* and *pdhA* genes (Kan^r, Erm^r)	This study
lpdA-pdhA+pCU-*bkd*	JE2Δ(*pdhA*+*lpdA*)complemented with pCU-*bkd* (Kan^r, Erm^r, Cam^r)	This study
lpdA-pdhA+pCU-*pdh*	JE2Δ(*pdhA*+*lpdA*)complemented with pCU-*pdh* (Kan^r, Erm^r, Cam^r)	This study
Plasmids		
pGEM-T	An *E. coli* cloning plasmid	Promega
pCU1	A shuttle vector (Amp^r in *E. coli*, Cam^r in *S. aureus*)	[36]
pCU-*bkd*	Plasmid pCU1 containing a 5.1-kb DNA fragment containing all four genes of the *S. aureus bkd* locus	[23]
pCU-*pdh*	Plasmid pCU1 containing a 5.9-kb DNA fragment containing all four genes of the *S. aureus pdh* locus	This study

Kan^r, kanamycin resistant; Erm^r, erythromycin resistant; Cam^r, chloramphenicol resistant.

obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). NE1724 contains a transposon insertion in the gene coding for the pyruvate dehydrogenase E1 alpha subunit (*pdhA*). An *lpdA : pdhA* (BKD : PDH-deficient) double mutant was generated by transducing the *lpdA* mutation in to NE1724.

Creation of complemented stains

For complementation with the *pdh* locus, the entire locus starting 586 nt upstream of the first gene (*pdhA*) and terminating 410 nt after the fourth gene (*pdhD*) was PCR amplified (a 5886 bp amplicon) using appropriate primers (forward primer; 5′-GGTACCAGTTCGCGGTAAAGCAG TAAAGG-3′ and backward primer; 5′-TCTAGAGTGAG TTTTTACATCTTGAACTGC-3′) and *S. aureus* genomic DNA as the template. PCR was carried out using Stratagene EXL DNA polymerase (Stratagene, CA) as per the manufacturer's instructions. This amplicon was cloned in vector pGEM-T (Promega), from where it was subcloned in vector pCU1 [36] at *Kpn*I and *Xba*I sites. The *pdhA* mutant of *S. aureus* strain JE2 was subsequently transformed with this construct. For complementation of the *lpdA* mutant, the entire *bkd* locus comprising all four BKD encoding peptides on pCU1 described previously [23] was transduced into the *lpdA* mutant.

Analysis of fatty acid composition in the wild-type JE2 and various *S. aureus* mutants

To determine the membrane fatty acid composition, cultures of various mutants, complemented strains and wild-type *S. aureus* strains were grown in 50 ml TSB in a 250 ml flask at 37 °C to an OD_{600}=0.6. The bacterial cells were then harvested and processed for the identification of fatty acids by the MIDI microbial identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc. (Newark, DE, USA) as described previously [23].

Growth kinetics of wild-type *S. aureus* strain JE2 and its derivative mutants

Mid-exponential phase cultures (OD_{600}=0.6) were diluted 10-fold in an Erlenmeyer flask containing 50 ml fresh TSB, with a flask-to-medium volume ratio of 6 : 1. Bacterial growth was subsequently monitored by incubating the flask in a shaking incubator (220 r.p.m.) and measuring the turbidity (OD_{600}) of the liquid culture periodically using a Thermo Scientific Biomate 3 spectrophotometer.

Estimation of carotenoid pigments in wild-type *S. aureus* strain JE2 and its derivative mutants and complemented strains

The carotenoid production of wild-type and *S. aureus* mutant strains was determined as previously described [37]. The overnight cultures were diluted 1 : 100 in 50 ml TSB and incubated at 37 °C with shaking (220 r.p.m.) for 48 h. Cells from 5 ml of each culture were collected by centrifugation. The cells were washed three times with sterile, ice-cold distilled water and the cell pellet wet weight was determined. Cells were resuspended in 450 µl methanol and incubated at 55 °C for 3 min with intermittent vortexing. The cells were subsequently pelleted by centrifugation, and A_{465} of the carotenoid-containing methanol extract was measured.

Determination of membrane fluidity of wild-type *S. aureus* strain JE2 and its derivative mutants and complemented strains

Membrane fluidity was determined using fluorescence polarization as previously described [38], with some modifications. For these studies, overnight cultures were diluted 1 : 100 in a flask containing 50 ml fresh TSB and were grown to OD_{600}=0.6 at 37 °C in a shaking incubator (220 r.p.m.). Bacterial cells were collected by centrifugation and washed twice with PBS. The bacterial cells were then resuspended in PBS to an OD_{600} to approximately 1.0. Subsequently, 2.0 ml of PBS containing 3 µM 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich, St. Louis, MO) was added to 1.0 ml of the bacterial suspension. DPH was initially dissolved in tetrahydofuran (THF) and added to PBS at the time of use. To the control blank, 2.0 ml PBS with an equivalent amount of THF was added to 1.0 ml of bacterial cell suspension. Both test and blank samples were incubated at 37 °C for 1 h. Fluorescence polarization was then measured in quartz cuvettes using a PerkinElmer LS55 Spectrofluorometer with excitation and emission wavelengths of 360 and 426 nm, respectively. Vertically polarized 360 nm light excites the DPH dye, which emits fluorescent light at 426 nm and is detected through polarized light filters. Polarization was calculated as described [12, 38, 39].

Determination of antimicrobial susceptibility

The antibiotic MICs for the wild-type and different mutant strains of *S. aureus* were determined as previously described [40, 41].

qPCR analysis of *lpdA* transcript levels in PDH-deficient and *crtM* in BKD-deficient *S. aureus* strain JE2

qRT-PCR assays were used to investigate whether the gene encoding the LpdA polypeptide in the *pdhA* mutant and the gene encoding the CrtM polypeptide in the *lpdA* mutant were overexpressed. Cultures of *S. aureus* wild-type strain JE2 and derivative mutants were grown to OD_{600}=0.6. Total RNA was extracted from these cells as described previously [42]. cDNA from DNase-treated 0.5 µg of total RNA was synthesized in a 20 µl reverse transcription reaction containing random hexamers and SuperScript III reverse transcriptase (Invitrogen). All real-time PCR reactions were carried out with a Bio-Rad iCycler (iQ5 system). The transcript levels were quantified using specific *lpdA* (forward primer; 5′-CGTTCTCGGTG GAGGTAC-3′ and backward primer; 5′-GATTCACCA AGCCTTCTTGC-3′) and *crtM* primer pairs (forward primer; 5′-TTGAAACGGACGCTGAATTA-3′ and backward primer; 5′-AGCAGCGATTTAGTAGGAATAC-3′). Expression levels of *lpdA* and *crtM* were normalized to the expression of the gene encoding DNA gyrase using *gyrA*-specific primer pairs (forward primer; 5′-TCCACAAGTCGCACGTACAG-3′ and backward primer; 5′-GGAAGGCTTGCTACATCTAACG-3′)

based on a previous report [43, 44]. Changes in gene expression were calculated using the formula $2^{-\Delta\Delta Cq}$ as previously described [45].

Statistical analysis

Results are reported as the mean±SEM of at least three independent experiments unless stated otherwise. Two sample *t*-tests were carried out to compare the membrane fluidity and staphyloxanthin levels in the mutant to the wild-type *S. aureus*. Data were analysed utilizing SAS version 9.4 (SAS Institute Inc., Cary, NC). Statistical significance was set at $P \leq 0.05$.

RESULTS

Fatty acid compositions of *S. aureus* JE2 and its derivative mutants

The composition of major fatty acids present in the cytoplasmic membrane of wild-type JE2 and its various derivative strains is shown (Table 2). As expected, the total BCFA content in the *lpdA* mutant was significantly reduced (30.79 %) compared to the BCFA content of the wild-type (50.09 %) (Table 2). The *lpdA* mutant showed low levels of ante-iso fatty acids (11.56 %) compared to the wild-type JE2 (30.86 %), thus leading to a low ante-iso:iso ratio in the mutant (0.60) relative to the wild-type (1.60). Furthermore, the amounts of odd-iso and even-iso fatty acids were also found to be altered in the *lpdA* mutant. The wild-type JE2 had a higher odd-iso fatty acid content whereas the *lpdA* mutant showed higher even-iso fatty acids (Table 2). On the other hand, a mutation in *pdhA* leading to the inactivation of pyruvate dehydrogenase caused a drastic decrease in the SCFA content (only 19.10 %) and a drastic increase in BCFAs (79.65 %) (Table 2). However, in the *pdhA* knockout mutant, the ante-iso:iso ratio did not change like the *lpdA* mutant and was similar to the wild-type JE2, because the ante-iso and iso fatty acid contents both increased by proportional amounts (Table 2). The *lpdA : pdhA* double mutant had slightly higher amounts of BCFAs in comparison to the *lpdA* mutant. This double mutant showed an increase in both total ante-iso and total iso fatty acids, leading to an ante-iso:iso ratio similar to the *lpdA* mutant (Table 2).

In these studies, when the *lpdA* mutant of JE2 was complemented with the *bkd* locus *in trans*, it resulted in restoration of the BCFAs and other membrane fatty acids in the *lpdA* mutant (Table 2). The ante-iso and iso fatty acids in the BKD-complemented *lpdA* mutant increased to equal the wild-type JE2 levels. The iso fatty acids also increased to the wild-type amounts, but the odd-iso and even-iso pattern did not revert to the wild-type JE2 levels in the *lpdA*-complemented strain (Table 2). Complementation of the *lpdA* mutant with the *pdh* locus had effects similar to the complementation of this mutant with the *bkd* locus (Table 2). Also, when the *pdhA* mutant was complemented with the *pdh* locus, it resulted in the restoration of the membrane fatty acid composition similar to the profile of the wild-type JE2 (Table 2). In contrast, complementation of the *pdhA* mutant with the *bkd* locus had little to no impact and caused no change in the membrane fatty acid composition in the complemented strain (Table 2). Complementation with the *bkd* locus of the *lpdA : pdhA* double mutant resulted in a profile similar to the *pdhA* mutant in terms of a very high BCFA content (Table 2). Complementation of the *lpdA : pdhA* double mutant with the *pdh* locus increased the BCFA content relative to the *lpdA* and *lpdA : pdhA* double mutant, but it remained lower than the BCFA levels in wild-type JE2 (Table 2).

Growth characteristics of JE2 and isogenic mutants

In growth kinetic studies, growth of the *lpdA* and *pdhA* mutants was slower compared to the wild-type JE2 (Fig. 1). Growth of the *lpdA* mutant was slower compared even to the *pdhA* mutant (Fig. 1). Growth of the *lpdA : pdhA* double

Table 2. Fatty acid profiles of wild-type JE2 and isogenic *lpdA*, *pdhA* and *lpdA-pdhA* mutants and complemented strains

	% (wt/wt) of total fatty acids									
	Wild type	*lpdA*	*lpdA with*		*pdhA*	*pdhA* with		*lpdA : pdhA*	Δ*lpdA : pdhA* with	
Fatty acid			pCU-*bkd*	pCU-*pdh*		pCU-*bkd*	pCU-*pdh*		pCU-*bkd*	pCU-*pdh*
Ante-iso C15 : 0	28.16	11.56	26.72	24.19	37.09	37.41	29.23	10.84	34.50	23.29
Ante-iso C17 : 0	2.70	ND	2.88	2.99	10.00	11.16	2.86	ND	7.88	1.35
Ante-iso C19 : 0	ND	ND	ND	ND	2.30	2.69	ND	3.10	1.88	ND
Ante-iso	30.86	11.56	29.60	27.18	49.39	51.26	32.09	13.94	44.26	24.64
Straight even	45.34	63.98	49.80	47.50	19.10	20.55	47.83	58.87	22.51	56.52
Iso odd	14.76	3.08	6.91	5.72	26.26	24.11	12.11	ND	17.50	11.25
Iso even	4.47	16.15	13.69	19.60	4.00	2.64	5.28	23.61	13.99	5.27
Iso	19.23	19.23	20.60	25.32	30.26	26.75	17.39	23.61	31.49	16.52
Ante-iso : iso ratio	1.60	0.60	1.44	1.07	1.63	1.92	1.85	0.59	1.41	1.49
BCFA	50.09	30.79	50.20	52.50	79.65	78.01	49.48	37.55	75.75	41.16
SCFA	46.89	63.98	49.80	47.50	19.10	20.55	49.08	58.87	22.51	57.55

ND, none detected.

Values indicate the average of two independent experiments.

mutant was slowest at 37 °C (Fig. 1). Complementation of the *lpdA* mutant with *bkd* locus and *pdhA* mutant with *pdh* locus corrected the growth defect observed in these individual mutants (Fig. 1). When these mutants were cultured at 20 °C, the *pdhA* mutant showed some growth but the *lpdA* and *lpdA : pdhA* double mutants failed to grow (Fig. 2).

Cytoplasmic membrane fluidity of JE2 and isogenic mutants as determined by fluorescence polarization

Polarization values that are inversely correlated with the fluidity of the cytoplasmic membrane showed a nice correlation with the level of BCFAs in different membranes. The *pdhA* mutant that showed highest levels of BCFAs and the *lpdA* mutant with the lowest levels of BCFAs showed the lowest and highest polarization values, respectively, and these polarization values were significantly different to the polarization values for the wild-type JE2 ($P \leq 0.005$) (Table 3). The *lpdA : pdhA* double mutant with BCFAs similar to the wild-type JE2 showed polarization values comparable to that of the wild-type. The complemented strains, *lpdA* mutant with the *bkd* locus and the *pdhA* mutant with the *pdh* locus, exhibited polarization values that were similar to the wild-type levels (Table 3).

Staphyloxanthin (carotenoid) content of JE2 and isogenic mutants

It was frequently observed that the *lpdA* mutant colonies were slightly darker (yellow) and the *pdhA* and the *lpdA : pdhA* double mutants were white on TSA plates. In view of these observations, the carotenoid levels in these strains were quantified. Upon quantification, the level of carotenoids showed a significant increase in the case of the *lpdA* mutant (Fig. 3). The levels of carotenoids were very low in the case of the *pdhA* mutant and the *lpdA : pdhA* double mutant (only 14.6 and 18.8 % compared to the wild-type JE2) (Fig. 3). When carotenoids were measured in the complemented strains, the *lpdA* mutant with the *bkd* locus and the *pdhA* mutant with the *pdh* locus showed carotenoid levels similar to the wild-type levels (Fig. 3).

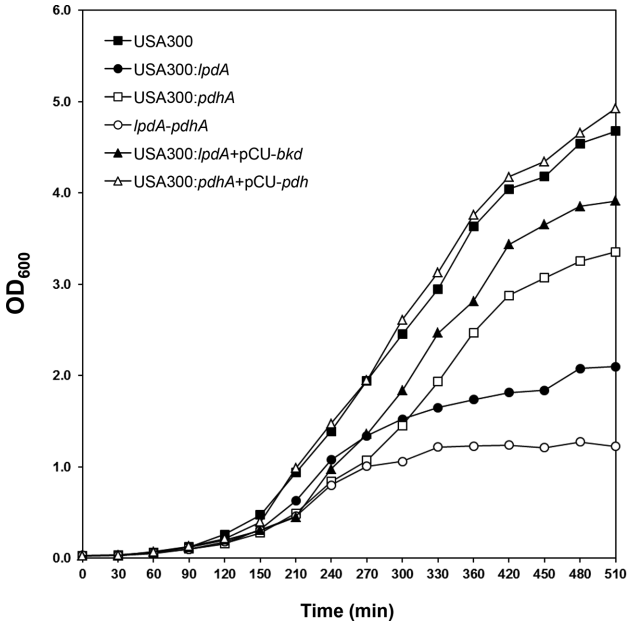

Fig. 1. Growth curve of the wild-type *S. aureus* strain JE2 and its derivative mutants in TSB at 37 °C. Values indicate the average of two independent experiments.

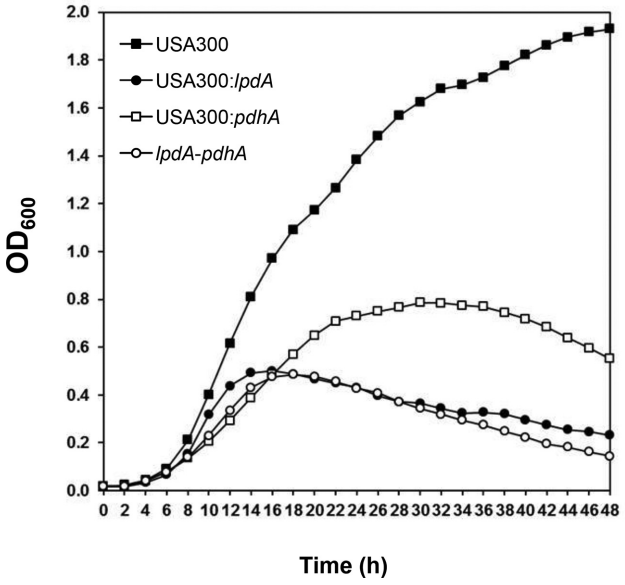

Fig. 2. Growth curve of the wild-type *S. aureus* strain JE2 and its derivative mutants in TSB at 20 °C. Values indicate the average of two independent experiments.

Antibiotic susceptibility of JE2 and isogenic mutants

In these studies, no difference was observed in the susceptibility of the *lpdA*, *pdhA* or *lpdA : pdhA* double mutants to oxacillin and bacitracin compared to wild-type JE2 (Table 4). A twofold reduction in MIC for vancomycin and D-cycloserine was observed only in the *lpdA : pdhA* double mutant. The *lpdA* mutant showed a twofold and the *lpdA : pdhA* double mutant showed a fourfold reduction in daptomycin MIC (Table 4).

Expression of *bkd* and *crt* locus genes in *pdhA* and *lpdA* mutants of *S. aureus* strain JE2

Even though there was a significant increase in the amount of staphyloxanthin produced in the *lpdA* mutant, in qPCR assays there was only a slight increase (1.38-fold) in expression of the the *crtM* gene (a gene of the staphyloxanthin pathway) in the *lpdA* mutant compared to its expression in the wild-type JE2 (Table 5). Similarly, a dramatic increase in the level of BCFAs was noted in the *pdhA* mutant, but in qPCR assays there was only a slight increase (1.25-fold) in expression of the *lpdA* gene (a gene of the BKD locus) in the

Table 3. Fluidity of *S. aureus* strains as measured by their relative polarization

Strain	Polarization value
Wild-type JE2	0.320±0.007
lpdA	0.374±0.016*
pdhA	0.274±0.001*
lpdA : *pdhA*	0.337±0.016
lpdA+pCU-BKD	0.317±0.011
pdhA+pCU-PDH	0.322±0.009

*P value ≤0.005.

pdhA mutant compared to its expression in the wild-type JE2 (Table 5).

DISCUSSION

Considering the well-developed abilities of *S. aureus* to adapt to its environment, where its membrane fatty acid composition plays a critical role, pathways that are likely to contribute to the production of BCFAs in this pathogen were investigated. The enzyme, BKD, acts on deaminated branched-chain amino acids and converts these to branched-chain acyl coenzyme A molecules. These acyl coenzyme A molecules then serve as precursors for BCFA synthesis through the activity of β-ketoacyl-acyl carrier protein synthase III (FabH) [28, 46–48]. β-keto-acyl carrier protein synthase II (FabF) is responsible for subsequent rounds of elongation until the acyl chain reaches 14 to 17 carbon atoms [28, 46–48].

Previously, we studied the role of BKD in a methicillin-susceptible *S. aureus* strain SH1000 and determined that the lack of BKD reduces the amount of BCFAs but that they are not completely eliminated [23]. Membrane fatty acid analysis in this study indicates that the amount of SCFAs is relatively higher in methicillin-resistant *S. aureus* strain JE2 (46.9 %) (Table 2) compared to strain SH1000 (32.4 %), as reported earlier [22, 23]. The ante-iso:iso fatty acid ratio (1.6) was also a little higher in strain JE2 than in strain SH1000 (1.4) [23]. However, TSB-grown bacterial cells were used for determining fatty acid composition in this study compared to *S. aureus* SH1000 cells grown in BHI medium in the previous study [23]. This factor is not expected to alter our conclusions, since *S. aureus* cells grown in TSB or BHI demonstrate a similar amount of BCFAs [22]. Overall, the fatty acid composition of the cytoplasmic membrane of the *lpdA* mutant of JE2 is similar to that of the *lpdA* mutant of SH1000 [23].

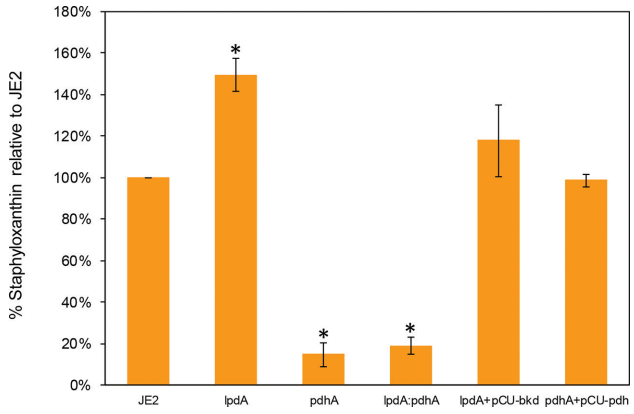

Fig. 3. Production of staphyloxanthin in the wild-type *S. aureus* strain JE2 and its derivative mutants. Values indicate the average of three independent experiments±standard error. 1 – Wild-type JE2; 2 – *lpdA*; 3 – *pdhA*; 4 – *lpdA* : *pdhA*; 5 – *lpdA*+pCU *bkd*; 6 – *pdhA*+pCU *pdh*.

The role of PDH in the production of BCFAs in this study was a bit unexpected. In *S. aureus* strain JE2, Tn disruption of *pdhA*, a gene responsible for one of the polypeptides of the PDH complex, led to a significant increase in the amount of BCFAs (78 %) and decrease in SCFAs in the cytoplasmic membrane compared to the wild-type bacterium, where the BCFAs account for about 50 %. Complementation of the mutant by the *pdh* locus helped restore the membrane fatty acid composition to that of the wild-type JE2. PDH acts on pyruvate and converts it to acetyl CoA, which then enters the tricarboxylic acid cycle [34]. Additionally, acetyl CoA can also lead to the production of butyryl CoA via the enzymes in the crotonyl CoA pathway [49]. FabH from *S. aureus* exhibits significant activity with butyryl CoA and also acetyl CoA [50], indicating that either compound could be the precursor for the synthesis of SCFAs. A comparison of the activity of PDH vs BKD [34] showed that BKD can also utilize pyruvate, albeit with lower efficiency than PDH, which helps to explain the low SCFA content in the membrane in the absence of functional PDH. The presence of both SCFAs and BCFAs in the membrane of the double mutant implies that another system is capable of supplementing the necessary precursors in the absence of both BKD and PDH. It is noteworthy that the proposed redundant system has a substrate specificity that resembles that of PDH, due to the higher amounts of even iso fatty acids and a similar ante-iso:iso ratio. The α-keto glutarate dehydrogenase (GDH) enzyme complex, with its homology to BKD and PDH complexes, was also considered to play a probable role in providing the precursors of SCFAs in the absence of BKD and PDH. However, the membrane fatty acid analysis of *S. aureus* JE2 lacking a functional GDH showed no alteration in composition compared to the wild-type JE2 bacterium (data not shown).

We postulated that PDH could account for the decreased BCFA that was shown in the absence of BKD, and thus we complemented the *lpdA* mutant with the *pdh* locus to investigate this hypothesis. Complementation with the *pdh* locus resulted in almost complete restoration of membrane BCFA content in the *lpdA* mutant, and a significant restoration of the BCFA content in the *lpdA* : *pdhA* double mutant. In addition, the iso even fatty acid content was determined to be higher in the strains complemented with the *pdh* locus *in*

Table 4. Susceptibilities of *S. aureus* parental strain JE2 and its isogenic mutants to cell wall- and membrane-active antibiotics

MIC values (μg ml^{-1}) indicate average of two independent experiments.

Strain	Antibiotic				
	Vancomycin	Oxacillin	Bacitracin	D-cycloserine	Daptomycin
Wild-type JE2	6.25	200	125	125	5
lpdA	6.25	200	125	125	2.5
pdhA	6.25	200	125	125	2.5
lpdA : pdhA	3.125	200	125	62.5	1.25

trans compared to even the wild-type levels. Kinetic characterization of PDH has shown its higher activity with α-keto iso valerate, a source of isobutyryl CoA (precursor of even-iso fatty acids) in comparison to α-keto iso caproate (source of odd-iso fatty acids). BKD on the other hand prefers α-keto iso caproate to α-keto iso valerate [34]. Thus, the membrane lipids of the *lpdA* mutant could reflect the intracellular acyl CoA pool resulting from the substrate specificity of PDH, while the membrane lipids of the *pdhA* mutant could reflect the substrate specificity of BKD.

The BCFA composition of the cell membrane in *S. aureus* seems to significantly impact cell growth. The BKD-deficient JE2 showed slower growth not only compared to the wild-type strain but even with respect to the PDH-deficient strain of JE2. The PDH-deficient strain is expected to have reduced ability to produce the energy needed for bacterial growth [51, 52]. An *lpdA : pdhA* double mutant showed the slowest growth in growth kinetic studies. When these strains were cultured at 20 °C, the *lpdA* and the *lpdA : pdhA* double mutant grew very poorly. These findings might also be indicative of reduced fitness of the mutants. A fitness issue with the *lpdA* mutant has also been documented in *S. aureus* SH1000 in regard to environmental stresses [23] and *Listeria monocytogenes* that showed poor survival in macrophages, in addition to its tolerance to unfavourable physiological conditions [53].

The membrane fluidity data in this study reflect the BCFA content in the cytoplasmic membrane. The *pdhA* mutant with highest BCFA level showed the greatest fluidity, and the *lpdA* mutant with smallest amount of BCFA in the cytoplasmic membrane showed the lowest fluidity. The *lpdA : pdhA* double mutant that showed BCFA content similar to the wild-type JE2 showed no significant change in membrane fluidity. Additionally, in a recent study it was documented that methyl-branched fatty acids reduce lipid condensation and the bi-layer thickness, resulting in the formation of kinks at the branching point that and a concomitant increase in membrane fluidity [54].

Table 5. Expression of *lpdA* and *crtM genes* in mutants of *S. aureus* strain JE2

Strain	Fold increase in expression of:	
	***crtM* gene**	***lpdA* gene**
lpdA	1.375±0.161	ND
pdhA	ND	1.252±0.113

ND, Not done.

Values indicate averages of three independent experiments±SEM.

Staphyloxanthin, a unique pigment in *S. aureus*, is not only important in its protection from host defence but also impacts membrane fluidity [55, 56]. The *lpdA* mutant appeared more pigmented during routine culturing on the TSA plates. Upon quantification, this mutant with low membrane BCFAs produced significantly elevated amounts of staphyloxanthin. In contrast, the *pdhA* mutant with a very high level of membrane BCFAs showed very little or no measurable production of staphyloxanthin. Elevated levels of staphyloxanthin contribute to reduced membrane fluidity [56], and the *lpdA* mutant had the least fluid membrane. In addition, acetyl CoA is not only a precursor of SCFAs but is also the first substrate in the mevalonate pathway that eventually yields carotenoids. In the absence of a functional PDH, the limited pool of acetyl CoA is funnelled towards fatty acid biosynthesis and thus is not available for staphyloxanthin production. In contrast, in the *lpdA* mutant, more acetyl CoA is likely funnelled into the staphyloxanthin pathway. This speculation is also supported by the fact that there was no increase in the expression of the genes of the staphyloxanthin pathway in the *lpdA* mutant.

The membrane fatty acid perturbations also seem to affect antibiotic tolerance of *S. aureus* to some extent. The *lpdA : pdhA* double mutant showed some sensitivity to the cell wall-inhibiting enzymes such as vancomycin and D-cycloserine, and also to a membrane-acting antibiotic daptomycin. This reduction in antibiotic tolerance may solely be due to the growth defects of the *lpdA* and *lpdA : pdhA* double mutants.

In summary, the PDH and the BKD enzyme complexes seem to have divergent effects on membrane fatty acid composition due to their substrate preferences and their specific roles in BCFA and energy metabolism. The lack of these enzymes causes significant perturbations in *S. aureus* membrane associated functions.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This manuscript has been prepared in accordance with the Ethics and Research Integrity policy of the Journal of Medical Microbiology.

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