

## Insertional Inactivation of Branched-Chain $\alpha$ -Keto Acid Dehydrogenase in *Staphylococcus aureus* Leads to Decreased Branched-Chain Membrane Fatty Acid Content and Increased Susceptibility to Certain Stresses<sup>∇</sup>

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*Staphylococcus aureus* is a major community and nosocomial pathogen. Its ability to withstand multiple stress conditions and quickly develop resistance to antibiotics complicates the control of staphylococcal infections. Adaptation to lower temperatures is a key for the survival of bacterial species outside the host. Branched-chain  $\alpha$ -keto acid dehydrogenase (BKD) is an enzyme complex that catalyzes the early stages of branched-chain fatty acid (BCFA) production. In this study, BKD was inactivated, resulting in reduced levels of BCFAs in the membrane of *S. aureus*. Growth of the BKD-inactivated mutant was progressively more impaired than that of wild-type *S. aureus* with decreasing temperature, to the point that the mutant could not grow at 12°C. The growth of the mutant was markedly stimulated by the inclusion of 2-methylbutyrate in the growth medium at all temperatures tested. 2-Methylbutyrate is a precursor of odd-numbered anteiso fatty acids and bypasses BKD. Interestingly, growth of wild-type *S. aureus* was also stimulated by including 2-methylbutyrate in the medium, especially at lower temperatures. The anteiso fatty acid content of the BKD-inactivated mutant was restored by the inclusion of 2-methylbutyrate in the medium. Fluorescence polarization measurements indicated that the membrane of the BKD-inactivated mutant was significantly less fluid than that of wild-type *S. aureus*. Consistent with this result, the mutant showed decreased toluene tolerance that could be increased by the inclusion of 2-methylbutyrate in the medium. The BKD-inactivated mutant was more susceptible to alkaline pH and oxidative stress conditions. Inactivation of the BKD enzyme complex in *S. aureus* also led to a reduction in adherence of the mutant to eukaryotic cells and its survival in a mouse host. In addition, the mutant offers a tool to study the role of membrane fluidity in the interaction of *S. aureus* with antimicrobial substances.

*Staphylococcus aureus* is an aggressive bacterial pathogen that is responsible for a variety of diseases, ranging from pyogenic skin infections to complicated life-threatening diseases, such as bacteremia and endocarditis (20, 28). It is estimated that 32.4% of the population in the community and up to 90% of health care professionals in the United States are colonized with *S. aureus* (21). In this context, the spread of methicillin-resistant *S. aureus* strains, and the more recent emergence of resistance to vancomycin, poses a significant threat in terms of our ability to control infections by this major human pathogen.

*S. aureus* encounters a wide range of environments that include thermal fluctuations and a nutritionally restricted milieu and must adapt to these conditions in order to survive. Cytoplasmic membranes are important barriers between bacterial cells and the environment, and it is essential for the bacteria to regulate the fluidity of their cytoplasmic membrane for the proper functioning of various membrane-associated processes. *S. aureus* has a complex fatty acid composition comprised of straight-chain saturated fatty acids (SCFAs), unsaturated fatty acids, and branched-chain fatty acids (BCFAs) (30,

35). BCFAs account for about 55 to 65% of the total fatty acids, and anteiso C15:0 is the major BCFA. BCFAs in general, and anteiso C15:0 in particular, are major determinants of membrane fluidity in *S. aureus*.

In *Bacillus subtilis*, two enzymes are critical in the synthesis of BCFAs: branched-chain  $\alpha$ -keto acid dehydrogenase (BKD) and  $\beta$ -ketoacyl-acyl carrier protein synthase III (FabH) (6, 19, 25). The synthesis of BCFAs begins with the transamination of isoleucine, valine, and leucine by branched-chain amino acid transaminase. The products of this reaction are subsequently decarboxylated by the BKD enzyme complex to produce short branched-chain acyl coenzyme A (acyl-CoA) derivatives 2-methylbutyryl-CoA, isobutyryl-CoA, and isovaleryl-CoA from isoleucine, valine, and leucine, respectively. These acyl-CoA precursors are then utilized by FabH to initiate BCFA biosynthesis.

BKD is a multisubunit enzyme complex that has been studied for several bacteria, e.g., *Pseudomonas aeruginosa* (22), *Pseudomonas putida* (34), *B. subtilis* (18), and *Listeria monocytogenes* (38). The purified enzyme complex is composed of four polypeptides, a dehydrogenase (E1 $\alpha$ ), a decarboxylase (E1 $\beta$ ), a dihydrolipoamide acyltransferase (E2), and a dihydrolipoamide dehydrogenase (E3) (8, 38). Genes encoding these four polypeptide components are organized in a cluster and are coregulated. A comprehensive search of the *S. aureus* genome sequence ([http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list\\_uids=610](http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=610)) identified a locus consisting of four genes which encode pro-

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TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Characteristics <sup>a</sup>	Source or reference
<b>Bacteria</b>		
<i>S. aureus</i> RN4220	Restriction-minus derivative of <i>S. aureus</i> strain 8325-4	16
<i>S. aureus</i> SH1000	<i>S. aureus</i> strain 8325-4 with functional <i>rsbU</i>	12
VKS102	<i>S. aureus</i> SH1000 with mutation in the <i>lpd</i> gene of BKD (Kan <sup>r</sup> )	This study
VKS103	SH1000Δ <i>lpd</i> containing pCU- <i>bkd</i> plasmid (Kan <sup>r</sup> and Cam <sup>r</sup> )	This study
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyra96 relA1 thi Δ(lac-proAB) F' (traD36 proAB<sup>+</sup> lacI<sup>q</sup>ΔM15)</i>	36
<b>Plasmids</b>		
pGEMT	Cloning vector for <i>E. coli</i> (Amp <sup>r</sup> )	Promega
pCU1	Shuttle vector (Amp <sup>r</sup> in <i>E. coli</i> and Cam <sup>r</sup> in <i>S. aureus</i> )	2
pGEM- <i>lpd</i>	Plasmid pGEMT containing a 2.2-kb DNA fragment encompassing the <i>S. aureus lpd</i> gene	This study
pGEM- <i>bkd</i>	5.1-kb DNA fragment containing all four genes of the <i>S. aureus bkd</i> locus	This study
pGEM- <i>lpd</i> -Kan	1.5-kb Kan <sup>r</sup> gene was inserted at the HindIII site of construct pGEMT- <i>lpd</i>	This study
pCU- <i>bkd</i>	Plasmid pCU1 containing a 5.1-kb DNA fragment containing all four genes of the <i>S. aureus bkd</i> locus	This study

<sup>a</sup> Kan<sup>r</sup>, kanamycin resistant; Cam<sup>r</sup>, chloramphenicol resistant; Amp<sup>r</sup>, ampicillin resistant.

teins with significant sequence homology with the four subunit proteins of the BKD enzyme complex of *B. subtilis* and *L. monocytogenes*. To investigate the roles of this locus in *S. aureus* physiology, a BKD-deficient strain of *S. aureus* was created by insertional inactivation of the first gene (dihydrolypoamide dehydrogenase; *lpd*) of the *bkd* locus. Further studies with this mutant demonstrated that the lack of a functional BKD enzyme complex in *S. aureus* leads to an alteration in membrane fatty acid composition, decreased membrane fluidity, enhanced susceptibility to alkaline pH and hydrogen peroxide stressors, reduced adherence to eukaryotic cells, and reduced survival in a murine host.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmid constructs used in this study are shown in Table 1. *S. aureus* was grown in tryptic soy broth or agar (TSB or TSA; Becton Dickinson) or in brain heart infusion (BHI) medium (Becton Dickinson). *Escherichia coli* cells were grown in Luria-Bertani broth or agar. When needed, ampicillin (50 μg ml<sup>-1</sup>), kanamycin (30 μg ml<sup>-1</sup> in the case of *E. coli*; 100 μg ml<sup>-1</sup> in the case of *S. aureus*), and chloramphenicol (10 μg ml<sup>-1</sup>) were added to the growth medium.

**DNA manipulation and analysis.** Plasmid DNA was isolated using a Qiaprep kit (Qiagen, Inc.); chromosomal DNA was isolated using a DNAzol kit (Molecular Research Center) from lysostaphin-treated *S. aureus* cells, per the manufacturer's instructions. All restriction and modification enzymes were purchased from Promega. DNA manipulation and Southern blot analysis were carried out using standard procedures. PCR was performed with a PTC-200 Peltier thermal cycler (MJ Research). Oligonucleotide primers were obtained from Sigma Genosys.

**Construction of the mutation in the first gene of the *S. aureus* BKD gene cluster.** A functional BKD enzyme is generated by the association of polypeptides encoded by individual genes of the four-gene BKD cluster (8, 38). To inactivate the BKD enzyme complex in *S. aureus*, the *lpd* gene, which is the first gene of the *S. aureus* BKD gene cluster, was disrupted by the insertion of a kanamycin resistance cassette in the coding region. To generate this mutant, two primers (forward primer 5'-CTACCGGTGAACCTTTGAGA-3' and reverse primer 5'-GGCAGAGAAAATGCGAGA-3') were used to amplify a 2,213-bp DNA fragment using genomic DNA from *S. aureus* strain SH1000 as the template. This amplicon represents a DNA fragment starting 409 nt upstream and spans the entire *lpd* gene of the *bkd* gene cluster. This amplicon was cloned in vector pGEM-T Easy (Promega) to generate the construct pGEM-*lpd*. The cloned fragment contained a unique HindIII restriction site into which a kanamycin resistance cassette (11) was cloned. The resulting construct, pGEM-*lpd*-kan, was used as a suicide plasmid to transform *S. aureus* strain RN4220 (a restriction-minus strain) by electroporation (29). The transformants were selected on TSA plates containing kanamycin. The selection resulted in a single crossover and integration of the entire construct into the *S. aureus* chromosome.

Phage 80 $\alpha$  was propagated on these transformants and used to resolve the mutation in the *lpd* gene in *S. aureus* by performing transduction outcrosses as described previously (32, 33). The transductants were confirmed for a mutation in the *lpd* gene using PCR and Southern blotting. For genetic complementation studies, the entire *bkd* locus starting 225 nt upstream of the first gene (*lpd*) and terminating 133 bp after the fourth gene (*bkdB*) was PCR amplified (5,058-bp amplicon) using appropriate primers (forward primer 5'-TGATTCAACCAT GTTGATT-3' and reverse primer 5'-TCAGGTGCAAGTGTACTG-3') and *S. aureus* SH1000 genomic DNA as the template. PCR was carried out using Stratagene EXL DNA polymerase (Stratagene, CA) per the manufacturer's instructions. This amplicon was cloned in vector pGEM-T from which it was subcloned in vector pCU1 (2). The *lpd* mutant of *S. aureus* strain SH1000 was subsequently transformed with this construct.

**Analysis of fatty acid composition in wild-type and *lpd* mutant *S. aureus*.** To determine the membrane fatty acid composition, cultures of the *lpd* mutant and its isogenic *S. aureus* parent strain were grown in 100 ml BHI medium at 37°C. Cells were harvested in the mid-exponential phase (optical density at 600 nm [OD<sub>600</sub>] of 0.5 to 0.7), and the cell pellet was washed three times with distilled water. The fatty acids in the bacterial cells (30 to 40 mg [wet weight]) were saponified, methylated, and extracted as described previously (38). The resulting methyl ester mixtures were separated by an Agilent 5890 dual-tower gas chromatograph. Fatty acids were identified by a Midi microbial identification system (Sherlock 4.5). This analysis was performed at Microbial ID, Inc. (Newark, DE).

**Growth kinetics of wild-type *S. aureus* and its isogenic *lpd* mutant.** Mid-exponential phase cultures (OD<sub>600</sub> = 0.6) were diluted 50-fold in an Erlenmeyer flask containing 50 ml fresh BHI medium with a flask-to-medium volume ratio of 6:1. All supplements (0.1 mM 2-methylbutyrate, isobutyrate, or isovalerate) were added to the medium as filter-sterilized solutions. In parallel flasks, the following stress conditions were imposed through the appropriate modifications of BHI medium: 8.8 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), low pH (pH 5.5), high pH (pH 9.5), and NaCl (1.5 M). Bacterial growth was subsequently monitored by incubating the flask in a shaking incubator (250 rpm) and measuring the turbidity of the liquid culture at OD<sub>600</sub> by using a Beckman DU-70 spectrophotometer.

**Determination of membrane fluidity of the *lpd* mutant and its isogenic wild-type strain SH1000.** Membrane fluidity was measured as recently described (3, 4). In brief, overnight grown cultures were used to inoculate fresh TSB and incubated at 37°C with shaking (250 rpm) until an OD<sub>600</sub> of 0.6 ± 0.05 was reached. The bacteria were pelleted by centrifugation at 4°C and washed twice with 0.85% NaCl. The cells were resuspended in 0.85% NaCl containing 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma, MO) to an OD<sub>600</sub> of 0.3 ± 0.05. DPH specifically labels and fluoresces within the hydrophobic regions of the lipid bilayer but does not fluoresce in aqueous environments (3, 4). Fluorescence polarization was subsequently measured using an SLM Aminco 8000C spectrofluorometer (SLM Aminco, SLM Instruments, Inc., IL). Excitation of the fluorescent probe was accomplished with vertically polarized monochromatic light at 360 nm for DPH, with emission intensity quantified at 426 nm, using a detector oriented either parallel to or perpendicular to the direction of the polarized excitation source. The experiment was performed three times, and the mean polarization values were compared for statistically significant differences by using the Student *t* test.

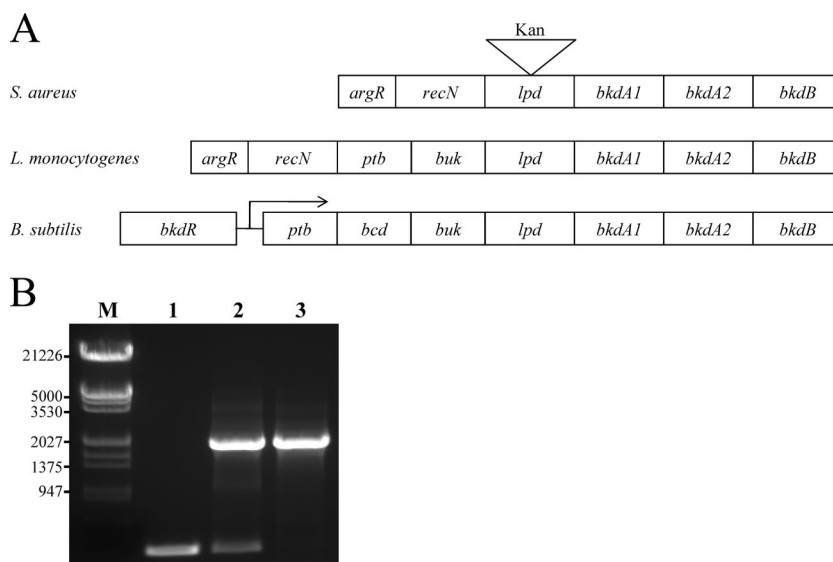


FIG. 1. (A) Schematic organization of the *bkd* gene cluster in three gram-positive bacteria. *argR*, arginine repressor; *recN*, DNA repair protein; *ptb*, phosphate acetyl/butyryltransferase family protein; *bcd*, leucine dehydrogenase; *buk*, butyrate kinase; *bkdA1/bkdAA*, BKD E1 $\alpha$  subunit; *bkdA2/bkdAB*, BKD E1 $\beta$  subunit; *bkdB*, dihydrolipoamide acetyltransferase. *bkdR* encodes a SigL-dependent regulator that regulates the expression of *bkd* genes in *B. subtilis*. *bkdR* is absent in the cases of *S. aureus* and *L. monocytogenes*. Instead, in these species, a gene encoding a DNA repair protein (RecN) is present upstream of *bkd* genes. (B) Construction and confirmation of mutation in the *lpd* gene in *S. aureus*. A kanamycin resistance gene has been inserted at the HindIII site of the *lpd* gene. Primers P1 (5'-TATACAATCACCAGCTGCA-3') and P2 (5'-ACAGTTATAGAAGCAGGTGA-3') were used in PCRs that allowed amplification of a 336-bp product in the case of the genomic DNA template from wild-type *S. aureus* (lane 1). The same primers amplified an ~1.8-kb amplicon in the case of an *lpd* mutant due to insertion of a 1.5-kb kanamycin resistance cassette (lane 3). The presence of two bands when genomic DNA from the merodiploid was used as the template suggests the presence of wild-type and mutated *lpd* genes (lane 2). Lane M, EcoRI/HindIII digest of  $\lambda$  DNA.

**Measurement of solvent tolerance.** Solvent tolerance was determined using the plate overlay method as previously described (24). Briefly, 20  $\mu$ l of overnight-grown cultures ( $\sim 10^6$  cells) of strain SH1000 and the *lpd* mutant were spotted separately onto BHI agar and BHI agar supplemented with 0.1 mM 2-methylbutyrate in glass petri dishes. The plates were incubated at 37°C, and the spots were allowed to dry for 45 min. Around 5 ml of toluene was directly pipetted onto the top of each agar plate surface in a well-ventilated laboratory area. The plates were incubated at room temperature for 8 h, and toluene was then poured off from the plates. The plates were inverted, and different sets were incubated at 25°C and 37°C for 24 h.

**Autolysis assays.** Autolysis assays were performed as previously described (15, 26). Briefly, wild-type and *lpd* mutant cultures of *S. aureus* SH1000 were grown to an OD<sub>600</sub> of 1.0 at 37°C in PYK medium (0.5% Bacto peptone, 0.5% yeast extract, 0.3% K<sub>2</sub>HPO<sub>4</sub> [pH 7.2]). After one wash with cold water (8,500  $\times$  g, 4°C, 15 min), cells were suspended in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.05% Triton X-100 to a OD<sub>600</sub> of 1.0. The flasks were incubated at 37°C with shaking (150 rpm), and the subsequent decline in the turbidity of the bacterial cell suspension was measured spectrophotometrically at 600 nm every 30 min for 10 h. Autolysis of the *lpd* mutant was also analyzed in terms of total and membrane-bound autolysin in the mutant compared to that for wild-type *S. aureus* strain SH1000. The total autolysins were extracted after the bead beating of bacterial cells in 0.25 M phosphate buffer (pH 7.0) using a BioSpec Mini-Beadbeater after growth in PYK medium to an OD<sub>600</sub> of 1.0. The membrane autolysins were extracted by exposing the bacterial cells to five freeze-thaw cycles in 0.25 M phosphate buffer (pH 7.0). The cells were vortexed vigorously after each thaw. The samples were analyzed for the presence of autolysins by a zymographic method using autoclaved *S. aureus* 8325-4 cells as described previously (15, 26).

**Adherence assays.** The relative adherence of *S. aureus* strain SH1000 and its derivative *lpd* mutant were determined with a mixed infection of A549 human lung epithelial cells (ATCC CCL 185) as recently described (17). Wild-type *S. aureus* strain SH1000 and its derivative *lpd* mutant were grown in BHI medium to an OD<sub>600</sub> of 0.3. Bacterial cells were washed three times in PBS and mixed in F-12K medium (ATCC). The resulting mixture was biased for mutant cells to better determine if there was any appreciable decrease in the adherence of the mutant compared to that of wild-type cells. A549 cells were cultured in F-12K medium supplemented with 10% heat-inactivated calf serum at 37°C in a hu-

midified 5% CO<sub>2</sub> atmosphere. For adherence assays, approximately  $5 \times 10^5$  bacterial cells were added to the monolayers of A549 cells ( $\sim 2 \times 10^5$  cells/well) to give an approximate multiplicity of infection of 2.5:1 (bacteria/A549 cells). The plate was centrifuged at  $100 \times g$  for 5 min to facilitate contact between bacteria and A549 cells. After 1 h of incubation, nonadherent bacterial cells were removed by washing the epithelial cell monolayer three times with warm sterile PBS. Next, epithelial cells were dispersed by the addition of 150  $\mu$ l of 0.25% trypsin-1 mM EDTA (Sigma) and then lysed by the addition of 400  $\mu$ l of 0.025% Triton X-100. The numbers of bacterial CFU adhering to the epithelial cells were determined by plating of diluted epithelial cell lysates on TSA plates with and without kanamycin. The fraction of *lpd* mutants that adhered to the A549 cells was then calculated and compared to the fraction of *lpd* mutant cells in the mixed culture used for adherence assays. Each experiment was conducted in triplicate.

**Survival of the wild-type and *S. aureus lpd* mutant cells in a murine systemic infection model.** In vivo survival experiments were carried out as described recently (33). Briefly, *S. aureus* strain SH1000 and its isogenic *lpd* mutant were grown to mid-log phase (OD<sub>600</sub> = 0.6) in BHI medium and subsequently washed three times with BHI medium. Wild-type and the *lpd* mutant cells were subsequently combined in BHI medium (72%:28% mixture of mutant/wild-type), and 0.25 ml of this suspension, containing  $1.15 \times 10^7$  bacteria, was injected into the peritoneal cavity of Swiss white Hla(ICR)CVF female mice (16 to 20 g) with a 26-gauge needle fitted to a 1-ml syringe. At 4, 8, and 18 h, three mice were euthanized by CO<sub>2</sub> asphyxiation. The liver and spleen were aseptically removed and homogenized in 2 and 1 ml of BHI medium, respectively, using a glass tissue grinder fitted with a glass pestle. Tissue homogenates were serially diluted, plated on TSA plates with or without kanamycin and allowed to grow overnight by incubation at 37°C. The bacterial colonies growing in the presence of kanamycin were used to calculate the fraction of *lpd* mutants relative to wild-type bacteria in the infected tissues and compared to the fraction of *lpd* mutants in the mixed suspension that was used to inject mice.

## RESULTS

**Genetic organization of the *bkd* gene cluster and construction of a BKD-null mutant in *S. aureus*.** The genetic organization of the *bkd* gene cluster in *S. aureus* is shown in Fig. 1A. A

TABLE 2. Fatty acid profiles of wild-type SH000, the *lpd* mutant strain, and a complemented strain

Fatty acid	% (wt/wt) of total fatty acids <sup>a</sup>					
	Wild type		<i>lpd</i> mutant		Complemented strain	
	BHI	BHI + 2MB	BHI	BHI + 2MB	BHI	BHI + 2MB
Iso C13:0	<1.0	<1.0	1.28 ± 0	<1.0	<1.0	<1.0
Iso C14:0	4.7 ± 0.8	<1.0	8.7 ± 1.3	1.3 ± 0.6	5.6 ± 5.2	<1.0
C14:0	3.6 ± 0.4	1.7 ± 0.4	13.6 ± 0.9	7.3 ± 2.3	5.9 ± 5.9	5.8 ± 0.1
Iso C15:0	12.0 ± 1	11.3 ± 0	4.6 ± 0.5	4.2 ± 1.3	10.7 ± 5.5	6.3 ± 0.1
Anteiso C15:0	29.4 ± 0.4	40.1 ± 2.8	14.1 ± 1.1	37.1 ± 0.9	27.9 ± 8.2	42.8 ± 3.3
Iso C16:0	1.5 ± 0	<1.0	2.4 ± 0.6	<1.0	2.8 ± 0.6	<1.0
C16:0	7.3 ± 0.9	5.0 ± 0.6	18.8 ± 0.3	12.3 ± 1.2	10.2 ± 6.1	11.9 ± 1.2
Iso C17:0	2.3 ± 1.0	3.5 ± 1.2	<1.0	<1.0	2.9 ± 3	<1.0
Anteiso C17:0	4.0 ± 2.5	8.2 ± 4.4	<1.0	3.6 ± 1.4	4.9 ± 5.1	3.5 ± 0.5
C17:0	1.4 ± 0	<1.0	1.4 ± 0.4	1.1 ± 0	1.0 ± 0.6	1.1 ± 0.1
C18:0	15.7 ± 3.9	14.1 ± 4.6	17.4 ± 3.0	16.8 ± 2.8	13.7 ± 0.9	13.3 ± 0.7
C19:0	2.8 ± 1.1	2.0 ± 1.1	1.3 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.1 ± 0.2
C20:0	8.4 ± 4.8	7.3 ± 2.9	6.6 ± 0.9	6.5 ± 0.7	5.5 ± 0.4	3.8 ± 0
Anteiso	37.6	49.9	16.1	41.4	33.5	47.6
Iso	25.9	18.9	19.3	8.5	26.2	10.8
Anteiso/iso ratio	1.4	2.6	0.8	4.9	1.3	4.4
SCFA	32.4	29.3	61.5	46.7	38.7	38.3
BCFA	63.5	68.8	35.4	50	59.7	58.4

<sup>a</sup> Shown are fatty acid profiles of wild-type SH000, its derivative *lpd* mutant, and the *lpd* mutant complemented in *trans* with all four genes of the *S. aureus lpd* gene locus after growth in BHI medium with and without 2-methylbutyrate (2MB), a precursor of odd-numbered anteiso fatty acids. Values are the average percentages of fatty acids from three independent experiments ± standard deviations. Fatty acids present in less than 1% of the total are not reported.

comparison of the *bkd* gene cluster in three gram-positive bacterial species indicates a similar genetic organization of the *bkd* structural genes but presents interesting differences in the genes upstream of this cluster (Fig. 1A). In *B. subtilis*, the *bkd* gene cluster contains seven genes, in contrast to what appears to be only four genes in the cases of *S. aureus* and *L. monocytogenes* (38). The seven-gene *bkd* cluster in *B. subtilis* is cotranscribed, and the expression of this locus is under the control of a regulatory protein, BkdR (8). Expression of *bkdR* itself is under the control of an alternative sigma factor, SigL (8). A *bkdR*-like gene is not present upstream of the *bkd* locus in the case of *S. aureus*. Rather, a gene (*recN*) encoding a putative DNA repair protein is present in the *S. aureus* chromosome immediately upstream of the first gene (*lpd*) of the *bkd* locus. To characterize the function of the *S. aureus* BKD, a kanamycin resistance cassette was inserted within the first gene of the *bkd* cluster to inactivate this enzyme complex. The production of a larger PCR product when genomic DNA from the resulting mutant was used as the template (Fig. 1B, lane 3) confirmed the insertion of a kanamycin gene cassette in the *lpd* open reading frame. Products of all four individual genes of the *bkd* cluster associate to form the functional BKD enzyme. It is speculated that a mutation in the *lpd* gene will have a polar effect on the expression of the entire *bkd* locus. Even if the mutant generated in this study is “leaky” for the expression of *lpd* downstream genes, the mutant bacteria will fail to generate an active BKD enzyme complex due to the lack of a functional Lpd.

**Analysis of fatty acid composition in the wild type and the *lpd* mutant of *S. aureus*.** The fatty acid compositions of strain SH1000 and its derivative *lpd* mutant are shown in Table 2. The fatty acid composition of strain SH1000 was typical for *S. aureus*, showing a complex mixture of BCFAs and SCFAs with anteiso C15:0 (29.4%) and C18:0 (15.7%) as the major fatty acids. Sixty-three percent of the total fatty acids were BCFAs,

and 32% were SCFAs; the anteiso/iso fatty acid ratio was 1.4. The *lpd* mutant strain was deficient in BCFAs (35.4%), and the percentage of SCFAs rose to compensate (61.5%). The anteiso/iso fatty acid ratio was 0.8 in the *lpd* mutant. When the *lpd* mutant strain was grown in the presence of 0.1 mM 2-methylbutyrate (a precursor of odd-numbered anteiso BCFAs), the BCFAs were restored to 50% of the total, with a major increase in anteiso C15:0 from 14.1% in the mutant grown in the absence of 2-methylbutyrate to 37.1% in its presence. The anteiso/iso fatty acid ratio was increased to 4.9 in the presence of 2-methylbutyrate. Interestingly, 2-methylbutyrate increased the BCFAs in SH1000 to 68.8%, anteiso C15:0 to 40.1%, and the anteiso/iso fatty acid ratio to 2.6. These results clearly show that the *lpd* mutant is deficient in BCFAs. In the growth experiments (see below), isobutyrate and isovalerate were less effective than 2-methylbutyrate in restoring the growth defect of the *lpd* mutant, consistent with the lower impact of iso than anteiso BCFAs on increasing membrane fluidity. Isobutyrate stimulated the production of the even-numbered iso fatty acids iso C14:0 and iso C16:0 in the *lpd* mutant (data not shown). The effects of 2-methylbutyrate on fatty acid composition are consistent with its effects on growth (see below). When cells of either SH1000 or the *lpd* mutant were grown at 25°C, the major changes in fatty acid composition of both strains were increases in anteiso C15:0 and total BCFAs and decreases in C18:0 and total SCFAs (data not shown). Similar findings were reported by Joyce et al. (13).

**The *lpd* mutant showed reduced growth at low temperatures that could be alleviated with short BCFA precursors.** A comparison of the growth kinetics shows that the growth of the *lpd* mutant was somewhat slower at 37°C than that of the wild-type strain SH1000 (Fig. 2C). However, growth of the *lpd* mutant was significantly slower at 20°C than that of wild-type *S. aureus* (Fig. 2B). When growth was monitored at 12°C, the wild-type SH1000 was able to grow after a long lag, but the *lpd* mutant

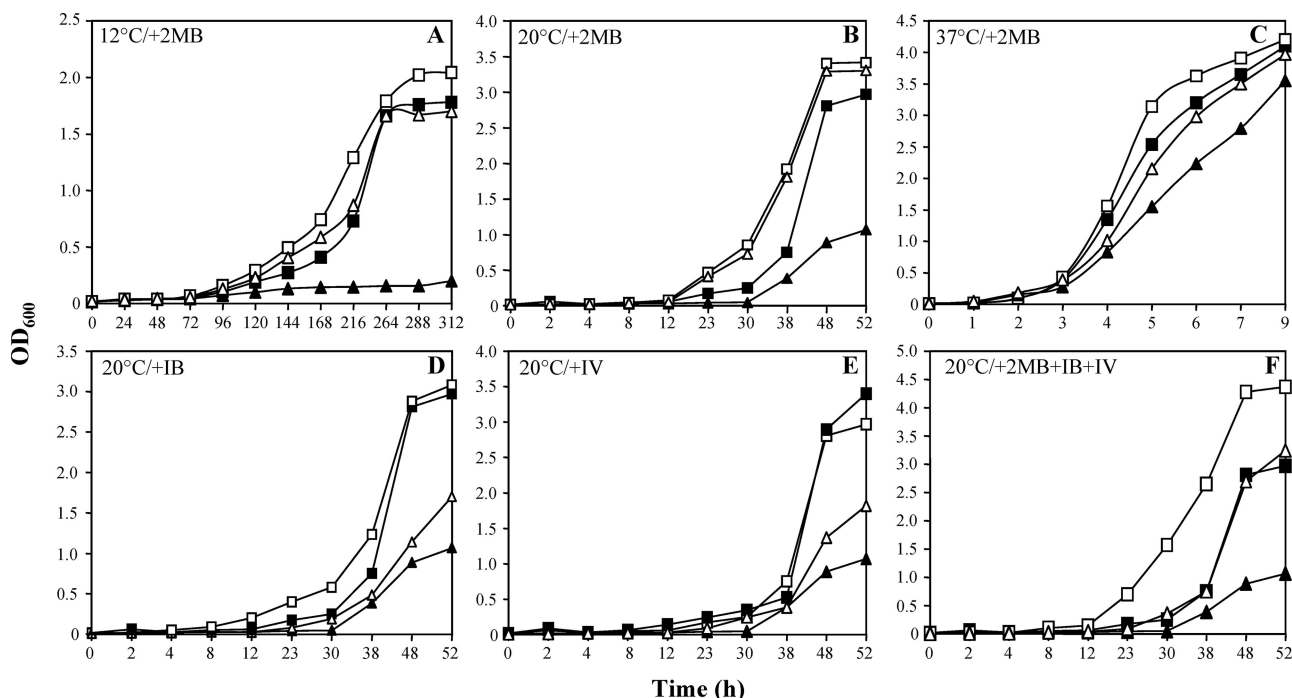


FIG. 2. Growth kinetics of the wild-type *S. aureus* strain SH1000 and its derivative *lpd* mutant with and without short-chain keto acid precursors (2MB, 2-methylbutyrate; IB, isobutyrate; IV, isovalerate). Growth temperatures and the precursors used are indicated. Closed squares, wild-type *S. aureus* strain SH1000; open squares, wild-type SH1000 with supplement; closed triangles, *lpd* mutant of strain SH1000; open triangles, *lpd* mutant with supplement.

failed to grow even after 312 h of incubation at this temperature in medium without added fatty acid precursor (Fig. 2A). These results suggest a critical role for the BKD enzyme complex during growth and survival of *S. aureus*, particularly at lower temperatures. To evaluate whether this defect in growth during cold conditions was indeed due to lack of a functional BKD, the media were supplemented with 2-methylbutyrate, isobutyrate, or isovalerate at 0.1 mM concentrations. Supplementation with these short-chain fatty acids allows the mutant bacteria to bypass the biochemical step that requires BKD activity and results in the synthesis of BCFAs. When supplemented with 0.1 mM 2-methylbutyrate, a precursor of odd-numbered anteiso fatty acids, the *lpd* mutant bacteria were able to grow at markedly increased rates at all temperatures (Fig. 2A to C) and especially at 20°C or 12°C. It is interesting that the growth of the wild-type *S. aureus* strain was also enhanced in the presence of 0.1 mM 2-methylbutyrate (Fig. 2A to C), which implies that the BHI growth medium may not provide an ideal environment for the synthesis of BCFA or that BKD activity is usually expressed at a low level in *S. aureus*. However, at 0.1 mM concentrations, neither isobutyrate nor isovalerate, which are precursors of even-numbered and odd-numbered iso fatty acids, respectively, were as effective in restoring the growth of the *lpd* mutant as 2-methylbutyrate (Fig. 2D and E). The combination of all three fatty acid precursors had a strong stimulatory effect on the growth of wild-type *S. aureus* at 20°C (Fig. 2F).

**A nonfunctional BKD enzyme leads to a less-fluid staphylococcal cell membrane.** In membrane fluidity measurements, a statistically significant difference ( $P < 0.004$ ) in the polariza-

tion value ( $0.390 \pm 0.004$ ) was recorded for wild-type *S. aureus* strain SH1000 cells compared to the polarization value of the *lpd* mutant cells ( $0.432 \pm 0.011$ ). These findings are in agreement with a higher polarization value for a less-fluid membrane (3, 4).

**Decreased toluene tolerance of the *lpd* mutant.** It has been reported that solvent tolerance in staphylococci is associated with an increased proportion of anteiso fatty acids and hence increased membrane fluidity, in contrast to the situation for gram-negative bacteria, where solvent tolerance is associated with decreased membrane fluidity (24). Hence, it was of interest to see whether the *lpd* mutant showed a decreased tolerance of toluene, and the results of these experiments are shown in Fig. 3. Strain SH1000 showed significant expanding ring-type growth at 25°C that was enhanced on BHI agar plates supplemented with 2-methylbutyrate. In comparison, growth of the *lpd* mutant was faint at 25°C on BHI agar, although its growth was enhanced on 2-methylbutyrate-supplemented agar. The results are consistent with decreased membrane fluidity correlating with increased solvent susceptibility.

**Response of the *lpd* mutant to various stress conditions.** The *lpd* mutant was also measured for growth defects compared to wild-type *S. aureus* under different stress conditions. No apparent difference in the growth kinetics of the *lpd* mutant was observed under acidic (BHI medium acidified to pH 5.5) or high-salt (BHI medium with an additional 1.5 M NaCl) conditions (data not shown). However, the growth of the *lpd* mutant was significantly slower at an alkaline pH (BHI medium, pH 9.5) than that of wild-type *S. aureus* strain SH1000 (Fig. 4A). In addition, in BHI medium with 8.8 mM  $H_2O_2$ , the

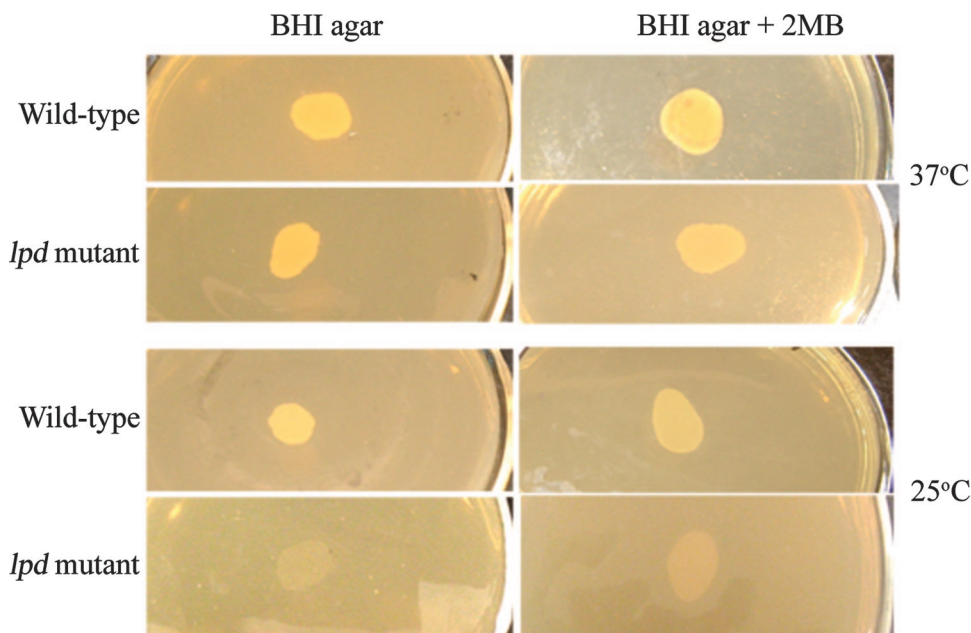


FIG. 3. Growth of SH1000 and the *lpd* mutant on toluene overlay agar plates. SH1000 and the *lpd* mutant were used to inoculate BHI agar with or without 2-methylbutyrate (2-MB), and cultures were incubated at 37°C and 25°C.

growth of the *lpd* mutant was severely retarded and no visible turbidity of the mutant was apparent by the time the wild-type *S. aureus* cultures reached stationary phase (Fig. 4B). These growth defects of the *lpd* mutant at an elevated pH and in the presence of H<sub>2</sub>O<sub>2</sub> were restored to a significant level when the mutant was complemented with the entire *bkd* locus on a plasmid in *trans* (Fig. 4A and B).

**Inactivation of BKD decreases autolysis of *S. aureus* cells.** In experiments aimed to investigate Triton X-100-stimulated autolysis, the *lpd* mutant cells demonstrated a lower rate of autolysis than the wild-type SH1000 cells (Fig. 5A). Interestingly, as is apparent from Fig. 5B, there was no appreciable difference between the total autolysin profiles for the *lpd* mutant (lanes 4) and for wild-type *S. aureus* strain SH1000 (lane 3). However, the zymographic pattern of the freeze-thaw extractable cell surface autolysins for the *lpd* mutant cells was signif-

icantly different from that for wild-type *S. aureus*. The number of autolysin bands was greater in wild-type *S. aureus* strain SH1000 (Fig. 5B, lane 1) than in its derivative *lpd* mutant (Fig. 5B, lane 2), and overall, the bands were of greater intensity in the SH1000 extract.

**Lack of a functional BKD impairs staphylococcal adherence to eukaryotic cells and the ability to survive in vivo.** In adherence assays, a mixture of *lpd* mutant and wild-type *S. aureus* bacteria ( $4.57 \times 10^5$  CFU) was added to the monolayers of A549. A total of  $(8.3 \pm 2.2) \times 10^4$  bacterial cells adhered (18.1% adherence) to A549 cells. Although the ratio of the *lpd* mutant cells to wild-type SH1000 in the mixture that was used for adherence was 68:32, the ratio of these two cell types in the bound fraction was 47:53. Additionally, whereas the relative adherence rate of the wild type cells was 30.2%, only 12.51% of the mutant cells were bound to A549 cells. The experiment

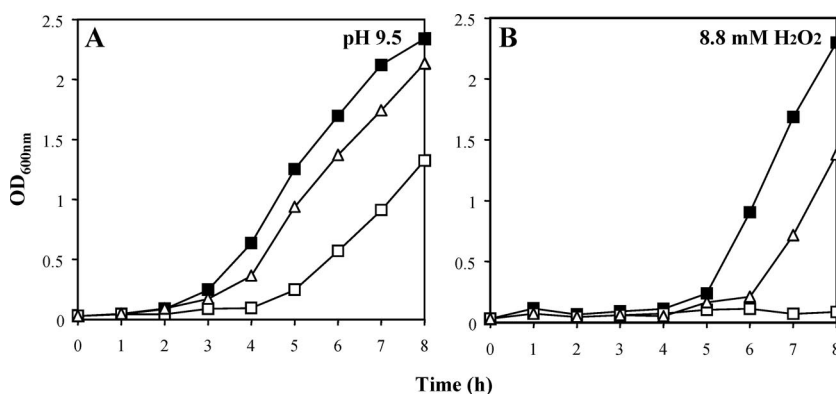


FIG. 4. Growth kinetics of the *lpd* mutant and its isogenic wild-type *S. aureus* under stress conditions. (A) Growth in BHI medium modified to pH 9.5. (B) Growth in BHI medium containing 8.8 mM H<sub>2</sub>O<sub>2</sub>. Closed squares, wild-type *S. aureus* strain SH1000; open squares, *lpd* mutant of strain SH1000; open triangles, *lpd* mutant of strain SH1000 complemented with a 5.1-kb wild-type *bkd* gene locus on plasmid pCU1.

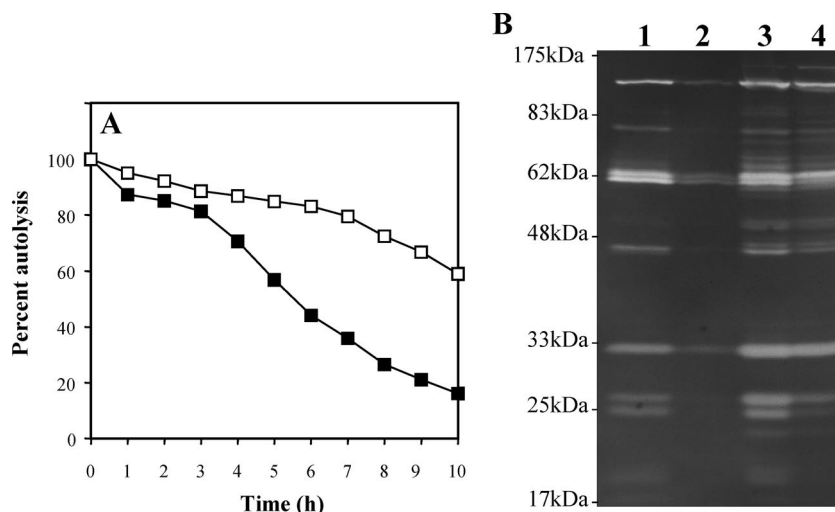


FIG. 5. (A) Autolysis of the *lpd* mutant (open squares) compared to autolysis of the isogenic wild-type *S. aureus* strain SH1000 (closed squares). (B) Autolysin profiles of freeze-thaw (lanes 1 and 2) and total (lanes 3 and 4) autolysin extracts from *S. aureus* strains against *S. aureus* 8325-4 cells. Lanes 1 and 3, wild-type *S. aureus* strain SH1000; lanes 2 and 4, *lpd* mutant of *S. aureus* strain SH1000. Equivalent amounts of protein samples were loaded for lane 1 and 2. Lanes 3 and 4 represent membrane-bound autolysin extracts from similar bacterial cell masses.

clearly suggests that differences in the BCFAs in the membrane affect staphylococcal adherence to eukaryotic cells.

Additionally, the role of BKD in *S. aureus* pathogenesis was examined. Mice were injected intraperitoneally with a mixture of wild-type SH1000 and the *lpd* mutant bacteria. Mice were sacrificed at 4, 8, and 18 h postinfection. In these experiments, the numbers of bacterial cells (total CFU  $g^{-1}$  tissue) in liver and spleen samples decreased with time (data not shown). However, the fraction of *lpd* mutant cells recovered from either the liver (Fig. 6A) or the spleen (Fig. 6B) in infected mice decreased with time, and at the same time, the fraction of wild-type cells increased. An increase in the population of wild-type cells compared to *lpd* mutant cells suggests that a decrease in BCFAs led to a disadvantage in terms of the survival of *S. aureus* cells in this animal model.

## DISCUSSION

Bacterial membrane fatty acids, as acyl chains of phospholipids and glycolipids, determine the fluidity or viscosity of the membrane, and modulation of fatty acid composition allows bacteria to survive in a wide range of physical and chemical environments (37). The total fatty acid composition of *S. aureus* is comprised of a complex mixture of BCFAs and SCFAs, which are major determinants of the biophysical properties of the membrane. BCFAs in general and anteiso C15:0 fatty acid, in particular, are believed to be major determinants of the fluidity of the *S. aureus* membrane. In order to further study the role of BCFA in the function of the *S. aureus* cytoplasmic membrane, we created a mutant in the *lpd* gene of the *bkd* gene cluster that plays a major role in the biosynthesis of

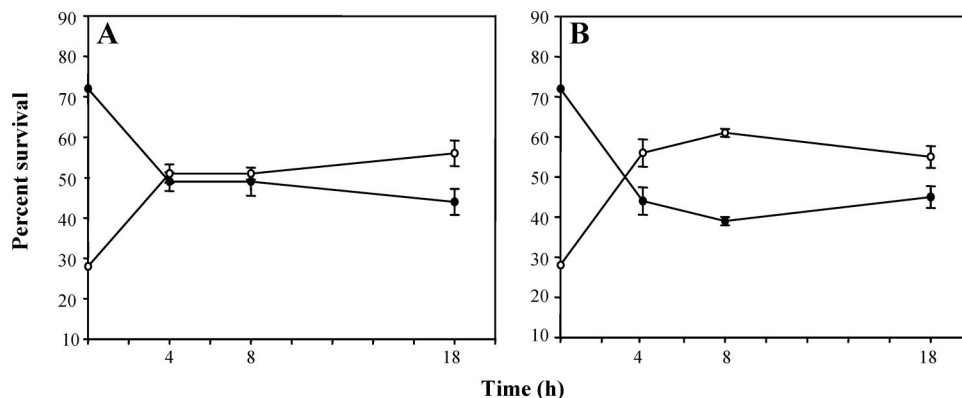


FIG. 6. Survival of the *lpd* mutant and the isogenic wild-type *S. aureus* strain SH1000 in mouse. Approximately  $1.15 \times 10^7$  CFU (28% wild-type and 72% *lpd* mutant) were injected intraperitoneally into mice. Three mice were sacrificed at 4, 8, and 18 h postinjection. Fraction of *lpd* mutant strain (closed circles) and isogenic wild-type strain (open circles) in the injected inoculum (time zero; y axis) and in the bacteria recovered from infected liver (A) and spleen (B), respectively.

BCFAs. The *lpd* mutant was defective in BCFAs and showed decreased membrane fluidity. Besides having a negative impact on growth at low temperatures, decreased membrane fluidity also correlated with lower tolerance of a variety of stresses and possibly in the insertion and display of cell surface proteins.

Studies with the BKD-inactivated mutant provided insight into the critical roles for this enzyme complex in staphylococcal growth. Inactivation of BKD led to a less-fluid membrane and a lower growth rate for the mutant. This level of growth reduction in the mutant became more dramatic at lower temperatures, and the mutant failed to grow at 12°C. Deficiencies of BCFAs in the *lpd* mutant produce a more-rigid (less-fluid) membrane that impairs the ability of the mutant to perform various essential membrane-associated processes. Restoration of the growth defect in the mutant with 2-methylbutyrate further supports the lack of a functional BKD enzyme complex in the mutant cells. It is evident from the fatty acid composition analysis (Table 2) that fatty acid anteiso C15:0 fatty acid, which is derived from 2-methylbutyryl-CoA, is the major BCFA in the staphylococcal cell membrane. This probably is the reason that other short-chain fatty acid precursors, isovalerate and isobutyrate, provided much less significant improvement in the growth of the BKD-inactivated mutant. Similar results have been noted with BKD mutants of *L. monocytogenes* (1, 38). *L. monocytogenes* differs from *S. aureus* in that *Listeria* normally contains almost no SCFAs. The *S. aureus lpd* mutant was not completely deficient in BCFA. Probably, the BCFAs that are present, which may represent close to a minimum requirement for BCFAs in the membrane lipids (7, 38), are produced via the pyruvate dehydrogenase and, possibly, the  $\alpha$ -keto glutarate dehydrogenase complexes, which have some activity with branched-chain  $\alpha$ -keto acids (14, 25).

Fluorescence polarization measurements using the probe DPH indicated that the membrane of the *lpd* mutant was less fluid than that of the parent strain. Consistent with this was the decreased solvent tolerance of the mutant, which could be increased by growth in the presence of 2-methylbutyrate. It has been shown that increased solvent tolerance in staphylococci is associated with increased membrane anteiso fatty acid content and fluidity (24).

The *lpd* mutant shows that intact BKD is critical for BCFA content and membrane fluidity in *S. aureus*, and this study provides additional interesting phenotypes associated with it compared to its wild-type counterpart. While no significant growth defect was noted when the *lpd* mutant was grown in the presence of high salt (1.5 M NaCl) or low pH (5.5), a much lower growth rate was observed for the mutant compared to the wild-type *S. aureus* at alkaline pH (9.5) or in the presence of 8.8 mM H<sub>2</sub>O<sub>2</sub>. It is speculated that the lack of BCFAs, particularly anteiso 15:0 fatty acid, in the cytoplasmic membrane of the *lpd* mutant leads to an increased susceptibility of the membrane to hydroxylation or peroxidation, rather than a direct role of the BKD enzyme complex in protection of *S. aureus* cells from these adverse conditions. Evidence for a role of BCFAs in the tolerance of alkali stress in *L. monocytogenes* has been presented (10).

The *lpd* mutant bacteria showed decreased susceptibility to the action of autolysins. This decreased autolysis of the mutant cells was observed irrespective of whether the cells were grown at 20°C (data not shown) or 37°C. The *lpd* mutant cells were

also less susceptible than wild-type *S. aureus* strain SH1000 to the action of lysostaphin in a disc diffusion assay when the mutant bacteria were grown at 20°C (data not shown). However, this decreased susceptibility to lysostaphin was not apparent between the *lpd* mutant and the wild-type *S. aureus* when both types of cells were grown at 37°C (data not shown).

Another notable observation with the *lpd* mutant was its decreased ability to adhere to eukaryotic cell surfaces. Proper adherence to eukaryotic cell surfaces is an important stage during the colonization of a host by pathogenic bacterial species. In addition to its reduced adherence, a relative decrease in the survival of the *lpd* mutant was observed compared to the wild-type *S. aureus* in an in vivo competition assay in a murine model.

It was expected that the inactivation of the BKD enzyme complex would lead to reduced BCFAs and a decreased growth rate at lower temperatures. It is clear that the decrease in susceptibility to autolysins is not due to the downregulation of autolysins in the mutant. Similarly, a decrease in adherence of the *lpd* mutant to eukaryotic cells is unlikely to be due to reduced expression of surface adhesins in the mutant. It is likely that a reduction in BCFA content in the cell membrane of the *lpd* mutant leads to a decrease in membrane fluidity, which becomes more prominent at lower temperatures. This in turn leads to an alteration and/or positioning of autolysin and adhesion molecules displayed on the bacterial cell surface, leading to the observed phenotypes.

As alternatives to vertebrate animal models of *S. aureus* infection, a nematode killing assay has been developed to identify virulence genes also required for *S. aureus* infection of warm-blooded animals (9, 31), and *Drosophila melanogaster* has been used as a model host for *S. aureus* infection (23). The *Caenorhabditis elegans* and *D. melanogaster* assays are carried out at 25°C, whereas in vertebrate animal models of infection, the temperature is 37°C or higher. The membrane fatty acid composition of *S. aureus* is different at 25°C than at 37°C, and this may be a factor affecting the virulence of *S. aureus* at this temperature. The inclusion of 2-methylbutyrate in the growth medium markedly stimulates the growth of wild-type *S. aureus* at lower temperatures and boosts the amount of anteiso fatty acids in the membrane and its fluidity. It would be interesting to know whether this had any effect on the virulence of the organism for *C. elegans* and *D. melanogaster*.

A change in membrane fatty acid composition is an important strategy employed by *S. aureus* to adapt to changes in environmental conditions. In vitro resistance of *S. aureus* to thrombin-induced platelet microbicidal protein is also associated with alterations in cytoplasmic membrane fluidity (4). A decrease in membrane fluidity increased resistance to oleic acid killing in *S. aureus* (5). However, in another staphylococcal species, *Staphylococcus haemolyticus*, the organism increased its BCFA content, particularly that of anteiso C15:0, and decreased its SCFA content, particularly that of C20:0, and hence increased its membrane fluidity, when grown in the presence of the membrane active solvent toluene (24). A comparison of the fatty acid composition of methicillin-sensitive and methicillin-resistant *S. aureus* strains has revealed differences in the proportions of anteiso and iso fatty acids in the cytoplasmic membranes of these two types of strains (27). The *lpd* mutant described here provides a useful tool for further



evaluation of the role of membrane fluidity in the interaction of *S. aureus* with a wide variety of antimicrobial agents. In addition, perturbation of cell membrane fatty acid composition may be an important target for the development of antimicrobial agents.

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