Fatty Acids Regulate Stress Resistance and Virulence Factor Production for *Listeria monocytogenes*

Yvonne Sun,^a Brian J. Wilkinson,^b Theodore J. Standiford,^c Henry T. Akinbi,^d and Mary X. D. O'Riordan^a

University of Michigan Medical School, Department of Microbiology and Immunology, Ann Arbor, Michigan, USA^a; School of Biological Sciences, Illinois State University, Normal, Illinois, USA^b; University of Michigan Medical School, Department of Internal Medicine, Ann Arbor, Michigan, USA^c; and University of Cincinnati College of Medicine, Cincinnati Children's Hospital Medical Center, Division of Neonatology, Department of Pediatrics, Cincinnati, Ohio, USA^d

Fatty acids (FAs) are the major structural component of cellular membranes, which provide a physical and chemical barrier that insulates intracellular reactions from environmental fluctuations. The native composition of membrane FAs establishes the topological and chemical parameters for membrane-associated functions and is therefore modulated diligently by microorganisms especially in response to environmental stresses. However, the consequences of altered FA composition during host-pathogen interactions are poorly understood. The food-borne pathogen *Listeria monocytogenes* contains mostly saturated branched-chain FAs (BCFAs), which support growth at low pH and low temperature. In this study, we show that anteiso-BCFAs enhance bacterial resistance against phagosomal killing in macrophages. Specifically, BCFAs protect against antimicrobial peptides and peptidoglycan hydrolases, two classes of phagosome antimicrobial defense mechanisms. In addition, the production of the critical virulence factor, listeriolysin O, was compromised by FA modulation, suggesting that FAs play a key role in virulence regulation. In summary, our results emphasize the significance of FA metabolism, not only in bacterial virulence regulation but also in membrane barrier function by providing resistance against host antimicrobial stress.

Biological functions that modulate the growth and virulence of pathogenic organisms, such as toxin secretion or nutrient acquisition, involve cellular membranes, which are composed primarily of phospholipids. For example, in *Vibrio cholerae*, exposure to surface-disrupting bile leads to rapid alterations in membrane phospholipids (21), which can modulate the activity of the type II secretion system (9a), critical for cholera toxin secretion. Therefore, perturbations in the membrane environment that influence membrane-associated functions can potentially affect the course of an infection. A major determinant in physical and chemical characteristics of membrane phospholipids is the composition of the constituent fatty acids (FAs), which differ by acyl chain length, extent of saturation, and branching patterns (61). *Listeria monocytogenes* is a Gram-positive bacterial pathogen with a cytoplasmic membrane highly enriched in branched-chain FAs (BCFAs) similar to *Staphylococcus* and *Bacillus* species (58), which represent major animal and plant pathogens. Some Gram-negative bacteria such as *Legionella* species also contain high levels of BCFAs (13). When grown in rich brain heart infusion (BHI) medium at 37°C, *L. monocytogenes* membrane contains very high levels of BCFAs with approximately 81 to 86% anteiso-BCFAs and 13 to 18% iso-BCFAs (28, 30, 62). Despite the similarity in their structures (Fig. 1), the antepenultimate methyl groups contribute to a markedly lower phase transition temperature for anteiso-BCFAs than the penultimate methyl groups in iso-BCFAs of the same chain length (29) and are believed to impart greater membrane fluidity in *L. monocytogenes* and facilitate propagation across wide range of temperatures (14, 27).

Both types of BCFAs are synthesized through a central pathway in *L. monocytogenes*, although the β -ketoacyl-acyl carrier protein synthase (FabH) that catalyzes the first condensation step in FA biosynthesis selectively utilizes precursors for anteiso-BCFAs (47). Typically, branched-chain amino acids are converted to α -keto acids and further decarboxylated to form corresponding coenzyme A (CoA) esters, which serve as primers for acyl chain

elongation by the branched-chain α -keto acid dehydrogenase (BKD) (Fig. 1). Alternatively, in a BKD-independent but poorly characterized pathway (59), carboxylic acids are converted to CoA esters, which can also serve as primers for chain elongation (Fig. 1). The BKD enzyme complex is composed of four subunits encoded by four open reading frames organized in a predicted operon structure. Two different transposon insertional mutants both exhibit significant reduction of BCFAs and severe growth defects under stress conditions such as low temperature and low pH (62). The general fitness defects, longer doubling time, and reduced maximal growth in rich media have prevented in-frame deletion of genes in the BKD operon, even after multiple attempts. However, after transducing the insertion into a clean isogenic wild-type background, we demonstrated with two independent transductants that deficiency in BKD resulted in strong attenuation in both tissue culture and mouse infection models. The BKD insertion mutants also exhibited a significant defect in production of listeriolysin O, the cytotoxin required for phagosomal escape (50).

As an intracellular pathogen, *L. monocytogenes* encounters diverse surface-disrupting stresses within the host. In the gastrointestinal tract, *L. monocytogenes* experiences membrane-disrupting activities of bile in the small intestine and pore-forming cationic antimicrobial peptides (CAMPs) produced by intestinal epithelial cells. Upon entry into host cells, *L. monocytogenes* must survive additional surface-disrupting agents in the phagosome, including

Received 11 January 2012 Accepted 9 July 2012

Published ahead of print 27 July 2012

Address correspondence to Yvonne Sun, sunyy@umich.edu.

Supplemental material for this article may be found at <http://jb.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00045-12

FIG 1 Schematics of FA synthesis precursors and their respective products in *L. monocytogenes*. Branched-chain amino acids—leucine, isoleucine, and valine (not shown)—are used as precursors in a BKD-dependent pathway for the synthesis of odd-number iso-BCFA, anteiso-BCFA, and even-number iso-BCFA (not shown), respectively. Alternatively, the carboxylic acids 2-methylbutyric acid (2MB) and butyrate can also be used as precursors in a BKD-independent pathway for the synthesis of anteiso-BCFA and straight-chain FAs, respectively. BKD, branched-chain α -keto acid dehydrogenase.

CAMPs and lysozyme, prior to escape into the cytosol. Based on the attenuated phenotype of the BKD[−] mutant, we hypothesized that BCFAs in *L. monocytogenes* facilitate resistance against surface-disrupting agents in the host phagosome and thus contribute to intracellular survival.

We show here that BCFAs in *L. monocytogenes* confer resistance against killing by CAMPs and peptidoglycan (PGN) hydrolases *in vitro*. BCFA-deficient bacteria were more susceptible to microbial killing in the macrophage phagosome, a compartment where CAMPs and PGN hydrolases are found. Nutritional supplementation that modified BCFA levels or composition in wild-type (WT) bacteria also reduced *L. monocytogenes* stress resistance and virulence. Taken together, our data indicate that exposure to FA modulation can have a profound effect on *L. monocytogenes* stress resistance and virulence regulation and should be further investigated for developing novel antimicrobial therapies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. monocytogenes* strains used in the present study include the wild-type (WT) strain 10403S, the BKD[−] mutant (MOR401), which harbors a Tn917 insertion in the gene encoding the E3 subunit of the BKD complex transduced from the *clb-2* mutant (50), the listeriolysin O-deficient (LLO[−]) mutant with an in-frame deletion of the *hly* gene (DP-L2161), and the BKD[−] LLO[−] mutant (MOR402), which was constructed by transducing the Tn917 insertion from the *clb-2* mutant into the LLO[−] mutant. Unless stated otherwise, bacteria were typically grown overnight from single colonies <1 week old with shaking (225 rpm) at 37°C in filter-sterilized brain heart infusion media (BHI; Difco lot 9103448) with or without supplementation (5 mM 2-methylbutyric acid [2MB], 250 mM sodium butyrate, or 100 mM leucine). HEPES-buffered BHI (25 mM, pH 7.0) was used for experiment with high concentrations of 2-methylbutyric acid (25 mM). The following conversion factors were used to normalize bacterial numbers from different growth conditions: at a culture optical density (OD) of 1.2, 1 ml of BHI, BHI + 250 mM butyrate, or BHI + 100 mM leucine culture each contains 2×10^9 , 1×10^9 , or 1.5×10^9 CFU, respectively.

For transcriptional analysis of *hly*, the LLO-encoding gene, WT bacteria from overnight cultures (50 ml) were collected by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS). Aliquots of bacterial PBS suspensions were added to fresh medium (3 ml) supplemented with or without 250 mM sodium butyrate or 100 mM leucine. Cultures were then incubated with shaking (225 rpm) at 37°C, and bacteria were harvested at 0.5, 2, and 8 h postinoculation by centrifugation and subsequently used for RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR analysis.

Macrophage infections. RAW 264.7 macrophages were maintained in RPMI medium 1640 (Gibco, catalog no. 21870) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and additional 2 mM L-glutamine. Infections of bone marrow-derived macrophages (BMDM) were carried out in Dulbecco modified Eagle medium (DMEM) high-glucose medium (Gibco, catalog no. 11965) supplemented with 10% heat-inactivated FBS and an additional 2 mM L-glutamine. Host cells (4×10^6 cells per plate) were plated onto coverslips (12 mm in diameter) in 24-well plates on the night prior to infections. Washed overnight cultures were used to infect host cells at multiplicity of infection (MOI) of 5 in the presence or absence of 2MB (5 mM) or leucine (100 mM). Butyrate (250 mM) was not included during infection because of its negative impact on macrophage viability. For RAW 264.7 macrophage infection, after the addition of bacterial suspensions, the plates were centrifuged at 1,200 rpm for 3 min, followed by incubation at 37°C for a total of 30 min of infection. For BMDM infection, the centrifugation was omitted. After infection, host cells were washed three times in PBS and incubated in medium containing gentamicin (10 μ g/ml) with or without 2MB (5 mM) or leucine (100 mM). The number of viable intracellular *L. monocytogenes* was determined by lysing host cells in sterile water and enumerating bacterial colonies on Luria-Bertani (LB) agar. To calculate the percent change in intracellular CFU, we used the following equation: % change = [(CFU at the final time point − CFU at the initial time point)/CFU at the initial time point] $\times 100$.

Survival assay. Overnight cultures were diluted in assay buffer (10 mM potassium phosphate buffer [pH 5.8], unless otherwise indicated) to approximately 4×10^3 CFU per ml. Diluted bacterial suspensions were added to compounds to be tested in 96-well plates, followed by incubation

at 37°C for 1 h. Stock solutions of polymyxin B (Calbiochem, catalog no. 5291), protamine (Sigma, catalog no. P4020), LL-37 (Anaspec, catalog no. 61302), mutanolysin (Sigma, catalog no. M9901), and human lysozyme (Sigma, catalog no. L1667) were prepared in assay buffer at concentrations of 10, 10, 1, 1, and 50 mg/ml, respectively. Synthetic CRAMP (CHI Scientific) was first dissolved in dimethyl sulfoxide to 1 mg/ml and then diluted in assay buffer to 10 µg/ml and stored at -20°C. The CFU were determined at 0 and 1 h postexposure by plating on LB agar. To calculate the percent survival, we used the following equation: % survival = $(CFU_{T=1h}/CFU_{T=0}) \times 100$.

BMDM isolation. Primary BMDM were isolated from the femurs of *Cnlp*^{+/+} and *Cnlp*^{-/-} C57BL/6 mice and *LysM*^{+/+} and *LysM*^{-/-} FVB/NJ mice. BMDM were differentiated by culturing bone marrow cells in DMEM (Gibco, catalog no. 11960) containing 20% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 55 µM β-mercaptoethanol, and 30% L929 conditioned medium for 6 days, during which fresh medium was added on day 3. Cells were harvested by washes with cold PBS on day 6 and used for infections on day 7 or frozen and stored in liquid nitrogen.

Plaque assay. L2 fibroblast cells were cultured in DMEM high-glucose medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids (Gibco catalog no. 11140), and 1 mM sodium pyruvate and added to six-well plates to approximately full confluence. Approximately 4×10^5 CFU of *L. monocytogenes* resuspended in L2 fibroblast tissue culture medium were added to each well. After a 30-min infection period, all wells were washed twice with PBS, and 3 ml of agarose overlay (L2 tissue culture medium with gentamicin and 0.7% agarose ± 100 mM leucine) was added to each well. Plaques were allowed to develop for 4 to 5 days prior to visualization by neutral red staining. Plaque sizes were measured in Adobe Photoshop ($n = 30$) and are expressed as percentages of the mean diameter of plaques formed by infection without leucine supplementation.

SDS-PAGE and immunoblotting analysis. Supernatant samples were normalized with fresh medium based on the OD at 600 nm. Proteins in the supernatant fraction were precipitated in 10% trichloroacetic acid, followed by one cold acetone wash. SDS-extractable surface fractions were obtained by resuspending bacterial pellets in SDS-PAGE sample buffer in volume normalized by OD. Lysate fractions were prepared by bead-beating suspensions of bacterial pellets normalized by OD. All protein sample fractions were heated at 95°C for 5 min and separated by SDS-PAGE, followed by immunoblotting with anti-LLO (αLLO; 1:10,000) antibody (Diatheva, catalog no. ANT0006).

Hemolytic assay. The supernatant samples were normalized by medium based on the OD, reduced by 5 mM dithiothreitol for 1 h at room temperature, and serially diluted in hemolysis assay buffer (125 mM NaCl, 35 mM Na₂HPO₄ [pH 5.5]) in 96-well plates. PBS-washed defibrinated sheep red blood cells were added to diluted supernatant samples at approximately 6.2×10^8 cells per well. The plates were incubated at 37°C for 30 min and centrifuged to pellet intact red blood cells. The supernatants were transferred to optically clear 96-well plates and analyzed for absorbance at 541 nm.

FA analysis. Bacteria were harvested from overnight cultures, washed with PBS to remove residual medium, and frozen in an ethanol-dry ice bath. Samples were sent for FA analysis using gas chromatography by Microbial ID (Newark, DE).

RNA extraction. RNA extraction was performed with a FastRNA Pro Blue kit (MP Biomedicals, catalog no. 6025-050) according to the manufacturer's protocol. Briefly, bacterial pellets were resuspended in 1 ml of RNApro solution and processed in lysing matrix B tubes (MP Biomedicals, catalog no. 6911-050) in the FastPrep instrument for 40 s at a setting of 6.0. The RNA was further treated with on-column DNA digestion to remove genomic DNA contamination using an RNeasy minikit (Qiagen, catalog no. 74104) according to the manufacturer's protocol. The concentration of final eluted RNA in RNase-free water was measured using a NanoDrop spectrophotometer (ND-1000).

TABLE 1 Primers used in this study

Primer	Sequence (5'-3')
hlyF	CCGGAACCTTACCACTTGTGAA
hlyR	ACTATATTTCCGATAAAGCGTGGTG
gyrAF	CCCAACTGCTGGGATGATTATGGGA
gyrAR	GGCAATACGTTCAACTAGGCGTGC
inlAF2	AAATCCTGTGGCACCACCAACAAC
inlAR2	GTTGTGCTGGCTGAATCCCAGTT

Synthesis of cDNA and quantitative PCR. Synthesis of cDNA was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT; Invitrogen, catalog no. 28025-013) according to the manufacturer's protocol with 1 µg of RNA and 1 µM reverse primer. A no-RT control was included for each sample where the M-MLV RT was replaced with RNase-free water. Quantitative PCRs were set up using Brilliant II SYBR green Low ROX QPCR master mix (Agilent, catalog no. 600830) with 1 µl of cDNA as a template and primer sets, each at a final concentration of 1 µM. Reactions (40 cycles of 95°C, 55°C, and 72°C each for 30 s) were monitored in a Stratagene Mx3000p and analyzed with MxPro QPCR software. The transcript levels of *gyrA*, a gene coding for DNA gyrase subunit A, were used as a normalizer. The sequences of primers used are shown in Table 1.

Statistical analysis. The statistical analyses for Fig. 3, 5, 6, and 7 were performed in Microsoft Excel with *P* values between each pairwise comparison calculated by two-tailed Student *t* tests. For Fig. 2 and 4, a two-way analysis of variance with the Bonferroni post test was performed using GraphPad Prism 5.0c to better determine the relationship between two groups of data sets with two independent factors.

RESULTS

BCFAs enhance survival of *L. monocytogenes* in phagosomes.

After uptake into phagocytic cells, *L. monocytogenes* encounters the antimicrobial arsenal of the host phagosome, including low pH, reactive oxygen species, and CAMPs, prior to escape into the cytosol. Based on the known role of BCFAs in environmental stress resistance (22, 62), we hypothesized that BCFAs would confer resistance to phagosomal killing. We infected RAW 264.7 macrophages with WT *L. monocytogenes* and the BKD-deficient mutant and measured bacterial survival at 2 h postinfection (hpi), a period during which WT bacteria have not yet begun rapid replication (Fig. 2A). The number of intracellular bacteria was equivalent between the two strains after 30 min of infection (data not shown), suggesting no difference in uptake. However, the BKD⁻ mutant, deficient in BCFAs (Table 2), exhibited significant loss of intracellular CFU compared to WT bacteria, indicating that the BKD⁻ mutant was more sensitive to killing than WT bacteria. Restoration of anteiso-BCFAs, the most prevalent type of BCFAs in *L. monocytogenes* by 2MB supplementation (Table 2) during infection caused no significant difference in survival of WT bacteria but significantly increased survival of the BKD⁻ mutant, suggesting that anteiso-BCFAs promote resistance to bacterial killing at early stages of infection.

During infection, *L. monocytogenes* can occupy two separate subcellular compartments: the phagosome or the cytosol. Measurement of the total intracellular CFU does not distinguish between bacteria in these compartments and consequently cannot be interpreted exclusively as phagosomal killing. Exit from the phagosome into the cytosol depends on the activity of LLO. Therefore, to address the role of BCFAs in *L. monocytogenes* survival specifically within the phagosomal compartment, we con-

FIG 2 BCFA enhance *L. monocytogenes* phagosomal survival during infection of RAW 264.7 macrophages. RAW 264.7 macrophages were infected with WT bacteria or the BCFA-deficient BKD⁻ mutant (A) or with the LLO⁻ mutant or the BKD⁻ LLO⁻ double mutant (B) from overnight cultures at an MOI of 5 for 30 min. The percent change in CFU reflects the percent difference in intracellular CFU determined at the indicated times postinfection (hpi) compared to 0.5 hpi. Averages of triplicates were plotted with error bars denoting the standard deviation. The data shown here are representative of at least three independent experiments. No significant difference between samples was denoted as “ns.” Significant differences are indicated by asterisks (***, $P < 0.005$; **, $P < 0.01$).

structed a mutant strain lacking both BKD and LLO and compared survival of the resulting BKD⁻ LLO⁻ double mutant and the LLO⁻ single mutant in infected macrophages. Both mutants remain inside phagosomes after entry due to deficiency in LLO. Survival of the BKD⁻ LLO⁻ double mutant was significantly lower than that of the LLO⁻ single mutant, a defect that was res-

cued by supplementation with 2MB during infection (Fig. 2B). Together, these data strongly support a role for BCFAs in resistance against phagosomal killing.

BCFA deficiency results in increased susceptibility to surface stress *in vitro*. The increased sensitivity of the BKD-deficient mutant to phagosomal defenses prompted us to investigate the role of BCFAs in resistance to specific mechanisms of antimicrobial stress. One such mechanism in macrophages is phagosome acidification. Although BCFA deficiency is associated with failure to grow at low pH (22), it was unclear whether acidic conditions could result in killing of BCFA-deficient bacteria. Therefore, we tested BCFA-dependent survival in acidic conditions *in vitro* by measuring CFU before and after 1 h exposure to citric acid and sodium phosphate solutions buffered at pH 5.0 or 7.0. The BCFA-deficient BKD⁻ mutant, despite exhibiting a growth defect in acidic medium, survived in pH 5.0 similarly to WT bacteria with or without 2MB supplementation (see Fig. S1 in the supplemental material). These results suggest that acidification of the phagosomal compartment is unlikely to be the primary cause of killing of BCFA-deficient bacteria.

We next hypothesized that decreased BCFAs altered the bacterial membrane and membrane-associated surface structures, which could render bacteria more susceptible to surface-disrupting compounds, such as CAMPs or lysozyme. Three different CAMPs with known anti-*Listeria* activities were tested in the present study. Human LL-37 and murine cathelin-related antimicrobial peptide (CRAMP) are cathelicidin-derived CAMPs found in many tissues and cell types, including macrophages (19, 35, 54).

TABLE 2 FA composition in *L. monocytogenes* can be manipulated through genetic mutation or metabolic supplementation^a

FA	FA composition (%)					
	WT <i>L. monocytogenes</i>				BKD ⁻ <i>L. monocytogenes</i>	
	BHI	BHI + 2MB	BHI + butyrate	BHI + Leu	BHI	BHI + 2MB
Straight FAs						
12:0	0.09	0.09	3.07	0.17	0.44	0.08
14:0	0.27	0.16	41.15	0.49	15.46	
16:0	0.69	0.53	24.39	0.45	43.88	3.02
18:0			0.17		1.83	
Total	1.05	0.78	68.78	1.11	61.61	3.1
Even iso FAs						
14:0 iso	0.45	0.26	1.72	0.85	10.81	0.61
16:0 iso	2.19	1.38	1.29	2.76	18.23	0.32
18:0 iso					0.52	
Total	2.64	1.64	3.01	3.61	29.56	0.93
Odd iso FAs						
13:0 iso			0.17	0.24		
15:0 iso	8.11	5.94	5.11	44.78	0.80	
17:0 iso	2.43	1.84	0.55	12.25	0.32	
Total	10.54	7.78	5.83	57.27	1.12	0
Anteiso FAs						
13:0 anteiso	0.16	0.16	0.24	0.08		0.19
15:0 anteiso	51.82	53.65	18.84	24.48	3.56	57.1
17:0 anteiso	33.5	35.55	2.55	12.65	1.63	38.14
19:0 anteiso	0.08	0.07		0.05		0.09
Total	85.56	89.43	21.63	37.26	5.19	95.52

^a BHI, brain heart infusion medium; 2MB, 5 mM 2-methylbutyric acid; butyrate, 250 mM sodium butyrate; Leu, 100 mM leucine. FA analysis was performed with bacteria harvested from overnight cultures by Microbial ID.

FIG 3 BCFA enhances *in vitro* resistance to surface-disrupting stresses. To identify potential mechanisms of phagosomal killing, we tested bacterial sensitivity *in vitro* to surface disrupting molecules likely to be present in phagosomes: LL-37, an antimicrobial peptide, and human lysozyme, a peptidoglycan hydrolase. Survival was determined by measuring CFU before and after 1 h of exposure to antimicrobial peptides LL-37 (A and B) or human lysozyme (C and D). To investigate the role of BCFA, 2MB was supplemented in growth media (B and D) to promote the synthesis of anteiso-BCFAs. Averages of three independent experiments are plotted with error bars denoting the standard deviation. Each independent experiment was performed with triplicates. Significant difference is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

Protamine, a CAMP enriched in arginines, is active against a wide range of bacteria and has been considered as a food preservative (25, 44). After incubation with bacteria for 1 h at 37°C, all three CAMPs decreased survival of the BCFA-deficient BKD⁻ mutant more than WT bacteria (Fig. 3; see Fig. S2 in the supplemental material). Notably, the BKD⁻ mutant was also more susceptible to polymyxin B (see Fig. S2B in the supplemental material), a cyclic CAMP normally only active against Gram-negative bacteria through interactions with lipopolysaccharide (16, 49). In all cases, survival of the BKD⁻ mutant was restored to WT levels by growing the mutant with 2MB, demonstrating that BCFA can protect *L. monocytogenes* against CAMPs *in vitro*.

Lysozyme is part of the defensive arsenal of professional phagocytes and catalyzes hydrolysis of the PGN backbone, resulting in loss of structural integrity and lysis of the target organism (17). *L. monocytogenes* normally evades lysozyme degradation by modifying its PGN through deacetylation of *N*-acetylglucosamine (8). However, BCFA-deficient *L. monocytogenes* displayed significantly decreased survival after 1 h of exposure to human lysozyme at 37°C compared to WT bacteria (Fig. 3C). Increased susceptibility of the BKD⁻ mutant was also observed with exposure to mutanolysin, a different *N*-acetylmuramidase insensitive to deacetylation modification (18) (see Fig. S2D in the supplemental material). The survival defect of the BCFA-deficient strain upon treatments with lysozyme or mutanolysin could be rescued by growing the mutant with 2MB supplementation (Fig. 3D; see Fig. S2D in the supplemental material), emphasizing the importance of BCFA in resistance against cell wall-disrupting agents. Our results imply that alterations in bacterial membrane FA composition have the potential to compromise cell wall structure and integrity. Together, these data show that disruption of BCFA synthesis renders *L. monocytogenes* highly susceptible to surface-dis-

FIG 4 BCFA-deficient *L. monocytogenes* were susceptible to phagosomal killing in BMDM deficient in CRAMP or lysozyme. BMDM were infected at an MOI of 5 for 30 min. The percent CFU change reflects the percent difference in intracellular CFU determined at the indicated hours postinfection (hpi) compared to 0.5 hpi. The data shown here are averages of three independent experiments. Each independent experiment was performed with triplicates. Significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

rupting molecules targeting either the bacterial membrane or cell wall.

To determine whether one particular antimicrobial mechanism in the phagosome was primarily responsible for killing BCFA-deficient *L. monocytogenes*, we assayed for bacterial intracellular survival in BMDM isolate from CRAMP- or lysozyme-deficient mice (*Cnlp*^{-/-} or *LysM*^{-/-}, respectively). The macrophages were infected with the LLO⁻ or the BKD⁻LLO⁻ mutant for 30 min, and intracellular CFU were determined at 30 min and 7 h postinfection. If CRAMP or lysozyme alone was the cause of killing, then we would predict rescue of the BKD⁻LLO⁻ mutant in either *Cnlp*^{-/-} or *LysM*^{-/-} BMDM. However, there was no significant difference in the survival of the BKD⁻LLO⁻ mutant inside wild-type and mutant macrophages (Fig. 4). The effective killing of BCFA-deficient *L. monocytogenes* observed in either *Cnlp*^{-/-} or *LysM*^{-/-} BMDM suggests that compromising a single antimicrobial factor in host cells alone is not sufficient to rescue BCFA-deficient bacteria from phagosomal killing. Interestingly, we noticed a significant decrease in survival of the LLO⁻ mutant in *Cnlp*^{-/-} BMDM compared to *Cnlp*^{+/-} BMDM, which suggests a potential role for CRAMP in promoting *L. monocytogenes* fitness inside phagosomes. Taken together, our data show that decreasing BCFA levels renders *L. monocytogenes* sensitive to more than one antimicrobial mechanism, resulting in a marked loss of bacterial survival.

Reduction of BCFA through metabolic supplementation in WT *L. monocytogenes* lowers resistance *in vitro* and in macrophages. Supplementation of specific FA precursors provides an alternative to genetic manipulation and is a more adaptable method for altering bacterial FA composition. Therefore, we tested whether metabolic supplementation with different FA precursors would affect WT bacteria stress resistance and influence fitness inside host cells. Growing *L. monocytogenes* with high levels of butyrate (250 mM) enriches the membrane with straight-chain FAs to a level similar to that found in the BKD⁻ mutant and decreases the proportion of anteiso-BCFA by 3- to 4-fold (Table 2). When exposed to LL-37 (Fig. 5A) or mutanolysin (see Fig. S3 in the supplemental material), butyrate-grown WT bacteria exhibited a significant decrease in survival compared to WT bacteria grown without supplementation. Thus, nutrient conditions that

FIG 5 Reduction of total BCFA through butyrate supplementation decreases resistance to LL-37 and intracellular CFU of WT *L. monocytogenes*. WT bacteria were grown in the presence of butyrate to enrich for straight-chain FAs. Survival of WT bacteria grown in BHI or in BHI + 250 mM sodium butyrate was determined by measuring CFU after one hour of exposure to antimicrobial peptide LL-37 (A) or between 0.5 and 2 hpi in RAW 264.7 macrophages (B). The data plotted represent averages of three independent experiments, with error bars denoting the standard deviation. Each independent experiment was performed with triplicates. Significant differences are indicated by asterisks (**, $P < 0.01$).

reduce BCFA content greatly compromise *L. monocytogenes* fitness *in vitro*.

The increased susceptibility to surface stress in butyrate-grown *L. monocytogenes* led us to hypothesize that such treatment would decrease survival of the bacterium in macrophages, as we had previously observed with the BKD⁻ mutant. Therefore, we infected RAW 264.7 macrophages with butyrate-grown WT *L. monocytogenes* and assayed for survival during early infection. Butyrate was only added during growth of the bacterial inoculum but not included in the cell culture medium during infection to eliminate any toxic effects of butyrate on macrophages. Butyrate-grown WT bacteria exhibited a significant loss of intracellular CFU after 2 h of infection in RAW 264.7 macrophages (Fig. 5B), suggesting that BCFA levels play an early role in determining the intracellular fate of *L. monocytogenes*.

Leucine supplementation compromises virulence but not CAMP resistance. Genetic mutation in BKD or butyrate supplementation reduces the total BCFA content in *L. monocytogenes* and causes a loss of resistance against surface stress and phagosomal killing. However, total BCFAs in *L. monocytogenes* are composed of anteiso- and iso-BCFAs, which have distinct physical properties such as melting temperature. We further dissected the contribution of anteiso- versus iso-BCFAs to stress resistance by disrupting the native ratio of these BCFAs. Both types of BCFAs are synthesized through the same biochemical pathway using different precursors. To test the contribution of anteiso-BCFA, we artificially increased the iso-BCFA content, thus reducing the anteiso- to iso-BCFA ratio, by growing *L. monocytogenes* in the presence of 100 mM leucine, a precursor for iso-BCFAs (Fig. 1, Table 2). Using our *in vitro* resistance assays and survival assays inside macrophages, WT bacteria grown with leucine remained resistant to LL-37 (Fig. 6A) but exhibited significantly reduced intracellular CFU after 2 h of infection in RAW 264.7 macrophages (Fig. 6B). In a longer macrophage infection experiment, leucine supplementation compromised intracellular growth and/or survival (Fig. 6C), as reflected by a significant increase in doubling time (Table 3). Similarly, leucine supplementation markedly reduced plaque size upon infection of an L2 fibroblast monolayer (Fig. 6D), suggesting that alterations in the BCFA ratio through leucine supplementation leads to long-term defects during infection. Together, our

FIG 6 Leucine supplementation in WT *L. monocytogenes* compromises virulence but not CAMP resistance. To further investigate whether specific BCFA composition is necessary to confer resistance, WT bacteria were cultured in the presence of leucine to decrease the ratio between anteiso- and iso-BCFA. (A) Resistance to LL-37 was determined by calculating survival of WT bacteria grown in BHI or in BHI + 100 mM leucine by comparing CFU before and after 1 h of exposure. (B) Resistance to macrophage killing was determined by calculating the percent CFU change between 0.5 and 2 hpi in RAW 264.7 cells. The data plotted represent averages of three independent experiments, with error bars denoting the standard deviation. Each independent experiment was performed with triplicates. (C) The effect of leucine supplementation on intracellular growth was determined by infecting RAW 264.7 macrophages and enumerating intracellular CFU during a time course of 12 hpi. Averages of triplicates were plotted, with error bars denoting standard deviation. The data shown represent at least two independent experiments. (D) We infected a monolayer of L2 fibroblasts with WT *L. monocytogenes* in the presence or absence of 100 mM leucine and compared the size of areas of clearance (plaques) caused by lysed cells. The data shown represent at least two independent experiments. Significant differences are indicated by asterisks (**, $P < 0.01$; ***, $P < 0.005$).

results suggest that iso-BCFAs are likely sufficient to provide resistance against surface stress *in vitro*, but a high anteiso- to iso-BCFA ratio is necessary for optimal fitness in the intracellular environment.

LLO production is sensitive to FA modulation. Bacterial virulence may be influenced by FAs through multiple mechanisms. We previously found that the BKD⁻ mutant was markedly deficient in producing LLO (49). However, because this mutant also

TABLE 3 Doubling times of *L. monocytogenes* intracellular growth in RAW 264.7 macrophages with or without leucine supplementation^a

Time (h) postinfection	Mean doubling time (min) ± SD		P
	No supplementation	With 100 mM leucine	
0.5–3	162.7 ± 11.0	861.8 ± 3,019.4	0.675
3–5	43.8 ± 5.7	99.5 ± 18.3	0.006
5–7	30.8 ± 2.6	55.7 ± 7.6	0.005
7–12	65.5 ± 9.1	100.5 ± 8.8	0.001

^a Doubling times were calculated for the growth curves shown on Fig. 6C using the following equation: time elapsed/[(log₁₀ second OD value – log₁₀ first OD value)/log₁₀ 2]. The values shown here are averages of triplicates in a single experiment but represent three independent experiments. The P values were calculated between each pairwise comparison by unpaired Student t test.

exhibited severe growth defects *in vitro*, we suspected the physiological consequences of permanently inactivating the BKD enzyme extended beyond changes in FA composition. To test whether temporary alterations in FA composition affect virulence factor production in WT *L. monocytogenes*, we assayed for LLO protein production and activity by using metabolic supplementation with different FA precursors. In order to see the greatest contrast between different culture conditions, we assayed for cumulative LLO production after overnight growth in the presence of butyrate or leucine supplementation by immunoblots and tested hemolytic activity of culture supernatants. Compared to a similar number of WT bacteria grown without supplementation, butyrate-grown WT bacteria produced no detectable LLO or hemolytic activity in the supernatant (Fig. 7A and B), indicating a requirement for BCFAs in LLO production. Decreasing the anteiso- to iso-BCFA ratio with leucine supplementation also resulted in a reproducible decrease in LLO production (Fig. 7A), and ~2-fold less hemolytic activity in the supernatant fraction (Fig. 7B). To investigate whether the loss of LLO in the supernatant fraction could potentially result from a secretion defect associated with altered membrane FA composition, we also collected SDS-extractable cell surface fractions and cellular lysates. LLO was not detected in either the surface fractions or cell lysates of butyrate-grown cultures, indicating that the absence of LLO in the supernatant was more likely due to inhibition of LLO synthesis. Similarly in leucine-grown cultures, there was no detectable LLO in the lysate fraction and a reduced level of LLO in the surface fraction. Thus, FA supplementation alters virulence regulation by *L. monocytogenes*.

To determine whether inhibition of LLO synthesis in WT bacteria grown with either butyrate or leucine occurred at the transcriptional level, we analyzed the transcript levels of *hly*, which encodes the LLO protein, over time. Bacteria from an overnight BHI culture were washed and reinoculated into fresh medium. The OD values were monitored and did not show significant differences between different conditions (Fig. 7C). We observed significantly less *hly* transcript in bacteria supplemented with butyrate or leucine as early as 30 min postinoculation (Fig. 7D). The level of *hly* transcript from bacteria in the presence of supplementations remained significantly lower throughout the time course. Therefore, we conclude that FA supplementation results in suppression of LLO synthesis at the transcriptional level.

We next tested whether other virulence determinants showed similar transcriptional suppression with butyrate or leucine supplementation. Internalin A, encoded by *inlA*, facilitates initial binding and uptake of bacteria into epithelial cells (7). Similar to *hly* transcript levels, *inlA* transcript levels showed a strong increase in control cultures 8 h postinoculation but were significantly lower in cultures supplemented with either butyrate or leucine (Fig. 7D). These results demonstrate that exposure of *L. monocytogenes* to FA precursors has a profound influence on specific virulence determinants in *L. monocytogenes*.

It is possible that butyrate or leucine could exert signaling functions independently from their effects on membrane FA composition. Therefore, we performed additional experiments to test whether the effects of supplementation were attributable to changes in FA composition. First, to determine whether the inhibition of LLO production with leucine supplementation was a result of signaling by branched-chain amino acids, we tested the effect of adding similar levels of isoleucine, which did not increase

total BCFA content but increased the proportion of anteiso-BCFAs (28), on LLO production. No inhibition of LLO production was associated with isoleucine supplementation (Fig. 7E), suggesting that regulation of LLO production by leucine was not a response to branched-chain amino acids in general. Second, we assayed for LLO production using bacteria grown with both butyrate and 2MB supplementation to test a potential signaling role for butyrate. Supplementation with 2MB (5 mM) by itself causes no changes in LLO production in WT bacteria (50). Supplementation with 25 mM 2MB in HEPES-buffered BHI completely restored anteiso-BCFA content in bacteria grown with 250 mM butyrate (Fig. 7G). However, it did not restore LLO production (Fig. 7E and F), suggesting that butyrate, unlike leucine, exhibits a potential signaling effect independent of alterations in FA composition.

DISCUSSION

Bacterial pathogenesis relies on numerous membrane-associated functions, such as secreting and anchoring virulence factors and surface modifications to evade host defense mechanisms. In the present study, we demonstrate that genetic disruption or exposure to FA precursors that lead to alterations in FA composition significantly compromise stress resistance and intracellular fitness. Disruption of the synthesis of BCFAs, the dominant class of membrane FA, through genetic mutation, causes decreases in membrane fluidity (14, 27), *in vitro* growth defects at low temperature and low pH (22, 62), and reduced intracellular growth and infection *in vivo* (50). Here, we establish a specific requirement for BCFAs in resistance against surface stresses and phagosomal killing. Furthermore, we show that metabolic supplementation with FA precursors not only alters FA composition but also compromises bacterial resistance to CAMPs and PGN hydrolases. Finally, we demonstrate that LLO production is sensitive to environmental FA precursors, suggesting a role for these molecules in precise tuning of virulence regulation.

Potential roles of membrane FA composition in resistance against CAMPs. In order to effectively design new anti-infective tools, it is important to understand the resistance mechanisms pathogens use to avoid killing by host defenses. CAMPs are part of the innate defense mechanism encountered by most bacterial pathogens. The antimicrobial mechanism of CAMPs includes pore formation in target membranes or disruption of intracellular functions that ultimately lead to bacterial death (60). Bacteria avoid the deleterious effects of CAMPs by secreting proteases, modifying surface structure to reduce surface negative charges, or exporting internalized CAMPs (31). During the course of infection in a host, *L. monocytogenes* initiates resistance mechanisms (10), such as modification of teichoic acids with D-alanine (1) and modification of negatively charged phosphatidylglycerol with positively charged lysine (51), to avoid interactions with CAMPs. However, we noticed a moderate but statistically significant decrease in survival of the LLO[−] mutant inside CRAMP-deficient macrophages compared to WT macrophages. This result introduces the possibility that even though *L. monocytogenes* develops resistance mechanism against killing by CAMPs, it may also sense CAMPs as a signal to stimulate virulence mechanisms similarly to the *Salmonella* PhoPQ signal transduction system (3). Alternatively, *L. monocytogenes* may indirectly rely on the initial damage caused by CRAMP as the signal to activate responses that promote intracellular survival. If true, alterations in FA composition may

FIG 7 Supplementation that decreases the proportion of anteiso-BCFA reduce the production of LLO and the level of *hly* transcript. The abundance and activity of LLO production was determined by immunoblotting (A) and hemolytic assay (B), respectively, with overnight cultures of WT *L. monocytogenes* grown in BHI without supplementation (“–”), with 250 mM sodium butyrate (“B” or “butyrate”), or with 100 mM leucine (“L” or “leucine”). Supernatant fractions from all samples were normalized with BHI based on the culture OD. For immunoblotting, proteins in the normalized supernatant were precipitated with 10% trichloroacetic acid (TCA) and analyzed by SDS-PAGE. “rLLO” denotes recombinant LLO as a positive control. For hemolytic assay, LLO activity in the normalized supernatant was serially diluted and tested against sheep red blood cells. A high absorbance reading indicates greater LLO activity. Averages of triplicates from one experiment were plotted, with error bars denoting standard deviation. The data shown here are representative of at least three independent experiments. (C) For transcriptional analysis of *hly*, the LLO-encoding gene, WT bacteria from overnight BHI culture were collected, washed, and added to fresh BHI medium supplemented with 250 mM butyrate or 100 mM leucine. The OD was monitored at the indicated sampling time points. Averages of three independent experiments were plotted, with error bars denoting standard deviation. (D) Bacterial samples were harvested at indicated time points postinoculation for RNA extraction, cDNA synthesis, and qPCR analysis. The transcript levels of virulence genes, *hly* and *inlA*, were normalized with the transcript level of *gyrA*, encoding a DNA gyrase subunit A. The data shown here are averages of three independent experiments with error bars denoting the standard deviation. Significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$). The effect of 2MB and isoleucine on LLO production was tested by immunoblotting (E) and hemolytic assay (F) with bacteria grown aerobically overnight in HEPES-buffered BHI (pH 7.0). “–” denotes no supplement, “B” denotes supplementation with butyrate (250 mM) only, “B2” denotes supplementation with butyrate (250 mM) and 2MB (25 mM), and “2” denotes supplementation with 2MB (25 mM) only. Hemolytic units were calculated for leucine- and isoleucine-supplemented samples, denoted by “Leu” and “Ile,” respectively, and are shown below the immunoblot. The data shown here are representative of at least three independent experiments. The hemolysis results shown in panel F are representative of at least three independent experiments. (G) FA analysis was performed by Microbial ID with bacteria harvested and washed from overnight aerobically grown cultures.

compromise not only bacterial defense mechanisms against CAMPs but may also alter signaling events important for fitness in the host environment.

Besides possible roles in modulating signaling events, membrane FA may serve as a direct determinant during physical interactions with CAMP. Studies using either cell free phospholipid reconstitutions or *in silico* molecular dynamics simulations agree

that branching of acyl chains decreases water permeability within the hydrophobic core (46, 53), which would imply a linear correlation between the extent of branching and CAMP resistance. However, based on studies with intact Gram-positive bacteria, there is an apparent lack of a consensus on how different FA constituents would facilitate or prevent CAMP membrane insertion. Our studies showed that *L. monocytogenes* with higher levels of

membrane BCFAs is more resistant to CAMPs, a result favoring a protective role for chain branching against CAMP insertion. These data are reminiscent of the observation that warnericin RK-resistant *Legionella pneumophila* strains also exhibit higher levels of BCFAs (57). An increasing degree of branching or unsaturation in FA acyl chains commonly correlates with increasing membrane fluidity (5, 14, 27). Therefore, these findings suggest a positive relationship between membrane fluidity and CAMP resistance. Indeed, *Staphylococcus aureus* strains resistant to thrombin-induced platelet microbicidal proteins, which are CAMPs found in blood, exhibited increased amounts of longer-chain, unsaturated FAs compared to sensitive parental strains (5). This is further supported by the observation that clinical isolates of methicillin-resistant *S. aureus* that are resistant to daptomycin, a CAMP approved by U.S. Food and Drug Administration for clinical use, all exhibited increased membrane fluidity (39). However, *S. aureus* strains that overproduce carotenoids exhibit reduced membrane fluidity but increased resistance to CAMPs (40) indicated a more complex relationship between membrane fluidity and CAMP susceptibility. Similarly, studies with *L. monocytogenes* spontaneous bacteriocin-resistant strains correlate resistance with decreased membrane fluidity in some cases (34, 38, 41) but with increased membrane fluidity in others (36, 55, 56), implicating other dominant players in CAMP resistance independent of membrane fluidity.

Potential roles of membrane FA composition in resistance against PGN hydrolases. In addition to defense against CAMPs, BCFAs also facilitate resistance against PGN hydrolases, suggesting that disruption in FA composition would likely lead to changes in the cell wall. These putative cell wall changes may occur in the peptidoglycan polymer itself or other associated components and modifications. *L. monocytogenes* is generally considered resistant to lysozyme, a common host defense mechanism, because of the ability to deacetylate cell wall *N*-acetylglucosamine residues by the peptidoglycan *N*-deacetylase PgdA. As a result, a *pgdA* mutant displays increased sensitivity to lysozyme killing and is attenuated *in vivo* (8, 45). Therefore, it is possible that the increased sensitivity to lysozyme exhibited by BCFA-deficient *L. monocytogenes* results from a deficiency in Pgd-related modification. Alternatively, because the peptidoglycan synthesis machinery contains multiple integral membrane proteins, whose activity is influenced by membrane fluidity (33), alterations in FA composition that change membrane fluidity may compromise peptidoglycan synthesis and overall peptidoglycan structure. If BCFA deficiency can compromise cell wall integrity, then CAMPs would have better access to target membranes, perhaps explaining the increased sensitivity of BCFA-deficient bacteria to polymyxin B, which is normally considered ineffective against Gram-positive bacteria in part because of the presence of an extensive cell wall as a protective barrier for the target membrane (16, 49). While a more detailed chemical analysis will be necessary to deduce possible structural defects in the cell wall, it is clear that disrupting bacterial membrane FA composition can elicit global alterations in surface structures affecting interactions with surface-disrupting compounds.

Membrane FA composition in virulence regulation. To further dissect the role of FA composition, we assayed for the effects of lowering the relative ratio between anteiso- and iso-BCFAs through supplementation of specific FA precursors. In WT bacteria typically enriched with anteiso-BCFA, we found that intracellular fitness but not resistance against surface stress was sensitive

to leucine supplementation, which resulted in a decreased anteiso- to iso-BCFA ratio. These results suggest that there may be an optimal FA composition for intracellular fitness. Based on our study, conditions that supported high anteiso-BCFA content also promoted the production of LLO. The exact mechanism by which FAs affect the transcription of *hly* is unclear and is under investigation. Based on similar inhibition seen with *inlA* transcription, we hypothesize that decreasing anteiso-BCFA content suppresses levels or function of PrfA, the major transcriptional activator of *hly*, *inlA*, and other virulence factors (12). In general, PrfA activity can be influenced by several physiological parameters such as carbon source (37) or temperature (26). Based on our study, alterations in FA composition may also modulate PrfA activity perhaps by changing FA flux, which serves as a signal for cellular differentiation in *Legionella pneumophila* (15). It remains to be determined whether and how exposure to environmental FA precursors may affect PrfA activity. Future investigation to determine specific signaling events that take place upon perturbations in FA composition may provide novel insight into the role of membrane properties in virulence regulation.

FAs as potential signaling molecules. The marked effects of butyrate and leucine on membrane FA composition may directly contribute to altered stress resistance and virulence regulation. Nevertheless, we also considered the possibility that butyrate or leucine or their derivatives act as intracellular signaling molecules that lead to surface modifications or virulence regulation independently from modifying membrane composition. Branched-chain amino acids such as leucine and isoleucine have long been recognized as signaling molecules for intracellular nutrient status in low-GC Gram-positive bacteria (48). The key transcriptional regulator CodY binds to branched-chain amino acids and represses the expression of genes involved in amino acid synthesis, sugar transport, and motility in *L. monocytogenes* (6). However, given the large number of genes in the CodY regulon, deletion of *codY* surprisingly did not cause significant changes in virulence (6). Similarly, both leucine and isoleucine are recognized by CodY, and yet they elicited different effects on LLO production. Therefore, although the consequences of leucine or isoleucine supplementation on CodY activity are unknown, it is unlikely that the leucine inhibits LLO production by acting as an inhibitory molecule through CodY signaling.

On the other hand, butyrate is produced as a fermentation product by the intestinal microbiota and is associated with diverse regulatory functions, including activating virulence in enterohemorrhagic *Escherichia coli* (42, 52) and Shiga toxin-expressing *E. coli* (24) and downregulating the expression of genes in *Salmonella* pathogenicity island I in *Salmonella enterica* serovar Typhimurium (11, 20, 32). In *L. monocytogenes*, although anteiso-BCFA levels were restored in butyrate-grown bacteria by 2MB, LLO production remained compromised, supporting a potential signaling role of butyrate independent of modulating FA composition. Although exposure to high levels of butyrate significantly reduced LLO production, virulence responses to intestinal levels of butyrate remain unknown. Future investigation into how straight-chain FAs modulate *L. monocytogenes* virulence will likely extend our understanding of the intestinal phase of *Listeria* infection.

Membrane FA composition as target for antimicrobial therapies. We have shown that manipulation of FA composition of *L. monocytogenes* by genetic or nutritional strategies has global consequences for bacterial stress resistance and virulence. It is likely

that *L. monocytogenes* and other pathogens have evolved a specific membrane FA composition to provide an optimal lipid environment for efficient membrane-associated functions during interactions with their host. Although *L. monocytogenes* can tolerate major changes in FA composition outside of host cells, these changes significantly attenuate *L. monocytogenes* in the host environment even when we singly remove host defense mechanism such as lysozyme or CAMPs. Therefore, our observations strongly support disrupting native FA composition as an effective antimicrobial strategy for several reasons. FA synthesis has been studied extensively as a bacterial process targeted for drug development. However, such efforts have been challenged by the variable efficacy of FA synthesis inhibitors in serum (4, 9, 43) and the emergence of resistant strains (23). As an alternative, we propose disrupting FA composition as another approach to cause multiple defects in target pathogens and render them more broadly susceptible to host defenses or conventional antibiotics. Because disrupting FA composition is not inherently lethal, it is less likely to induce the spontaneous generation of resistant strains. Furthermore, by disrupting FA composition, we may compromise bacterial virulence regulation and reduce infectivity, providing time for host defenses or conventional treatment regimens to clear infections.

ACKNOWLEDGMENTS

We thank Ronald G. Larson and Shihu Wang for helpful discussions on membrane architecture based on molecular dynamic studies. We are grateful to Kristin Burkholder and Sara Cassidy for critical reviews of the manuscript and to other members of the O'Riordan laboratory for helpful discussions. We also want to thank the Center for Statistical Consultation and Research in the University of Michigan for their help on statistical analyses.

This study was supported by NIH grant AI064540 (M.X.D.O.), USDA National Research Initiative Competitive Grant Program 2006-35201-17386 (B.J.W.), and USDA National Institute of Food and Agriculture Postdoctoral Fellowship 2011-67012-30682 (Y.S.).

REFERENCES

- Abachin E, et al. 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. *Mol. Microbiol.* 43: 1–14.
- Reference deleted.
- Bader MW, et al. 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* 122:461–472.
- Balemans W, et al. 2010. Essentiality of FASII pathway for *Staphylococcus aureus*. *Nature* 463:E3.
- Bayer AS, et al. 2000. *In vitro* resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. *Infect. Immun.* 68:3548–3553.
- Bennett HJ, et al. 2007. Characterization of *relA* and *codY* mutants of *Listeria monocytogenes*: identification of the CodY regulon and its role in virulence. *Mol. Microbiol.* 63:1453–1467.
- Bonazzi M, Lecuit M, Cossart P. 2009. *Listeria monocytogenes* internalin and E-cadherin: from structure to pathogenesis. *Cell. Microbiol.* 11:693–702.
- Boneca IG, et al. 2007. A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. *Proc. Natl. Acad. Sci. U. S. A.* 104:997–1002.
- Brinster S, et al. 2009. Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. *Nature* 458:83–86.
- Camberg JL, et al. 2007. Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids. *EMBO J.* 26:19–27.
- Camejo A, et al. 2009. *In vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLoS Pathog.* 5:e1000449. doi:10.1371/journal.ppat.1000449.
- Cardenal-Muñoz E, Ramos-Morales F. 2011. Analysis of the expression, secretion, and translocation of the *Salmonella enterica* type III secretion system effector SteA. *PLoS One* 6:e26930. doi:10.1371/journal.pone.0026930.
- de las Heras A, Cain RJ, Bielecka MK, Vázquez-Boland JA. 2011. Regulation of *Listeria* virulence: PrfA master and commander. *Curr. Opin. Microbiol.* 14:118–127.
- Diogo A, Verissimo A, Nobre MF, da Costa MS. 1999. Usefulness of fatty acid composition for differentiation of *Legionella* species. *J. Clin. Microbiol.* 37:2248–2254.
- Edgcomb MR, Sirimanne S, Wilkinson BJ, Drouin P, Morse RD. 2000. Electron paramagnetic resonance studies of the membrane fluidity of the food-borne pathogenic psychrotroph *Listeria monocytogenes*. *Biochim. Biophys. Acta Biomembr.* 1463:31–42.
- Edwards RL, Dalebroux ZD, Swanson MS. 2009. *Legionella pneumophila* couples fatty acid flux to microbial differentiation and virulence. *Mol. Microbiol.* 71:1190–1204.
- Evans M, Feola D, Rapp R. 1999. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant Gram-negative bacteria. *Ann. Pharmacother.* 33:960–967.
- Flannagan RS, Cosio G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat. Rev. Microbiol.* 7:355–366.
- Fliss I, Emond E, Simard RE, Pandian S. 1991. A rapid and efficient method of lysis of *Listeria* and other Gram-positive bacteria using mutanolysin. *Biotechniques* 453:456–457.
- Gallo R, et al. 1997. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse RID A-8931-2009. *J. Biol. Chem.* 272:13088–13093.
- Gantois I, et al. 2006. Butyrate specifically downregulates *Salmonella* pathogenicity island 1 gene expression. *Appl. Environ. Microbiol.* 72:946–949.
- Giles DK, Hankins JV, Guan Z, Trent MS. 2011. Remodeling of the *Vibrio cholerae* membrane by incorporation of exogenous fatty acids from host and aquatic environments. *Mol. Microbiol.* 79:716–728.
- Giotis ES, McDowell DA, Blair IS, Wilkinson BJ. 2007. Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 73:997–1001.
- Heath R, White S, Rock C. 2002. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. *Appl. Microbiol. Biotechnol.* 58:695–703.
- Herold S, Paton JC, Srimanote P, Paton AW. 2009. Differential effects of short-chain fatty acids and iron on expression of *iha* in Shiga-toxinogenic *Escherichia coli*. *Microbiology* 155:3554–3563.
- Johansen C, Gill T, Gram L. 1995. Antibacterial effect of protamine assayed by impedimetry. *J. Appl. Microbiol.* 78:297–303.
- Johansson J, et al. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* 110:551–561.
- Jones S, Drouin P, Wilkinson B, Morse P. 2002. Correlation of long-range membrane order with temperature-dependent growth characteristics of parent and a cold-sensitive, branched-chain-fatty-acid-deficient mutant of *Listeria monocytogenes*. *Arch. Microbiol.* 177:217–222.
- Julotok M, Singh AK, Gatto C, Wilkinson BJ. 2010. Influence of fatty acid precursors, including food preservatives, on the growth and fatty acid composition of *Listeria monocytogenes* at 37 and 10°C. *Appl. Environ. Microbiol.* 76:1423–1432.
- Kaneda T. 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* 55:288–302.
- Keeney K, Colosi L, Weber W, O'Riordan M. 2009. Generation of branched-chain fatty acids through lipoate-dependent metabolism facilitates intracellular growth of *Listeria monocytogenes*. *J. Bacteriol.* 191:2187–2196.
- Koprivnjak T, Peschel A. 2011. Bacterial resistance mechanisms against host defense peptides. *Cell. Mol. Life Sci.* 68:2243–2254.
- Lawhon S, Maurer R, Suyemoto M, Altier C. 2002. Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. *Mol. Microbiol.* 46:1451–1464.
- Lee PP, Weppner WA, Neuhaus FC. 1980. Initial membrane reaction in peptidoglycan synthesis: perturbation of lipid-phospho-N-acetylmuramyl-pentapeptide translocase interactions by n-Butanol. *Biochim. Biophys. Acta Biomembr.* 597:603–613.
- Martínez B, Rodríguez A. 2005. Antimicrobial susceptibility of nisin resistant *Listeria monocytogenes* of dairy origin. *FEMS Microbiol. Lett.* 252:67–72.
- Mendez-Samperio P. 2010. The human cathelicidin hCAP18/LL-37: a

- multifunctional peptide involved in mycobacterial infections. *Peptides* 31:1791–1798.
36. Mendoza F, Maqueda M, Galvez A, Martinez-Bueno M, Valdivia E. 1999. Antilisterial activity of peptide AS-48 and study of changes induced in the cell envelope properties of an AS-48-adapted strain of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 65:618–625.
 37. Milenbachs AA, Brown DP, Moors M, Youngman P. 1997. Carbon source regulation of virulence gene expression in *Listeria monocytogenes*. *Mol. Microbiol.* 23:1075–1085.
 38. Ming X, Daeschel M. 1993. Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott-A. *J. Food Prot.* 56:944–948.
 39. Mishra NN, et al. 2011. *In vitro* cross-resistance to daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob. Agents Chemother.* 55:4012–4018.
 40. Mishra NN, et al. 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *Antimicrob. Agents Chemother.* 55:526–531.
 41. Naghmouchi K, Drider D, Hammami R, Fliss I. 2008. Effect of antimicrobial peptides divergicin M35 and nisin A on *Listeria monocytogenes* LSD530 potassium channels. *Curr. Microbiol.* 56:609–612.
 42. Nakanishi N, et al. 2009. Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli*. *Microbiology* 155:521–530.
 43. Parsons JB, Frank MW, Subramanian C, Saenkhom P, Rock CO. 2011. Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 108:15373–15383.
 44. Potter R, Truelstrup Hansen L, Gill TA. 2005. Inhibition of foodborne bacteria by native and modified protamine: importance of electrostatic interactions. *Int. J. Food Microbiol.* 103:23–34.
 45. Rae CS, Geissler A, Adamson PC, Portnoy DA. 2011. Mutations of the *Listeria monocytogenes* peptidoglycan *N*-deacetylase and *O*-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways. *Infect. Immun.* 79:3596–3606.
 46. Shinoda W, Mikami M, Baba T, Hato M. 2004. Molecular dynamics study on the effects of chain branching on the physical properties of lipid bilayers. 2. Permeability. *J. Phys. Chem. B* 108:9346–9356.
 47. Singh AK, et al. 2009. FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. *FEMS Microbiol. Lett.* 301:188–192.
 48. Stenz L, et al. 2011. The CodY pleiotropic repressor controls virulence in Gram-positive pathogens. *FEMS Immunol. Med. Microbiol.* 62:123–139.
 49. Storm DR, Rosenthal KS, Swanson PE. 1977. Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* 46:723–763.
 50. Sun Y, O'Riordan MXD. 2010. Branched-chain fatty acids promote *Listeria monocytogenes* intracellular infection and virulence. *Infect. Immun.* 78:4667–4673.
 51. Thedieck K, et al. 2006. The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. *Mol. Microbiol.* 62:1325–1339.
 52. Tobe T, Nakanishi N, Sugimoto N. 2011. Activation of motility by sensing short-chain fatty acids via two steps in a flagellar gene regulatory cascade in enterohemorrhagic *Escherichia coli*. *Infect. Immun.* 79:1016–1024.
 53. Tristram-Nagle S, et al. 2010. Structure and water permeability of fully hydrated diphytanoyl PC. *Chem. Phys. Lipids* 163:630–637.
 54. Turner J, Cho Y, Dinh N-N, Waring AJ, Lehrer RI. 1998. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* 42:2206–2214.
 55. Vadyvaloo V, et al. 2004. Cell-surface alterations in class IIa bacteriocin-resistant *Listeria monocytogenes* strains. *Microbiology* 150:3025–3033.
 56. Vadyvaloo V, Hastings JW, van der Merwe MJ, Rautenbach M. 2002. Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. *Appl. Environ. Microbiol.* 68:5223–5230.
 57. Verdon J, et al. 2011. Fatty acid composition modulates sensitivity of *Legionella pneumophila* to warnericin RK, an antimicrobial peptide. *Biochim. Biophys. Acta Biomembr.* 1808:1146–1153.
 58. Whittaker P, et al. 2005. Use of fatty acid profiles to identify food-borne bacterial pathogens and aerobic endospore-forming bacilli. *J. Agric. Food Chem.* 53:3735–3742.
 59. Willecke K, Pardee AB. 1971. Fatty acid-requiring mutant of *Bacillus subtilis* defective in branched chain α -keto acid dehydrogenase. *J. Biol. Chem.* 246:5264–5272.
 60. Yeaman MR, Yount NY. 2003. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 55:27–55.
 61. Zhang Y-M, Rock CO. 2008. Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol.* 6:222–233.
 62. Zhu K, Ding X, Julotok M, Wilkinson BJ. 2005. Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso- $C_{15:0}$ fatty acid required for low-temperature growth of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 71:8002–8007.