The Transcriptional Response of *Listeria monocytogenes* during Adaptation to Growth on Lactate and Diacetate Includes Synergistic Changes That Increase Fermentative Acetoin Production^{∇†}

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The organic acids lactate and diacetate are commonly used in combination in ready-to-eat foods because they show synergistic ability to inhibit the growth of *Listeria monocytogenes*. Full-genome microarrays were used to investigate the synergistic transcriptomic responses of two *L. monocytogenes* strains, H7858 (serotype 4b) and F6854 (serotype 1/2a), to these two organic acids under conditions representing osmotic and cold stress encountered in foods. Strains were exposed to brain heart infusion (BHI) broth at 7°C with 4.65% water-phase (w.p.) NaCl at pH 6.1 with (i) 2% w.p. potassium lactate, (ii) 0.14% w.p. sodium diacetate, (iii) the combination of both at the same levels, or (iv) no organic acids as a control. RNA was extracted 8 h after exposure, during lag phase, to capture gene transcription changes during adaptation to the organic acid stress. Significant differential transcription of 1,041 genes in H7858 and 640 genes in F6854 was observed in at least one pair of the 4 different treatments. The effects of combined treatment with lactate and diacetate included (i) synergistic transcription differences for 474 and 209 genes in H7858 and F6854, respectively, (ii) differential transcription of genes encoding cation transporters and ABC transporters of metals, and (iii) altered metabolism, including induction of a nutrient-limiting stress response, reduction of menaquinone biosynthesis, and a shift from fermentative production of acetate and lactate to energetically less favorable, neutral acetoin. These data suggest that additional treatments that interfere with cellular energy generation processes could more efficiently inhibit the growth of *L. monocytogenes*.

Listeria monocytogenes is a psychrotolerant food-borne pathogen that is of particular concern to the ready-to-eat-meat and -seafood industries because its ability to grow at temperatures as low as −0.4°C (70) reduces the ability of refrigeration to control the pathogen's growth in foods. Because *L. monocytogenes* generally contaminates foods at low levels (27) and has a high infectious dose (69), the ability of the organism to grow in foods is critical for its ability to cause disease (16). Foods do not support growth if they are stored frozen, have a pH of ≤4.4, have a water activity of <0.92, or incorporate some type of growth inhibiting measure (21), e.g., chemical preservatives (32). Ready-to-eat meat and seafood products have been identified as high-risk foods for listeriosis, as these foods support growth of *L. monocytogenes* (22), but it has also been predicted that reformulation of U.S. deli meats with effective growth inhibitors, e.g., the organic acids lactate and diacetate, would result in a 2- to 8-fold reduction of listeriosis due to consumption of these products (55). One particular advantage to using the combination of lactate and diacetate for *L. monocytogenes* control in meat and seafood products is that these inhibitors have been shown to have greater-than-addi-

tive, i.e., synergistic, growth-inhibiting effects (65), although the mechanisms behind those synergistic effects are unknown.

The classic theory of how organic acids inhibit growth of bacteria and yeasts is that the undissociated portion of the acid in solution is able to freely diffuse across the cell membrane, and inside the cell the acid then dissociates into nondiffusible hydrogen cations and acid anions due to high intracellular pH. Accumulation of intracellular ions is thought to have many growth-inhibiting effects, including reduction of the proton motive force, reduction of intracellular pH, interference with microbial metabolism, and anion toxicity (9, 20, 37). Bacteria respond to organic acid stress by transcriptional activation of the general stress (3, 7) and oxidative stress (35) responses, membrane alterations (5) (possibly to reduce diffusion of organic acids or increase anion transport) (52), and metabolic changes (7, 44, 50). These organic acid responses appear to be distinct from the inorganic acid stress response caused by exposure to low pH from added HCl, which in *L. monocytogenes* includes activation of general stress responses, transcription of genes encoding proton pumps, and cell membrane modification (18, 59). However, few studies (see, e.g., references 7, 23, 24, and 50) have specifically studied the organic acid stress response separated from the inorganic acid stress response. Organic acids are used as growth inhibitors in mildly acidic foods because lower pH increases the concentration of inhibitory (undissociated) organic acid, thus compounding the effects of organic acid stress with inorganic acid stress.

The goal of this research was to investigate the transcriptomic response of *L. monocytogenes* to the combination of

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lactate and diacetate in a complex, stressful environment to better understand bacterial adaptation to these organic acids and the mechanisms behind their synergistic growth inhibition. To date, acid stress response studies of bacteria have focused on acid-adapted exponential-phase (7, 34) or stationary-phase (41) cells or on transcription differences during acid shock (exposures of less than 1 h) (3, 5). As the end goal of bacteriostatic growth-inhibiting treatments in foods is to indefinitely extend the lag phase, our work focused specifically on the transcriptomic response to organic acid stress under conditions approximating adaptation (8 h into lag phase) during refrigerated storage (7°C, microaerophilic conditions) of a food product (pH 6.1, 4.65% water-phase [w.p.] NaCl). Lag-phase transcription has only occasionally been studied in bacteria (51) and yeast (8) and not under stress conditions. After controlling for cold and osmotic stress, we found that *L. monocytogenes* strains H7858 and F6854 responded to lactate and diacetate stress with unique, large-scale changes to transcription, including many changes in transcription of genes encoding proteins involved in membrane transport and energy metabolism. One effect was increased acetoin production, at the expense of menaquinone-dependent respiration and fermentative production of organic acids.

MATERIALS AND METHODS

Strains and growth conditions. The *L. monocytogenes* strains used in this study were H7858 (FSL F6-366) and F6854 (FSL R2-559) (47). H7858 is a lineage I, serotype 4b strain, and F6854 is a lineage II, serotype 1/2a strain. Both are isolates from ready-to-eat meats and have been linked to human listeriosis cases (47); H7858 was isolated from deli meats linked to a multistate outbreak in 1998 to 1999 (11), and F6854 was isolated from hot dogs linked to a sporadic case in 1988 (10).

Strains were grown in chemically defined minimal medium (DM) (2) to stationary phase and then transferred to brain heart infusion (BHI) broth with organic acids, as previously described (65), to simulate pregrowth in a nutrient-limited (e.g., food processing) environment followed by transition to a nutrient-rich (e.g., food product) environment. Briefly, single colonies were used to inoculate 5 ml BHI broth for overnight (14 to 18 h) growth in a shaking incubator (230 rpm, 37°C). BHI cultures were used to inoculate 5 ml of DM, with 25 mM glucose, acclimated to 16°C at a 1:100 dilution, followed by incubation at 16°C without shaking until late log phase, defined as an optical density at 600 nm (OD_{600}) of 0.4 ± 0.02 (which represents about 24 h of growth). Late-log-phase cultures were used to inoculate 100 ml of DM at 16°C in 300-ml flasks (Bellco Glass Co., Vineland, NJ) at a 1:100 dilution. Cultures in DM were incubated for 72 h without shaking until early stationary phase. Early-stationary-phase cultures in DM were then used to inoculate 90 ml of BHI treatment medium at 7°C with a 1:10 dilution, for a final inoculum of $\sim 2 \times 10^8$ CFU/ml. Cultures were then incubated at 7°C for 8 h to allow for induction of a lag-phase transcriptomic response before RNA extraction; for a temporal perspective, 8 h represents 5% of the overall 160-h lag phase observed in cells treated with this combination of lactate and diacetate (65). Treatment media were prepared as previously described (65) to contain 4.65% water-phase (w.p.) NaCl and either no organic acids as a control (CTRL), 0.14% w.p. sodium diacetate (SDA) (Macco Organiques, Inc., Valleyfield, Quebec, Canada), 2% w.p. potassium lactate (PL) (Purasal Hi Pure P-Plus; Purac America, Inc., Lincolnshire, IL), or the combination of 0.14% w.p. SDA and 2% w.p. PL (PLSDA) (Purasal OptiForm PD-Plus; Purac America, Inc.). BHI media were adjusted to pH 6.0 with HCl before autoclaving (for a pH after 9:10 dilution with DM of pH 6.1) and stored at 4°C until use.

RNA extraction. After 8 h of incubation in treatment medium, the 100-ml treatment cultures were added to 10 ml of chilled 10% phenol-ethanol solution in 250-ml polypropylene centrifuge bottles (Nalgene) to stop RNA transcription. Cells were then pelleted by centrifugation (10,000 rpm, 10 min) at 4°C and suspended in 5 ml TRI reagent (Ambion, Austin, TX) for mechanical lysis by bead beating (in screw cap tubes with 3 ml of 0.1-mm acid-washed zirconium beads; 5 min homogenization in a Mini-Beadbeater-8 [BioSpec Products, Inc., Barlesville, OK]), followed by RNA extraction according to the TRI reagent

FIG. 1. The dye-swapped, single-loop design hybridizes a single biological replicate of both the PL and SDA treatments to both the CTRL and PLSDA treatments (4 hybridizations), using opposite dye labels for each sample, and a second biological replicate is hybridized with dye assignments swapped (4 more hybridizations) to balance labeling effects. The design was repeated twice for each *L. monocytogenes* strain, H7858 and F6854, comprising 32 total hybridizations over 4 biological replicates per strain. Arrow bases and arrowheads represent Cy3- and Cy5-labeled cDNA. Boxes contain the factor levels used for ANOVA model A (i.e., CTRL, SDA, PL, or PLSDA), and indicated above and below the boxes are the factor levels used for ANOVA model B (i.e., lactate or diacetate).

manufacturer's recommendations. RNA samples were treated with RQ1 DNase (70 U/100 μ l RNA; Promega, Madison, WI), followed by cleanup using the Qiagen RNeasy minikit (Qiagen, Valencia, CA). RNA was quantified using a NanoDrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE), and samples with an A_{260}/A_{230} ratio of >1.8 and an A_{260}/A_{280} ratio of >1.9 were considered acceptable. Any samples with an A_{260}/A_{230} ratio of <1.8 or an A_{260}/A_{280} ratio of <1.9 were precipitated in 3 volumes ethanol and 1/10 volume sodium acetate at -80°C , washed with ethanol, and hydrated in nuclease-free water. The RNA integrity of all samples was measured on a Bioanalyzer (Agilent Technologies, Santa Clara, CA), and only samples with an RNA integrity number of >8.0 were used.

cDNA labeling and microarray hybridization. cDNA labeling and array hybridization were performed essentially as previously described (49) using the JCVI PFGR Microbial RNA Aminoallyl Labeling for Microarrays standard operating procedure (SOP no. M007; <http://pfgc.jcvi.org/index.php/microarray/protocols>) to label 6 μ g total RNA as input for indirect labeling with Cy3/Cy5 fluorescent dyes. JCVI PFGR *L. monocytogenes* full-genome microarrays (version 3) were hybridized using the JCVI PFGR Hybridization of Labeled DNA and cDNA Probes standard operating procedure (SOP no. M008), and slides were scanned with a GenePix 4000B array scanner (Molecular Devices, Sunnyvale, CA).

Statistical analysis of microarray data. Microarrays were hybridized using two complete replicates (4 biological replicates for each strain) of a dye-swapped, single-loop hybridization scheme (Fig. 1) which allowed for maximum statistical power to estimate the comparisons of interest, i.e., the effects of lactate and diacetate treatment and their interaction (26). For all arrays, fluorescence of Cy3 and Cy5 channels were extracted as median intensity values, with no background correction, and imported in the statistical platform R (v2.2.1). The software package MAANOVA (v0.98-7; <http://www.bioconductor.org/packages/bioc/1.7/src/contrib/html/maanova.html>) was used to spatially normalize the intensity values of each array by printing block using LOWESS normalization, and normalized values were averaged for each of three replicates per probe.

Analysis of variance (ANOVA) was chosen to analyze the array data, as this technique allows for appropriate specification of the sources of variation in complex hybridization designs (17). The array data were fit to two mixed-model designs where the first, model A, was designed to capture the effect of each organic acid treatment individually using a single treatment factor with four levels (the levels are designated CTRL, SDA, PL, and PLSDA) and the second, model B, was designed to model the interaction between diacetate and lactate using two factors, diacetate and lactate, each with two levels, e.g., no diacetate and diacetate. Model A is specified as $y = \text{treatment} + \text{array} + \text{sample} + \text{dye} + E$, where y is the transcript level of a gene, treatment is the fixed effect of organic acid treatment (CTRL, SDA, PL, and PLSDA), the random effects are the specific array hybridized, the RNA sample, and the Cy3 or Cy5 dye label, and E is the residual error in the model. Model B is specified as $y = \text{diacetate} + \text{lactate} + \text{diacetate} \times \text{lactate} + \text{array} + \text{sample} + \text{dye} + E$, where

the fixed effects diacetate and lactate represent the effect of either acid, the diacetate $\times$ lactate term captures the interaction between acids, and the other parameters are the same as in model A. Throughout this paper, use of the organic acid abbreviations (e.g., SDA) refers to results from model A and use of acid names (e.g., diacetate) refers to results from model B. Tests for statistical significance of treatment, lactate, diacetate, and the interaction effects were based on 1,000 permutations of sample labels and *P* values adjusted using Benjamini-Hochberg step-up correction for multiple comparisons. The transcript target of a probe was considered significantly differentially transcribed for an effect if the signal for that probe had an adjusted *P* value of <0.05 with an absolute fold change of >1.5 between at least one pair of treatment conditions across the treatment effect or for the diacetate, lactate, or interaction effects. To address reproducibility between and among the replicate array data, the contributions of the three random effects (microarray slide, biological sample, and experimental error) were plotted (see Fig. S1 in the supplemental material), and only the array effect contributed to the variance to a greater degree than the random error.

Probe annotation. According to the BLAST-based annotation provided by JCVI, there are cases where multiple probes target the same locus in a single strain, as well as cases where a single probe targets multiple loci in one strain. For each strain, the set of all probes with reported BLAST hits to the target strain was filtered to a subset of relevant probes containing what we determined was the best BLAST hit to each targeted locus, according to the following criteria. First, all probes without a BLAST hit to H7858 or F6854 were dropped (all BLAST hits to these 70-nucleotide [nt] oligonucleotides contained at least 56 nt at an 89.9% match to the target and an E score of $\leq 1.1 \times 10^{-7}$). Next, for all loci targeted by more than one probe, only those probes that were the best BLAST match to those loci were retained, where the best match was defined as the maximum nucleotide length and percent match of any probe to that locus. After this process, a subset of $\sim 3,000$ probes remained for each strain. Most loci were targeted by only 1 probe, though there were cases where up to 4 probes targeted the same locus. In total, 2,991 loci, of 3,117 total, were probed in H7858 (≥ 65 nt; percent match, $\geq 89.9\%$), and 2,902 loci, of 3,028 total, were probed in F6854 (≥ 61 nt; percent match, $\geq 89.9\%$).

To facilitate comparison between the results for H7858 and F6854 as well as comparison to other published work, the open reading frames (ORFs) targeted by the probes were annotated according to orthologous genes in the EGD-e reference genome wherever possible, as determined by orthologous protein sequences using OrthoMCL (39). When a probe targeted an ORF with an ortholog in EGD-e, the probe was annotated with the EGD-e locus identifiers; if the ORF had more than one EGD-e ortholog, all EGD-e locus IDs were included as hits for a probe; and if there was no EGD-e ortholog, the strain-specific locus IDs, were maintained. In some cases, annotating two different strain-specific ORFs as the same EGD-e locus IDs created additional cases where multiple oligonucleotides probed the same locus. If any of the redundant probes matched less well (as indicated by a shorter nucleotide match or lower percent match) to the H7858 or F6854 ORF orthologous to the EGD-e locus ID in the new annotation, it was dropped; otherwise, duplicates were retained until the next level of screening. Probes annotated with an EGD-e locus ID were assigned operon membership as previously reported (68).

Using the *a priori* methods described above, it was possible to generate a list of probes that best matched each strain's ORFs and to annotate those ORFs, wherever possible using EGD-e locus IDs, yet this list still contained multiple probes to the same gene. Once the microarray data were collected and each probe analyzed for significance, the following two procedures were applied to collapse the redundant list of probes to a list of unique parameter estimates for each gene probed in a strain. First, any gene with transcripts targeted by more than two probes was considered significantly differentially transcribed only if all probes for that gene had signals that were significantly differentially transcribed and the parameter estimates for all significant comparisons did not conflict (e.g., a gene was not considered significantly differentially transcribed if the target of probe for that gene showed induction by lactate treatment and the other showed repression by lactate treatment). Second, the ANOVA parameter estimates for all the probes for a gene were averaged to calculate single estimates for each gene.

Statistical analysis of gene transcription results. Quality threshold (QT) analysis (30) implemented in Multiexperiment Viewer (v4.4) (60) was used to cluster all genes for each strain that were significant in the model A treatment test based on their $\log_2$ ANOVA parameter estimates for the effects of CTRL, SDA, PL, and PLSDA. The minimum quality threshold was set at a diameter of 0.6 for the absolute difference in estimates, so genes showing similar patterns of upregulation and downregulation cluster separately.

Gene set enrichment analysis (GSEA) (67) was used to test for statistically

significant positive or negative transcription of genes functionally related by membership in role categories or previously published regulons. Role categories were downloaded from the Comprehensive Microbial Resource Center (<http://cmr.jcvi.org/>). GSEA was also used to test for genes under the control of 9 known global regulators in *L. monocytogenes*, σ^B (56), σ^H (14), σ^L (14), CodY (6), PrfA (43), VirR (40), VirS (40), CcpA (42), and HprK (42). The gene sets for σ^B , σ^H , σ^L , PrfA, and VirR contained only positively regulated genes, the set for CodY contained only negatively regulated genes, the sets for CcpA and HprK were divided into one set each for positively and negatively regulated genes, and the set for VirS contained both positively and negatively regulated genes. All pairwise comparisons of treatment effects (model A), as well as the diacetate, lactate, and interaction effects (model B), were tested for enrichment, with significance defined as a *P* value of <0.05 and a false-discovery rate (FDR) of $<30\%$.

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was used to confirm gene transcription patterns suggested by microarray analysis. Primers and TaqMan minor-groove-binding probes were designed using Primer Express v.1.0 (Applied Biosystems, Foster City, CA) to match a consensus sequence for strains H7858 and F6854 unless previously reported primers and probes were available (see Table S1 in the supplemental material). qRT-PCR was performed essentially as previously described (49), using cDNA reverse transcribed with random hexamers (Applied Biosystems) from 1 μ g RNA and target gene copy numbers absolutely quantified using genomic DNA standard curves (12). cDNA was synthesized from the CTRL and PLSDA treatments of the same RNA samples used to hybridize the microarrays. Due to insufficient RNA yields from replicate 3 of F6854, only replicates 1, 2, and 4 were used; all 4 replicates of H7858 were used. As both housekeeping genes previously used to normalize target gene mRNA transcript levels (12) showed significant differential transcription in response to PLSDA treatment compared to CTRL (*spoB* by qRT-PCR, *gap* by microarray), target mRNA copy numbers were normalized to total input RNA at the cDNA synthesis stage. To normalize variance, mRNA copy numbers were $\log_{10}$ transformed and transcript levels were analyzed (in JMP, v. 7.0; SAS Institute Inc., Cary, NC) using a separate ANOVA for each strain and gene with the formula $y = \text{treatment} + \text{replicate} + E$, where y is the $\log_{10}$ copy number, treatment is either CTRL or PLSDA, replicate is included as a blocking factor, and E is the error term.

Acetoin production assay. The acetoin produced by *L. monocytogenes* cultures treated with acetate and lactate under aerobic (shaking, 230 rpm) and microaerophilic (static) conditions was measured with a Voges-Proskauer reaction, with modifications (58). Cultures of H7858 and F6854 were pregrown to stationary phase in DM, as described above, and inoculated 1:10 into 90 ml BHI treatment medium prewarmed to 37°C, followed by incubation with or without shaking.

Treatment media were formulated as in the array experiment, with an additional treatment of 0.74 mM acetoin (Sigma). All combination of treatments were tested (CTRL, SDA, PL, Act, PL-SDA, SDA-Act, PL-Act, and PLSDA-Act) in biological triplicate for each strain and each oxygen level (i.e., with and without shaking). Acetoin production was measured after 5.5 h of incubation by adding a 100- μ l sample to 10 μ l L-arginine (10 mg/ml in dH₂O; Sigma), 50 μ l 1-naphthol (10 mg/ml in ethanol; Sigma), and 25 μ l 7 M KOH. After 4 h of incubation at room temperature, absorbance at 520 nm was measured (using a NanoDrop spectrophotometer) and compared to a standard curve in CTRL medium (using 10 mM to 0.31 mM acetoin in 2-fold serial dilutions), which was linear for A_{520} versus [acetoin]^{0.5}. The calculated acetoin produced in each treatment was normalized to the optical density (OD₆₀₀) of the cultures after 5.5 h of incubation, and the results were analyzed (in JMP) by oxygen condition with the ANOVA model [acetoin] = diacetate $\times$ lactate $\times$ acetoin $\times$ strain + date + E , where the fixed effects diacetate, lactate, acetoin, and strain represent the effects of the three metabolites in the treatment medium and the strains tested and these are fully crossed to estimate all possible interactions. Date is a fixed-effect blocking term, and E is error. The effect of date was significant as a blocking factor and was subtracted from the data before calculating the average and standard deviation of the data for visual presentation.

Microarray data accession number. Raw and normalized microarray data are available at the NCBI Gene Expression Omnibus (GEO) under database accession number GSE25195.

RESULTS

Treatment with organic acids causes unique, large-scale changes to *L. monocytogenes* transcription. Microarray data were analyzed by two ANOVA models. Model A tested for gene transcript level differences between each of the four or-

ganic acid treatments (CTRL, SDA, PL, and PLSDA). Model B tested for gene transcript level differences consistent with the diacetate and lactate treatments having independent, additive effects or with diacetate and lactate interacting and having synergistic or antagonistic effects. In total, there were 1,416 genes that showed significant (adjusted *P* value of <0.05), differential transcription (fold change, >1.5) in at least one of the two strains (see Workbook S1 in the supplemental material) in at least one of the ANOVA models. In strains H7858 and F6854, 1,041 and 640 genes, respectively, were differentially transcribed; among these genes, 265 were differentially transcribed in both strains. Two major results from these gene counts, which combine ANOVA models A and B, are (i) that H7858 differentially transcribes about twice as many genes as F6854 in response to organic acid stress and (ii) that only a minor fraction of the differentially transcribed genes are shared. Therefore, we will focus on comparing and contrasting groups of genes with shared transcription patterns or functional similarities and highlight those shared responses with mechanistic or phenotypic support.

Focusing on differential gene transcription between treatments (model A), there were 983 and 662 genes that were significantly differentially transcribed in H7858 and F6854, respectively, between at least one pair of treatments (e.g., between PL and CTRL). Differentially transcribed genes clustered into 12 and 7 quality-threshold clusters for H7858 and F6854, respectively; these clusters grouped into 12 distinct transcription patterns (Fig. 2 and Table 1; lists of genes by cluster pattern are given in Workbook S1 in the supplemental material). Patterns A and G contained the majority of differentially transcribed genes from H7858, and genes with these patterns showed increased (pattern A) or decreased (pattern G) transcript levels in response to single-inhibitor treatments (SDA and PL) and a greater response to PLSDA; similar transcription profiles were not identified in F6854. Patterns B and H contained the majority of differentially transcribed F6854 genes and a smaller number of genes from H7858, and genes with these patterns showed differential transcription in response to PL treatment and a greater response to PLSDA but a minimal response to SDA treatment.

H7858 and F6854 show unique, mostly synergistic, responses to diacetate and lactate treatment. Testing of the additive effects of diacetate and lactate and their interaction on transcript levels (model B) showed that each strain had a large number of, mostly unique, gene transcripts with a significant interaction effect (Table 2) (474 genes in H7858 and 209 in F6854; 54 shared), where the interaction means that the effect on gene transcription of diacetate in the presence of lactate is different than the effect of diacetate when lactate is not present. These interactions cause either (i) synergy, where the effect of the combined treatment is increased beyond the additive effect of both treatments (e.g., outliers with transcription pattern A or G [Fig. 2]) or where only the combination treatment causes differential transcription (e.g., transcription pattern J), or (ii) antagonism, where the combined treatment causes less than an additive effect (e.g., transcription pattern K) or where the combination actually has the opposite effect (e.g., transcription patterns C and F). The presence of so many genes with significant interaction effects indicates that these

phenotypically synergistic organic acid treatments (65) also have synergistic effects on gene transcription.

Considering the effects of diacetate or lactate on transcript levels of genes with no significant interaction effects, approximately 200 genes in H7858 were differentially transcribed in response to either diacetate or lactate (Table 2). Transcript levels of 133 of those genes were affected by both lactate and acetate, showing an additive effect of organic acid treatment. F6854 had about 200 genes with significant changes in transcript level in response to lactate but showed no genes differentially transcribed in response to acetate. The presence of a significant diacetate effect only in H7858 is consistent with the GI treatment QT clusters (QTCs) (Fig. 2, from model A) where the majority patterns for H7858 (A and G) showed a response to the SDA treatment but the majority patterns for F6854 (B and H) did not. The gene expression patterns suggest that the genes involved in the transcriptional response of F6854 to diacetate treatment alone are the same genes that are affected by the interaction of acetate and lactate. The growth phenotypes of 13 strains of *L. monocytogenes*, including H7858 and F6854, show that the effects of diacetate and the interaction of diacetate and lactate contribute similar amounts to changes in lag phase and growth rate, both to a lesser extent than lactate alone (65), making it plausible that the acetate and interaction effects could largely overlap in some strains.

Organic acid treatment alters transcription of genes encoding membrane systems involved in ion transport or permeability. Two large groups of differentially transcribed genes plausibly related to the organic acid stress response include genes encoding (i) systems involved in cation transport and (ii) ABC transporters of metal or of unknown function. Among 21 genes annotated as cation and iron-carrying compound transporters in either H7858 or F6854, 14 showed lower transcript levels in one or both strains exposed to GI treatments compared to the control (13 in PLSDA versus CTRL and 1 in PL versus CTRL) (Table 3). Three of the genes with increased transcript levels are members of the *mnh* operon, encoding an Na^+/H^+ antiporter; these genes showed increased transcript levels in H7858 in either PL versus CTRL or PLSDA versus CTRL comparisons. Two of the multidrug and toxic compound efflux (MATE) pump genes are in operons with putative transcriptional regulators that function as repressors (57) and that have functionally consistent, i.e., opposite, differential transcription; this consistent transcription pattern suggests that the transporters are regulated by the repressors. For example, *lmo2087*, which encodes a MATE pump, shows very strong decreases in transcript levels (25- and 8-fold for PLSDA versus CTRL in H7858 and F6854, respectively), and transcript levels of a putative TetR family repressor, *lmo2088*, are 2- to 3-fold higher in both strains in PLSDA versus CTRL. MATE pumps are Na^+/H^+ -coupled drug antiporters, which are frequently involved in multidrug resistance, osmotic stress, and pathogenicity (36), making it possible that these genes may play a role in either maintenance of pH homeostasis or anion extrusion.

Another functionally relevant group of differentially transcribed genes encode ABC transporters (see Workbook S1 in the supplemental material); differential transcript levels observed include the following: (i) decreased transcript levels (PLSDA versus CTRL) for 5 genes encoding iron complex transporters in one of the two strains, including 1 potential

FIG. 2. Quality threshold clusters (QTCs) of the genes showing differential transcription by organic acid treatment (ANOVA model A). Each subplot indicates a cluster of significant genes for either H7858 or F6854. Clusters are grouped by similar differential transcription pattern, i.e., A to F for clusters with increased transcription and G to L for clusters with decreased transcription, with group details shown in Table 1. Gray lines are individual gene ANOVA regression parameters, the black line is the average value for the cluster, and the dotted lines represent the 25th and 75th quantiles.

TABLE 1. Groups of genes with similar transcription patterns across organic acid treatments as determined by ANOVA model A

Transcription pattern ^b	Strain(s)	No. of genes with ^a :		Selected differentially transcribed genes ^c	
		Increased transcription	Decreased transcription	Increased transcription	Decreased transcription
A, G	H7858	271	377	Ribosomal protein genes (<i>rpmJ</i> , <i>rpsHS</i> , <i>rplPC</i>), toxic ion resistance genes (lmo1967 and lmo1977), mannose ABC transporter genes (lmo1997, lmo2001 [<i>manSL</i>]), regulator gene lmo2003, zinc-containing alcohol dehydrogenase genes (lmo0773, lmo2573)	Biosynthesis of cofactor genes (lmo1556 [<i>hemC</i>], lmo0224, lmo0225 [<i>folPB</i>]), fatty acid metabolism genes (lmo1806, lmo1807 [<i>acpP</i> , <i>fabG</i>]), iron ABC transport genes (lmo1957, lmo1959), oxidative stress genes (lmo0983 [<i>gpxA</i>], lmo0669 [<i>ydaD</i>]), glycolysis genes (<i>pgk</i> , <i>gap</i> , <i>gapR</i>), pyruvate dehydrogenase gene (lmo1053 [<i>pdhB</i>]), two-component response regulator genes (lmo1021, lmo1377, lmo2500), ABC-2 transport protein gene lmo0742
	F6854	0	0		
B, H	H7858	32	45	Pentose phosphate epimerase gene (lmo0499 [<i>rpe</i>]), cytochrome <i>d</i> oxidase subunit gene (lmo2718 [<i>cydA</i>])	lmo0001 (<i>dnaA</i>), lmo0243 (<i>sigH</i>), <i>bkd</i> operon member lmo1372 (<i>lpdA</i>), potassium sensor gene lmo2679 (<i>kdpD</i>), putative acetyltransferase genes (lmo0353, lmo0652)
	F6854	201	295	lmo0433 (<i>inlA</i>), two-component system genes (lmo2500 and -1 [<i>phoRP</i>]), sugar ABC transporter genes (fructose transporter genes lmo0357, lmo0426, and lmo2137 [<i>fruA</i>]), prophage genes (lmo2283, lmo2284, lmo2286, and lmo2293), zinc-containing alcohol dehydrogenase genes (lmo0773, lmo2573)	Motility genes (<i>cheA</i> , <i>flgDE</i> , <i>fliM</i>), lmo0894, lmo0896 (<i>rsbWX</i>), cation transporter genes (lmo2087, lmo2575, lmo0990), menaquinone biosynthesis genes (<i>menBDF</i>), peroxidase gene (lmo0983 [<i>gpxA</i>]), acetate kinase genes (lmo1168 and lmo1581), ABC-2 transport protein gene lmo0742
	H7858 and F6854	7	7	Regulatory protein gene (lmo0168 [<i>abrB</i>]), DNA damage-inducible protein gene (lmo1975 [<i>dinP</i>]), oxidoreductase gene (lmo2163 [<i>gfo</i>]), ATP-binding ABC component gene (lmo2192 [<i>oppF</i>]), glucose-6-phosphate isomerase gene (lmo2367 [<i>pgi</i>])	2 prophage genes (lmo0113, lmo0124), glucose kinase gene (lmo1339 [<i>gki</i>])
C, I	H7858	31	40	Acetolactate synthase gene (lmo2006 [<i>alsS</i>]), MATE pump gene lmo2725, TetR-like repressor gene lmo2088	lmo1985 (<i>ilvN</i>), lmo2483 (<i>hprK</i>)
	F6854	14	8	Fructose phosphotransferase system component gene (lmo2135 [<i>fruA</i>]), transcription antiterminator gene (lmo2138), lmo1055 (<i>pdhD</i>)	lmo1531 (<i>queA</i>)
	H7858 and F6854	0	2		Glutamine synthetase repressor gene lmo1298, putative ABC permease gene lmo1746
D, J	H7858	0	0		
	F6854	12	80	Transcriptional regulator gene (lmo2493 [<i>czzA</i>])	Amino acid genes (lmo1985 [<i>ilvN</i>], lmo1587 [<i>argF</i>], lmo1591 [<i>argC</i>]), fatty acid gene (<i>bkd</i> operon member lmo1373), nucleotide metabolism gene (lmo0192 [<i>purR</i>]), energy metabolism gene (lmo1171 [<i>adhE</i>])
E, K	H7858	24	13	Hemolysin genes (lmo0202 [<i>hly</i>], lmo2738), pyruvate dehydrogenase repressor gene (lmo0948), transcriptional regulator genes (H7858_1021.1 and H7858_0866)	Potassium-transporting ATPase subunit gene (lmo2680 [<i>kdpE</i>]), MerR family transcriptional regulator gene (lmo2728)
	F6854	0	0		
F, L	H7858	36	114	lmo0714 (<i>fliG</i>) in the CodY gene set, lmo1246 (<i>deaD</i>), lmo1566 (<i>citC</i>), acetyl-CoA carboxylase gene (lmo1573 [<i>accD</i>]), lmo2582 (<i>vicK</i>)	Regulatory protein genes (lmo0893 [<i>rsbV</i>], lmo0895 [<i>sigB</i>], lmo1280 [<i>codY</i>]), lactate dehydrogenase gene (lmo0210 [<i>ldh</i>])
	F6854	0	0		

^a Genes were considered significantly differentially expressed if they had a fold change of >1.5 and adjusted (for multiple comparisons) *P* value of <0.05. Genes in a cluster were described as having increased transcription if the average transcription expression of the cluster increased from control (CTRL) treatment to the combination treatment (PLSDA).

^b Transcription patterns in the same row of Fig. 2 are presented together. This combines patterns that show the same relative trends in either increased or decreased differential transcription across treatments.

^c Genes that appeared relevant due to membership in an overrepresented group (e.g., functional groups, regulated operons, etc.) or in a group functionally related to pathways elaborated in the text were included.

TABLE 2. Genes significantly differentially regulated in response to diacetate and lactate effects and the interaction of the organic acids as determined by ANOVA model B

Organic acid effect	Strain(s)	No. of genes with ^a :		Selected differentially transcribed genes or gene sets ^b		
		Increased transcription	Decreased transcription	Gene sets	Increased transcription	Decreased transcription
Acetate	H7858	79	116	Positive enrichment for transcription and 16 transcriptional regulators (lmo2243 [<i>adaA</i>], lmo2128 [<i>malR</i>]), most also with significant lactate effects; glycolysis-related genes increased (<i>fbp</i> , <i>pdhA</i> , mannose-6-phosphate isomerase H7858_2275) and decreased (<i>pfk</i> , <i>gap</i> , <i>pgk</i>)		
	F6854	0	0			
	H7858 and F6854	0	0			
Lactate	H7858	100	114	Positive enrichment for transcription (lmo1496 [<i>greA</i>]) and protein fate (low-temp requirement lmo0215, peptidase lmo1393)		
	F6854	105	108	Positive enrichment for the PrfA regulon (lmo0205 [<i>plcB</i>], lmo0422 [<i>inlA</i>])		
	H7858 and F6854	15	12		lmo1770 (<i>purQ</i>), lmo2085 (<i>sdrD</i>), phosphotransferase system IIA lactose/cellobiose component gene lmo2685, ribosomal protein genes (<i>rpmAF</i>), lmo2524 (<i>fabZ</i>)	lmo0967 (<i>relA</i>), lmo2243 (<i>adaA</i>), lmo2662 (<i>rpiB</i>), lmo1806 (<i>acpP</i>), lmo2727 (<i>hlp</i>)
Interaction	H7858	157	317	Positive enrichment for the CodY regulon (lmo0565 [<i>hisH</i>], lmo0689 [<i>cheV</i>], lmo0714 [<i>flhG</i>])		
	F6854	58	151	Positive enrichment for the PrfA regulon (<i>inlA</i>) and purine biosynthesis ; negative enrichment for the Hpr gene set (lmo2192 [<i>oppF</i>], lmo0593)		
	H7858 and F6854	8	32		lmo0002 (<i>dnaN</i>), lmo0890 (<i>rsbS</i>), lmo1975 (<i>dinP</i>), zinc transporter gene lmo1446 (<i>zurM</i>), oxidoreductase gene lmo2163 (<i>gfo</i>)	Biosynthesis-related genes (lmo1547 [<i>mreC</i>], lmo2526 [<i>murA</i>], lmo1591 [<i>argC</i>], lmo2622 (<i>rplN</i>))

^a Genes were considered significantly differentially expressed if they had a fold change of >1.5 and adjusted (for multiple comparisons) *P* value of <0.05. Genes in a cluster were described as having increased transcription if the presence of the organic acid had a numerically positive effect on gene transcription.

^b Bold phrases describe sets of genes that were significantly enriched (*P* < 0.05, false-discovery rate, <30%) by GSEA. Specific genes or gene groups that appeared relevant due to membership in a overrepresented group (e.g., functional groups, regulon, regulated operons, etc.) or in a group functionally related to pathways affected by organic acid treatments were included.

operon in H7858 (lmo1957 and lmo1959); (ii) increased transcript levels (PLSDA versus CTRL) for 2 genes encoding zinc transport proteins in F6854 (lmo0157 and lmo1446, where lmo1446 shows a significant positive interaction in both strains); and (iii) decreased transcript levels, in H7858, for 8 of the 10 significantly differentially transcribed genes annotated in KEGG as ABC-2 type (efflux) or uncharacterized ABC transporters. The last set of genes includes lmo0742, which encodes an ABC-2 type ATP-binding protein (possibly a multidrug or sodium ion efflux protein), with ~8-fold-decreased transcript levels in both strains (PLSDA versus CTRL).

Organic acid treatments affect energy generation mainly by shifting fermentation toward acetoin production. Many of the changes in transcription due to organic acids suggest alterations in the end products of central metabolism, particularly in H7858, as supported by decreased transcript levels of genes encoding proteins involved in production of organic acids

through fermentation and increased transcript levels of genes encoding proteins involved in acetoin synthesis (Fig. 3). *pdhB*, encoding a pyruvate dehydrogenase complex subunit involved in the formation of acetyl coenzyme A (acetyl-CoA) and ethanol from pyruvate, had 3-fold-lower transcript levels in H7858 (PLSDA versus CTRL). While *pta*, encoding an enzyme for the formation of acetyl-phosphate from acetyl-CoA, is not differentially transcribed, two genes encoding acetate kinases (i.e., *ackA1* [lmo1168] and *ackA2* [lmo1581]), which convert acetyl-phosphate to acetate, had 1.5- to 2-fold-lower transcript levels (Table 1, transcription pattern H) in PLSDA than in CTRL in F6854. qRT-PCR of *ackA1* confirmed decreased transcript levels in PLSDA versus CTRL in F6854 (*P* = 0.003, 16-fold decrease) and suggested that transcript levels were also decreased in H7858 (*P* = 0.08, 14-fold decrease). For genes encoding enzymes involved in the synthesis of lactate, microarray data show that transcript levels of *ldh*, encoding lactate

TABLE 3. Genes encoding cation- and iron-carrying compounds differentially transcribed in either H7858 or F6854^a

ANOVA model A treatment effect (fold) for:											ANOVA model B interaction effect (fold) ^b for:	
Locus	Protein description	Symbol	H7858				F6854				H7858	F6854
			Transcription pattern	SDA vs	PL vs	PLSDA vs	Transcription pattern	SDA vs	PL vs	PLSDA vs		
				CTRL	CTRL	CTRL		CTRL	CTRL	CTRL		
LMOF6854_0506	Magnesium and cobalt transport protein CorA, putative		No probe ^c				H	-1.1	-1.3	-1.6	—	—
lmo0541	Iron compound ABC transporter, substrate-binding protein		— ^d	—	—	—	H	1.0	-1.2	-1.9	—	—
lmo0641	Cation-transporting ATPase, E1-E2 family		G	-1.5	-1.4	-3.1	—	—	—	—	-1.5	—
lmo0818	Cation-transporting ATPase, E1-E2 family		No probe				J	1.0	1.0	-1.6	—	-1.6
LMOH7858_0876	Cation transport ATPase, E1-E2 family		L	1.2	1.0	-2.5	No probe				-2.7	—
lmo0990	MATE efflux family protein	<i>dinF</i>	—	—	—	—	H	-1.2	-1.2	-1.9	—	—
lmo1073	Hemin ABC transporter, hemin-binding protein, putative		No probe				H	-1.1	-1.1	-1.7	—	—
lmo1100	Cation transport ATPase, E1-E2 family		L	1.1	1.1	-2.2	No probe				-2.6	—
lmo1846	MATE efflux family protein		G	1.0	-1.2	-2.0	—	—	—	—	—	—
lmo1848	Iron (chelated) ABC transporter, permease protein	<i>pstC</i>	A	1.1	1.1	1.7	B	1.1	1.4	2.2	—	—
lmo1852	Copper ion-binding protein	<i>copP</i>	—	—	—	—	J	1.0	1.0	-2.1	—	—
lmo2087	MATE efflux family protein, putative		G	-1.8	-1.3	-25.3	H	-1.3	-1.6	-8.2	-10.9	-4.1
lmo2088	Transcriptional regulator, TetR family		C	1.9	1.3	2.2	B	1.2	1.2	2.9	—	—
lmo2105	Ferrous iron transport protein B	<i>feoB</i>	L	1.8	2.2	1.3	—	—	—	—	-2.9	—
lmo2378	Na ⁺ /H ⁺ antiporter, MnhA component	<i>mnhA</i>	L	1.6	2.0	1.2	—	—	—	—	-2.8	—
lmo2379	Na ⁺ /H ⁺ antiporter, MnhB component	<i>mnhB</i>	A	1.1	1.2	2.4	—	—	—	—	1.9	—
lmo2382	Na ⁺ /H ⁺ antiporter, MnhE component	<i>mnhE</i>	A	1.0	1.1	1.5	—	—	—	—	—	—
lmo2575	Cation efflux family protein		L	1.1	1.0	-1.5	H	-1.3	-1.7	-13.3	-1.7	—
lmo2600	Cobalt ABC transporter, ATP-binding protein	<i>czcD</i>	G	-1.1	-1.1	-1.8	B	1.2	1.2	2.3	—	—
lmo2601	Cobalt ABC transporter, ATP-binding protein		L	1.2	1.0	-2.1	—	—	—	—	-2.5	—
lmo2680	Potassium-transporting ATPase, C subunit	<i>kdpC</i>	K	1.1	-1.7	-1.1	Unassigned	1.3	-1.7	1.0	—	—
lmo2725	MATE efflux family protein		C	1.1	1.0	2.0	—	—	—	—	2.0	—
lmo2726	Transcriptional regulator, MarR family, putative		G	-1.5	-1.2	-3.7	H	-1.1	-1.2	-2.3	-2.0	-2.0

^a Genes assigned the JCVI subrole cation- and iron-carrying compounds and the two differentially transcribed putative regulators in the same operon as genes with this subrole.^b This interaction parameter estimate represents the effect of lactate in the presence of diacetate versus the effect of lactate without diacetate present.^c There was no valid oligonucleotide probe on the array for the locus in the indicated strain.^d —, the gene was not significantly differentially transcribed in the indicated ANOVA model.

FIG. 3. The transcription profile in response to the combination treatment with lactate and acetate shows a shift toward fermentative production of acetoin and away from aerobic respiration and the production of lactate and acetate. Boxes contain compounds or complexes; italic text indicates genes, with strain and direction of differential transcription labeled (for the lactate acetate treatment compared to control); roman text outside boxes (e.g., ADP) indicates reaction by-products; and arrows indicate reaction direction. Heavy lines correspond to reactions with increased transcription; dark gray lines and the heavy box correspond to increased acetoin production shown in Fig. 4.

dehydrogenase, were decreased (Table 1, transcription pattern L) in H7858, although qRT-PCR of *ldh* did not show significant transcript level differences in PLSDA compared to CTRL for either strain. In contrast to the decreased transcript levels of genes encoding proteins in pathways that produce organic acids, genes encoding proteins in a pathway that produces acetoin from pyruvate (74) had increased transcript levels H7858. Specifically, transcript levels of *alsS*, encoding alpha-acetolactate synthase, were significantly increased (Table 1, transcription pattern C), and transcript levels of *lmo1992*, encoding alpha-acetolactate decarboxylase, were significantly ($P < 0.01$) increased by 1.4-fold. Finally, 5 of 6 genes encoding proteins involved in menaquinone activity (*menABDFH*) had decreased transcript levels in either H7858 or F6854. As a deficiency in menaquinone synthesis causes *L. monocytogenes* to perform anaerobic metabolism even in the presence of oxygen (66), due to the loss of oxidative phosphorylation, decreased transcription of *men* genes further supports a switch to anaerobic fermentation of sugars.

To confirm that treatment with organic acids induces acetoin production, acetoin produced in response to treatment with lactate, acetate, and/or acetoin was measured for cells grown under both microaerophilic and aerobic conditions at 37°C. Under both conditions and across both strains, lactate and acetate significantly ($P < 0.01$) increased acetoin production, by 0.8 and 0.7 mM acetoin per OD₆₀₀ unit under microaerophilic conditions and 1.8 and 1.2 mM acetoin per OD₆₀₀ unit aerobically, for lactate and acetate, respectively (results for F6854 are shown in Fig. 4, and results for H7858 are shown in Fig. S2 in the supplemental material). While the incubation temperature for these experiments was higher than that used for the microarray experiments, the results are as expected based on the gene expression data. Treatment with 0.74 mM acetoin also significantly ($P < 0.01$) reduced further acetoin production under microaerophilic conditions, by 1.3 mM acetoin per OD₆₀₀ unit. This self-repression of acetoin production suggested that acetoin may have use as a growth inhibitor

FIG. 4. Organic acid treatment increases acetoin production in *L. monocytogenes* F6854. Acetoin production was measured after 5.5 h of microaerophilic (A) or aerobic (B) incubation in medium with 0.71 mM associated potassium lactate (x axis) and/or 0.77 mM associated sodium diacetate (◆, with diacetate; ◇, without diacetate) and/or 0.74 mM acetoin (dashed lines, with acetoin; solid lines, without acetoin) and normalized to the measured optical density at 600 nm. Points are averages from three biological replicates, and error bars indicate ± 1 standard deviation. Results for H7858 were similar and are shown in Fig. S2 in the supplemental material.

under microaerophilic conditions, but comparison between the final OD₆₀₀ measurements for all 8 treatments showed that the cultures containing acetoin grew to levels similar to those of the corresponding cultures of treatments without acetoin (results not shown), indicating that acetoin does not have growth-inhibiting properties in this system. Significant ($P = 0.03$) differences in acetoin production between bacterial strains were detected only in aerobic cultures, with H7858 estimated to produce 0.4 mM more acetoin per OD₆₀₀ unit than F6854 under the same treatment conditions.

Organic acid stress alters virulence and metabolic regulation pathways in *L. monocytogenes*. Organic acid treatment induced transcription of the PrfA virulence regulon in F6854. Gene set enrichment analysis (GSEA) identified the PrfA regulon as positively enriched across every comparison except PL versus SDA. For example, for the PLSDA versus CTRL comparison, *actA* showed 5.7-fold upregulation (adjusted $P = 0.18$), *plcB* showed significant 2.7-fold upregulation (adjusted $P = 0.01$), and *inlA* showed 4.2-fold upregulation (adjusted $P < 0.01$).

Additional support for increased PrfA activity is provided by the microarray-based evidence for a reduction in Hpr serine phosphorylation, which has been linked to increased PrfA activity in *L. monocytogenes* (19). Hpr is phosphorylated on a serine residue (Hpr_{Ser-P}) by the kinase function of HprK when fructose bisphosphate and ATP concentrations are high, inducing Hpr_{Ser-P}-activated carbon catabolite repression activity of CcpA (19), a regulatory protein which may induce genes in pathways for acetoin production and repress those for lactate and acetate production (42). Our array data suggest that interaction of lactate and diacetate reduces the regulatory activity of HprK in F6854. In F6854, in the presence of lactate and acetate, GSEA identified a significant decrease in transcript levels for a set of genes whose transcription was previously shown to decrease in a $\Delta hprK$ strain, suggesting that the combination treatment partially mimics the effect of *hprK* deletion. HprK-dependent genes that were significantly differentially transcribed between PLSDA and CTRL include

the following: lmo0593, encoding a formate/nitrite transporter; lmo1985 (*ilvN*), encoding acetolactate synthase; and lmo2192 and lmo2195 (*oppFB*), encoding an oligopeptide ABC transporter ATP-binding protein and a permease *oppFB* (Table 1). A decrease in metabolite pools of ATP is also likely consistent with reduced HprK kinase activity (19). The fact that organic acid treatment shifts cells away from ATP-producing acetate production (presented above) and causes lower growth rates (65) suggests a reduction in the ATP pool, which would be expected to reduce Hpr_{Ser-P} production. Two lines of transcript evidence suggest this reduced phosphorylation of Hpr during organic acid exposure. *hprK* transcript levels were significantly lower in H7858 exposed to diacetate (transcript pattern I), and qRT-PCR confirmed decreased transcript levels in H7858 and F6854 ($P = 0.01$ and 0.01 [5- and 15-fold decreases], respectively).

In H7858, some genes known to be regulated by the nutrient state-sensing repressor CodY had significantly higher transcript levels in the presence of lactate and diacetate than in the presence of lactate alone. These genes include the following: lmo0561 and lmo0565 (*hisEH*), encoding histidine biosynthesis enzymes; lmo2647, encoding a putative creatininase; lmo2824 (*serA*), encoding a serine biosynthesis enzyme; lmo0689 (*cheV*), encoding a chemotaxis protein; lmo2469, encoding an amino acid permease; and lmo0714 (*fliG*), encoding a flagellar motor switch protein (Table 2, interaction effect). GSEA provided additional support for activation of the CodY regulon in the presence of the lactate and diacetate combination, as 23 of the 83 genes known to be regulated by CodY were determined to be positively enriched for this strain across the interaction comparison (model B). Transcript levels of *codY*, measured by microarray analysis, were decreased 2-fold by lactate with diacetate present compared to lactate without diacetate (the interaction in model B) in H7858. While qRT-PCR of *codY* showed no significant repression in H7858 ($P = 0.05$, 8-fold repression), the gene was significantly repressed ($P = 0.01$, 23-fold repression) in F6854.

DISCUSSION

Our data reported here indicate that the organic acids lactate and diacetate, which are often used to inhibit *L. monocytogenes* growth in ready-to-eat foods, induce a variety of metabolic responses in lag-phase *L. monocytogenes*, including a nutrient-limiting stress response, changes to membrane transport, and possible induction of virulence gene transcription. One potential mechanism behind the growth inhibition facilitated by these organic acids is that these acids strongly inhibit fermentative production of organic acids, causing the cell to produce energetically less favorable acetoin.

Metabolic and virulence responses are induced by organic acids during adaptation to a complex, stressful environment. While the CodY regulon, which coordinates a nutrient-limiting response, is induced during cold growth (4°C versus 37°C) (12, 13), we observed that H7858 shows further induction of CodY-regulated genes, as well as decreased transcript levels of *codY*, in response to organic acid treatment. Similarly, the *Bacillus subtilis* response to potassium sorbate treatment was shown to be similar to the response to nutrient-limited conditions, as indicated by relief of CodY and AbrB repression (5). One

possible explanation is that organic acids create an energy-limiting state that lowers the concentrations of GTP and hence the concentration of branched-chain amino acids that bind to CodY, decreasing CodY repression (64).

Complementary to the CodY regulon induction in H7858, the PrfA regulon is induced in F6854 in response to organic acid treatment. Further, a gene set previously reported to be downregulated in $\Delta hprK$ mutant (42) showed lower transcript levels in F6854 exposed to organic acids, with *hprK* transcription decreased in both organic acid-exposed H7858 and F6854. In *L. monocytogenes* the PrfA regulon is induced in an HprK knockout strain (42), and relief of CodY repression is important for virulence (6). Our data thus suggest that organic acids may induce a coordinated metabolic and virulence gene transcription response, although the specific targets and mechanisms will require further study. Previous work has shown that pregrowth to stationary phase in the presence of levels of NaCl, diacetate, and/or lactate similar to those used in this study could alter the invasiveness of *L. monocytogenes* in a pregrowth pH-dependent manner (25), while exposure to lactate and acetate in liver pate did not significantly induce *prfA* or *inlA* transcription (48). Acetate is also a pH-dependent signal for virulence gene expression in *Salmonella* in the context that short-chain fatty acid profiles mimicking host ileal, but not colonic, conditions induce expression of genes involved in invasion (38). Thus, exposure to lactate and diacetate may contribute to virulence gene-inducing conditions typically encountered by *L. monocytogenes* in the host ileum.

Many of the stress responses known to be induced by the control conditions (cold and salt stress) were not further affected by organic acid treatment. The alternative sigma factor SigB is a global stress response regulator in *Listeria* that is important for survival under multiple stresses, including strong acid, osmotic, cold, and oxidative stress, (12, 15). Gene set enrichment analysis of our microarray data did not detect significant changes to the SigB regulon, suggesting that organic acid stress does not induce the SigB regulon during lag-phase adaptation beyond the already well-described general stress response. This is in contrast to work by Bowman et al. (7) with exponentially growing cells, which showed different induction of SigB-regulated genes by organic (acetic) acid stress at pH 5.0 than by strong (HCl) acid stress at the same pH. The different growth phases likely contribute to the different responses, as entry into stationary phase induces *sigB* transcription (4). Osmotic stress response systems, which likely were induced by the 0.8 M NaCl in the treatment media used here (as 0.5 M KCl has previously been shown to induce an osmotic stress response [33]), were not further induced by organic acid stress.

There also was little indication in our data that an oxidative stress response was induced by organic acid treatment. Oxidative stress responses have been shown to be induced by both strong and weak acid stress in *L. monocytogenes* (7) and *Bacillus cereus* (44) and strong acid stress in *B. subtilis* (73), though all these studies were conducted using aerobic growth conditions. Under aerobic growth conditions, the organic acid sorbate caused oxidative DNA damage in yeast cells, resulting in increased mutagenesis, but this effect was not observed under anaerobic growth conditions (54). Our experiment was conducted under microaerophilic conditions, and transcription

of menaquinone biosynthesis genes essential for aerobic respiration (63) was downregulated in response to organic acid treatment, suggesting that the cells were shifting toward anaerobic growth, which may reduce the potential for oxidative damage. In our microarray data, known oxidative stress response genes were not differentially regulated (such as for lmo1443) or were downregulated (e.g., for a gene encoding a glutathione peroxidase [lmo0983] and for SigB-regulated lmo0669 [15], encoding an oxidoreductase), suggesting that exposure to organic acid does not induce the oxidative stress response. In *Escherichia coli* the global oxidative stress response regulator *soxS* was transcribed for only 10 min after exposure to chlorine (71), so if the oxidative stress response in *L. monocytogenes* was similarly short term, transient induction would not have been detected at the 8-h incubation time point used in this study.

Combined organic acid treatment does not elicit an inorganic acid stress response. *L. monocytogenes* uses at least three systems to adapt to inorganic acid stress, the glutamate decarboxylase (GAD) system, the arginine deaminase (ADI) system, and the F_1F_0 ATPase (18, 59). In the GAD system, glutamate is decarboxylated to γ -aminobutyrate by glutamate decarboxylase (*gadD1*, *gadD2*, or *gadD3*), consuming an intracellular proton, and then γ -aminobutyrate is exchanged for extracellular glutamate through an antiporter (encoded by *gadT1* or *gadT2*). None of the *gad* genes were differentially transcribed in our data set, which is consistent with the observation that a *gadD1 gadD2* knockout mutant was able to grow in the presence of acetic (30 mM), benzoic (3 mM), and sorbic (4 mM) acids as well as the wild-type strain (29). In addition, we found none of the genes in the ADI system to be differentially transcribed in response to organic acid stress, and we found no clear trends in the transcription of genes encoding F_1F_0 ATPase components. It has been shown that *L. monocytogenes* does not induce these three systems in response to acetic acid stress (7), and our data show that these systems additionally are not utilized in response to either lactate or the combination of lactate and acetate stress, suggesting that the adaptive response to additional organic acid stress is fundamentally different from that to strong acid stress.

Membrane modifications are likely important to the organic acid stress response. Our data show that two classes of differentially regulated transporters, multidrug resistance efflux pumps (especially the MATE transporters with associated TetR family repressors) and ABC transporters, likely have critical, but uncharacterized, roles in *L. monocytogenes* resistance to organic acid stress. Transporters are important in the yeast organic acid stress response, where *Saccharomyces cerevisiae* strongly downregulated membrane transporters in response to acetic acid stress (1). In *Acetobacter aceti*, resistance to acetic acid was reduced upon deletion of a putative ABC transporter (46). Further, yeast has at least one known multidrug resistance pump, PdrR, responsible for transporting carboxylic anions from the cell (28, 53, 61), which was later shown to confer resistance to sorbate and benzoate but not acetate, (28), suggesting that transporters of weak acid anions have structural specificity. Paradoxically, deletion of transporters upregulated by sorbic acid stress in *B. subtilis* (5) and sublethal HCl in *B. cereus* (45) caused increased resistance to those same stresses. These studies with yeasts and bacteria suggest that

transporters may be involved in energetic export of lactate and diacetate from the cell or may convey resistance to the organic acids by some undescribed mechanism such as reducing the diffusion of associated acids into the cell, as has been shown in yeasts (9, 52).

The synergistic growth-inhibitory effect of diacetate and lactate may be due to induction of an energetically less favorable fermentation. When *L. monocytogenes* EGD-e is grown on rich medium under aerobic or anaerobic conditions, acetoin and acetate production ceases and lactate is produced. Further, the aerobic production of acetoin and acetate can be eliminated through a defect in menaquinone synthesis (66). These previous findings imply that lactate production (which uses pyruvate as a terminal electron acceptor) can be a substitute for menaquinone-dependent respiration. Our array data show that organic acid treatment reduces transcription of both NAD^+ -regenerating pyruvate oxidation pathways (i.e., menaquinone biosynthesis and lactate production) and ATP-generating acetate production and increases transcription of the pathway that produces neutral acetoin (Fig. 3). Increased production of acetoin from pyruvate prevents the additional acidification of the cytoplasm from lactate or acetate production when treatment with those organic acids creates an already acidified environment, but it does so at the expense of fermentation pathways with energetically favorable by-products such as NAD^+ and ATP. Coupled with the reduction in the ability to respire through menaquinone, these data suggest that organic acids may inhibit the growth of *L. monocytogenes* partially by interfering with the fermentation balance. It is tempting to speculate that the observed synergy between lactate and diacetate may be due to anion-specific end product inhibition of lactate and acetate synthesis pathways.

Our findings are consistent with previous studies on the effects of organic acids on other bacteria. For example, it has been shown that the transcriptional response of *Lactobacillus plantarum* to a combination of lactic acid and lower growth rate includes increased transcription of alternative fermentation pathways that produce malate, acetate, and ethanol end products instead of lactate (50). *B. cereus* strains decreased transcription of *ldhA*, a gene involved in lactate production, only when treated with 2 mM undissociated lactate and not with 2 mM acetate. Further, treatment with nonbactericidal concentrations of both organic acids, as well as HCl, induced acetoin biosynthesis (44). Following strong acid stress, *B. subtilis* induced acetoin production and dehydrogenases (e.g., *adhA*) and decarboxylases (e.g., *ilvH*), which may contribute to pH homeostasis and acid consumption, respectively, while *ldh* transcription was induced by basic conditions (73). It is important to note that posttranslational effects of end product inhibition on enzyme activity, e.g., lower activity of Ldh in the presence of high concentrations of lactate (31), would not be measured by transcriptomic data.

Conclusions. It is important to view the conclusions from this mechanistic study of organic acid growth inhibitors in the broad context of food safety. One approach to reducing foodborne illness from ready-to-eat foods is to use additives, such as organic acids, as a backup safety measure to slow or stop growth of pathogenic bacteria in the rare cases where those foods are contaminated with pathogens. However, in light of growing evidence suggesting interactions between the bacterial

stress response and virulence (72), it becomes critical to understand the mechanisms of action of new growth-inhibiting strategies to minimize unintended consequences of their use. Mechanistic studies, such as this one, can both help to avoid ineffective or dangerous treatments and provide a basis for a positive contribution to safety through rational development of new growth inhibitors that exploit adaptive mechanisms for further growth inhibition synergy and hence improved food safety.

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