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A novel RofA-family transcriptional regulator, GadR, controls the development of acid resistance in *Listeria monocytogenes*

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ABSTRACT Stomach acid provides a significant innate barrier to the entry of the food-borne pathogen *Listeria monocytogenes* into the human gastrointestinal tract. A key determinant of acid resistance in this bacterium is the conserved glutamate decarboxylase system, GadD2 (encoded by the *gadT2D2* operon), which helps to maintain the intracellular pH during exposure to gastric acid. In this study, we identified a premature stop codon in a gene located immediately downstream of the *gadT2D2* operon that was highly linked to an acid-sensitive phenotype. When this open reading frame was restored through homologous recombination, an acid-resistant phenotype was restored. Through a series of genetic, transcriptomic, and survival experiments, we established that this gene, which we designated *gadR*, encodes a transcriptional regulator of the *gadT2D2* operon. GadR belongs to the RofA family of regulators, primarily found in streptococci, where they are involved in regulating virulence. The data further showed that *gadR* plays a critical role in the development of acid resistance in response to mild acid exposure, a response that is known as the adaptive acid tolerance response (ATR). A deletion analysis of the *gadT2D2* promoter region identified two 18-bp palindromic sequences that are required for the GadR-mediated induction of *gadT2D2*, suggesting that they act as binding sites for GadR. Overall, this study uncovers a new RofA-like regulator of acid resistance in *L. monocytogenes*, which plays a significant role in both growth phase-dependent acid resistance and ATR and accounts for previously observed strain-to-strain differences in survival at low pH.

IMPORTANCE The ability to survive the acidic conditions found in the stomach is crucial for the food-borne pathogen *Listeria monocytogenes* to gain access to the mammalian gastrointestinal tract. Little is currently known about how acid resistance is regulated in this pathogen and why this trait is highly variable between strains. Here, we used comparative genomics to identify a novel RofA-family transcriptional regulator, GadR, that controls the development of acid resistance. The RofA family of regulators was previously found only in a small group of bacterial pathogens, including streptococci, where they regulate virulence properties. We show that *gadR* encodes the dominant regulator of acid resistance in *L. monocytogenes* and that its sequence variability accounts for previously observed differences between strains in this trait. Together, these findings significantly advance our understanding of how this important pathogen copes with acid stress and suggest a potential molecular target to aid its control in the food chain.

The bacterium *Listeria monocytogenes* is a well-studied member of the *Bacillota* phylum (formerly the *Firmicutes*) due to its impact on food safety and its behavior in the host as a facultative intracellular pathogen. Ingestion of food contaminated with this bacterium is associated with a risk of infection, termed listeriosis, especially in immunocompromised individuals, where the mortality rate can be as high as 30% (1–3). The organism is particularly problematic for producers of ready-to-eat foods as it has the ability to grow at refrigeration temperatures and in some foods preserved at low pH and/or low water activity (4–6). Its robust response to acid stress also increases the risk that the pathogen can survive transit of the acidic conditions in the stomach and thereafter invade the epithelium of ileum (7, 8), which is the first stage of pathogenesis during an infection (3). While much has been learned about the mechanisms that contribute to the acid stress response in this pathogen (4, 9–11), the regulatory mechanisms that control the expression of these systems are much less well understood.

Almost three decades ago, an adaptive response to acid was described in *L. monocytogenes*, whereby a mild acid stimulus (pH 4.0–6.0) triggers the development of high levels of acid resistance to normally lethal acid conditions (pH 3.0) (12, 13). This response, which was named the adaptive acid tolerance response (ATR), requires *de novo* protein synthesis but the mechanisms underpinning its regulation have never been properly elucidated. Interestingly, entry to the stationary phase was also found to promote acid resistance in *L. monocytogenes* independently of pH (12). The discovery that Sigma B (SigB), an alternative sigma factor that controls the general stress response in *L. monocytogenes*, plays an important role in acid resistance (14–17) initially suggested that it might play a role in regulating the stationary phase acid resistance and the ATR. SigB is activated in the stationary phase, it responds to mild acid stress, and it plays a role in transcribing key components involved in protection against acid stress, including the glutamate decarboxylase and arginine deiminase pH homeostasis systems (17–19). Furthermore, mutants lacking SigB have an acid-sensitive phenotype in the stationary phase and a reduced ATR (14, 16, 19). However, *sigB* mutants are still capable of developing acid resistance in the stationary phase and inducing a significant ATR (16, 19), suggesting that other regulatory factors must be involved in regulating the acid stress response of this pathogen.

Two glutamate decarboxylases (GadD2 and GadD3) are present in most strains of *L. monocytogenes*, and a third (GadD1) is present in some lineage II strains (Fig. 1A). The corresponding genes for these decarboxylases are encoded at three distinct genetic loci (20–23). They contribute to acid stress protection by helping to maintain the intracellular pH, because the decarboxylation reaction consumes protons (24–26). Two of these systems are genetically coupled with glutamate/gamma-aminobutyrate (Glu/GABA) antiporters (GadD1T1 and GadT2D2) that allow uptake of glutamate in exchange for the product of the decarboxylation reaction, GABA. GadD3 is not co-expressed with an antiporter and appears to use intracellular glutamate independently of a Glu/GABA exchange mechanism (27, 28). GadD1T1 is reported to contribute to growth under acidic conditions (21), whereas GadT2D2 is the dominant system with respect to acid resistance (23, 29). Little is known about the transcriptional regulation of these two systems, although the transcription of *gadD3* is under SigB control (30, 31). SigB also controls the expression of succinate semialdehyde dehydrogenase (Lmo0913), which is required in the GABA-shunt pathway that generates succinate and Glu from GABA and α-ketoglutarate (15, 32). There are significant strain-to-strain differences in the behaviors of the GAD systems in *L. monocytogenes* (23, 28). In particular, the well-studied lab strains EGD-e and 10403S, both of which belong to lineage II serotype 1/2a, produce and secrete different amounts of GABA in response to acidification. Specifically, 10403S secretes GABA in response to acidification, whereas EGD-e does not, although *gadD1T1*, *gadT2D2*, and *gadD3* are conserved in these two strains (23). Furthermore, the deletion of each of the three GAD systems from these two strains produces different phenotypes with respect to acid resistance; notably, deletion of *gadD2* produces an acid-sensitive phenotype in 10403S but has no effect on EGD-e (23). It is striking that the well-studied
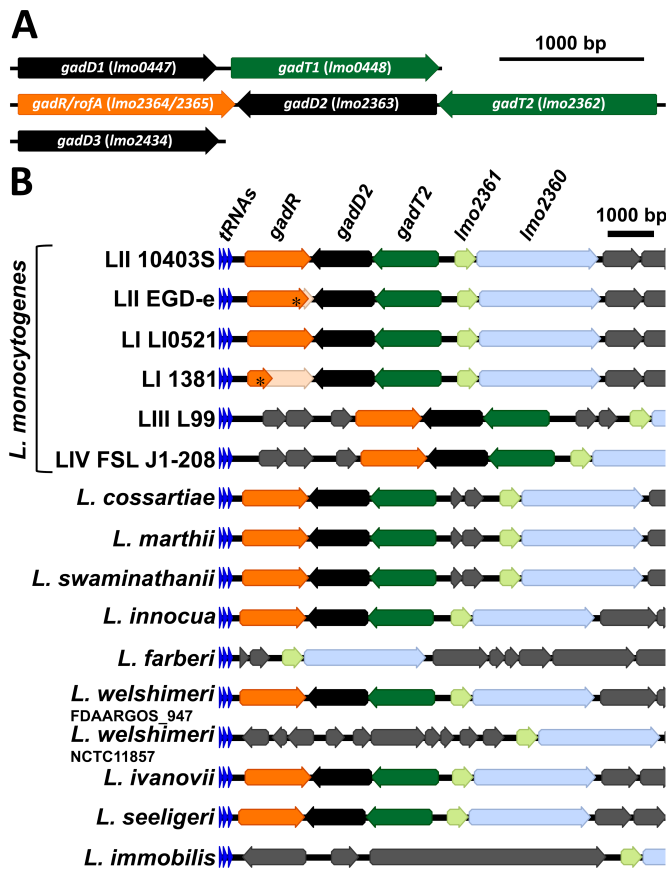
FIG 1 The *gadT2D2-gadR* gene cluster is highly conserved in the *Listeria senso stricto* clade. (A) Operon structures of *gadD1T1*, *gadT2D2-gadR*, and *gadD3* are depicted, using sequences extracted from strain 10403S. (B) Gene arrangements downstream of the conserved *tRNA-Gly* are depicted for all species from *Listeria senso stricto* clade species reported by Carlin et al. (33). *L. monocytogenes* lineages I–IV are abbreviated as LI–LIV. The PMSCs in *gadR* are highlighted with asterisks and the untranslated regions of *gadR* are faded.

lab strain EGD-e is especially sensitive to acid, the genetic basis for which has remained unclear (23, 28, 29).

We have recently described the phylogenetic and phenotypic characterization of a collection of 168 lab, food, environmental, and clinical isolates of *L. monocytogenes* with respect to their acid tolerance (growth at pH 4.9), acid resistance (survival at pH 2.3), and salt tolerance (growth in NaCl 0.8–1.5 M) (34). This collection is both phylogenetically and phenotypically diverse, with wide variabilities in acid tolerance and acid resistance across individual strains. Some phenotypic outliers were identified as harboring lesions in the *sigB* operon, which explains compromised general stress response and acid-sensitive phenotypes (34). One strain identified in that study, a lineage I strain designated 1381, displayed reduced acid tolerance (no growth at pH 4.9) and reduced acid resistance (poor survival at pH 2.3) although there was no evidence of mutations affecting the SigB system (34). We have recently shown that the growth defect in strain 1381 at low pH, but not the poor survival phenotype (at pH 2.3), is caused by a mutation in the *mntH* gene, which encodes a manganese transporter belonging to the NRAMP family (35). However, this mutation does not account for the decreased acid resistance in this strain (35). Thus, the genotype of this strain could help provide new insights into the mechanisms underpinning acid resistance in *L. monocytogenes*.

In the present study, we used comparative genomics to help further elucidate the genetic basis for the reduced acid resistance observed in this strain. Our findings reveal the presence of a hitherto unknown transcriptional regulator, GadR, that plays a crucial

role in the regulation of acid resistance by modulating the expression of the GadT2D2 in this pathogen. The presence of a mutation within the *gadR* gene is shown to solely account for the differences in acid resistance between the two well-studied lab strains, EGD-e and 10403S. Overall, this study identifies a key regulatory component of adaptive acid resistance in *L. monocytogenes* and raises the possibility that acid resistance, and thus virulence, might be controlled through a strategy that specifically targets this regulator.

RESULTS

Identification of a RofA-like regulator that positively influences acid resistance

The acid-sensitive and acid-intolerant CC2 strain 1381 is closely related to the chromosomally sequenced reference strain LI0521 (35), facilitating an investigation into the genetic basis of these unusual phenotypes. There are four genes intact in strain LI0521, which are truncated by the presence of premature stop codon (PMSC) in strain 1381, including *mntH* (35). Among the other three is a putative RofA-like transcriptional regulator, located downstream of and oriented convergently with the acid-resistance operon *gadT2D2* (Fig. 1). Notably, this gene was also truncated in the widely studied lab strain EGD-e (L374*) and thus mis-annotated as two open reading frames (ORFs), *lmo2365* and *lmo2364* in that strain (36). Bioinformatic analysis revealed that this *gadT2D2-rofA* gene cluster is conserved in most species from *Listeria sensu stricto* clade (Fig. 1B) (33) but absent from the *Listeria sensu lato* clade (data not shown). These observations suggested that this RofA-like transcriptional regulator might be the cognate regulator of the *gadT2D2* operon and, therefore, we designated the full ORF as *gadR*. Interestingly, in addition to strains 1381 and EGD-e, CC7 strain 1147 and all five CC18 strains from the previously characterized strain collection (*n* = 168) are predicted to be *gadR*⁻ based on the presence of PMSCs within the ORF (data not shown). All these *gadR*⁻ strains survived poorly at pH 2.3 (34), suggesting a possible role of GadR in acid resistance.

To test whether GadR influences acid resistance and *gadT2* transcription, full-length *gadR* was cloned from CC2 strain 1380 and introduced into strain 1381 using an IPTG-inducible integrative expression vector pIMK3, generating construct pIMK3::*gadR* (37) (Table 1). The acid resistance of strain 1381 pIMK3::*gadR* during the stationary phase was enhanced in an IPTG-induced fashion compared to strain 1381 (Fig. 2A). The transcript levels of *gadT2* were positively correlated with the presence of a functional copy of *gadR* (from pIMK3::*gadR*) (Fig. 2B and C). The pIMK3 vector did not affect acid resistance or *gadT2* transcription (Fig. 2A and B). These results demonstrated that GadR positively influences both acid resistance and *gadT2* transcription. When the pIMK3::*gadR* plasmid was transformed into the *mntH*⁺ strain 1381R1 (35) (Table 1), the positive effects on both acid resistance and *gadT2* transcription were very similar to that observed in the *mntH*⁻ parental strain (Fig. 2A and B), suggesting that the effect of GadR on acid resistance is independent of MntH.

GadR controls acid resistance by activating GadT2D2 expression independently of SigB

To test whether altered *gadT2D2* transcription is exclusively responsible for GadR-mediated acid resistance, three mutant strains were constructed in a genetic background where the *gadR* was intact (lab strain 10403S); they carried deletions either in the *gadR* gene (Δ*gadR*), the *gadT2D2* locus (Δ*gadT2D2*), or both (Δ*gadT2D2R*) (Table 1). The ability of these strains to survive at pH 2.3 was compared following growth to the stationary phase. All three mutants exhibited a very similar decrease in acid resistance compared to the parental strain 10403S (Fig. 3A), suggesting that GadT2D2 expression is solely responsible for the GadR-mediated acid resistance. The GadR-mediated acid resistance in strain EGD-e (*gadR*⁻) was restored by repairing the point mutation in *gadR* (*374L)
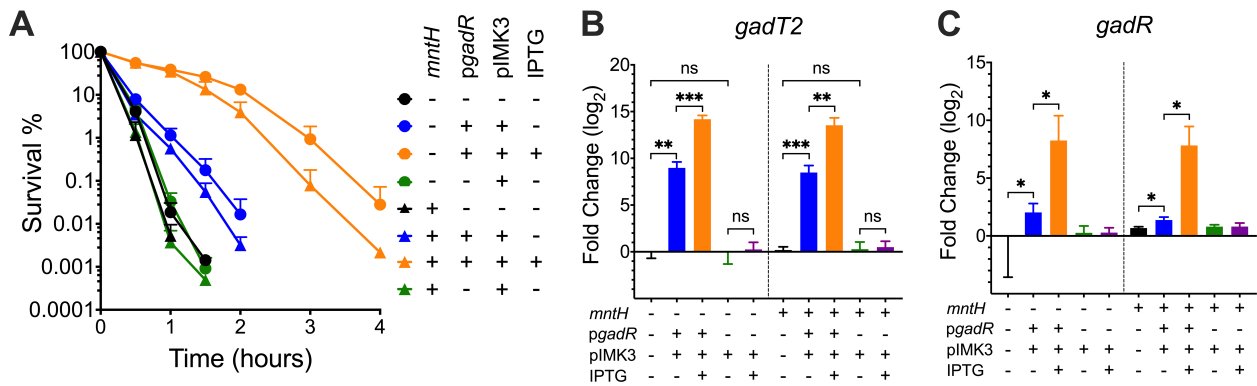
FIG 2 GadR positively influences acid resistance and *gadT2* transcription, independently of the manganese transporter MntH. (A) Stationary phase cultures of strains 1381 (*mntH*) and 1381R1 (*mntH*⁺) either with or without *gadR* complementation (p*gadR*) and 1 mM IPTG induction were challenged at pH 2.3 and survival was recorded over 4 h. Transcription of *gadT2* (B) and *gadR* (C) was measured for these strains after overnight incubation for 18 h. Transcript levels relative to the *gadR*⁻ parent strain 1381 are presented. Three independent experiments were performed with technical triplicates (A) or duplicates (BC). Statistically significant differences were determined by paired *t* test (two-tailed) (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

in the chromosome by homologous recombination (Fig. 3A and B). These observations demonstrated a prominent role for GadR in acid resistance and indicated that the *gadR* frameshift mutation in EGD-e explains the intrinsic acid sensitivity of this reference strain. To examine the relative contributions of GadR and the general stress response regulator SigB to acid resistance, Δ*sigB* was introduced into strains 10430S and EGD-e with or without *gadR* (Table 1), and the acid resistance of strains lacking either or both the *gadR* and *sigB* was compared to the strains that have functional SigB and GadR in both genetic backgrounds (strains EGD-e and 10403S). The strains EGD-e *gadR*⁻ Δ*sigB* and 10403S Δ*gadR* Δ*sigB* were extremely acid sensitive and, therefore, the cultures were grown to the stationary phase and exposed to pH 2.4 (rather than pH 2.3). In both genetic backgrounds, GadR made a more significant contribution than SigB to acid resistance, while the absence of both regulators resulted in extreme acid sensitivity (Fig. 3B). These results suggested that the combined effects of GadR and SigB account for most, if not all, of the acid resistance of *L. monocytogenes* at pH 2.4 in stationary phase. To elucidate possible crosstalk between GadR and SigB, the transcript levels of *gadT2*, *gadR*, and the SigB-dependent *gadD3* during stationary phase were measured for these strains (Fig. 3C through E). The results suggested that SigB has limited, if any, influence on GadR-mediated *gadT2* transcription. *gadR* transcription showed minor variations across these strains suggesting that it is controlled neither by GadR nor by SigB (Fig. 3D). The transcription of *gadD3* was confirmed to be SigB-dependent and was unaffected by *gadR* deletion (Fig. 3E), suggesting that GadR does not influence SigB activity and does not control *gadD3* transcription. To establish if the GadR-mediated *gadT2* transcription is associated with altered Glu/GABA antiporter activity, the ability to export GABA into the medium was examined for these strains during the stationary phase following an acid challenge at pH 3 for 1 h (which is not lethal to strains carrying either *gadR* or *sigB*, Fig. S1). The results showed that significant extracellular GABA was only detectable in the presence of both *gadR* and *gadT2D2* (Fig. 3F). Taken together, these results demonstrated that GadR promotes acid resistance by activating the expression of the GadT2D2 independently of SigB.

Adaptive acid resistance is highly GadR-dependent

Since GadR contributes significantly to acid resistance in the stationary phase, we reasoned that it might also participate in regulating the ATR in *L. monocytogenes*. To test this, the transcription of *gadT2*, *gadR*, *gadD1*, and *gadD3* was measured in exponentially grown cultures of strains EGD-e wildtype (WT) (*gadR*⁻) and the isogenic *gadR*⁺ derivative after 15 min exposure to a range of low pH conditions (pH 3–6.5, compared to pH
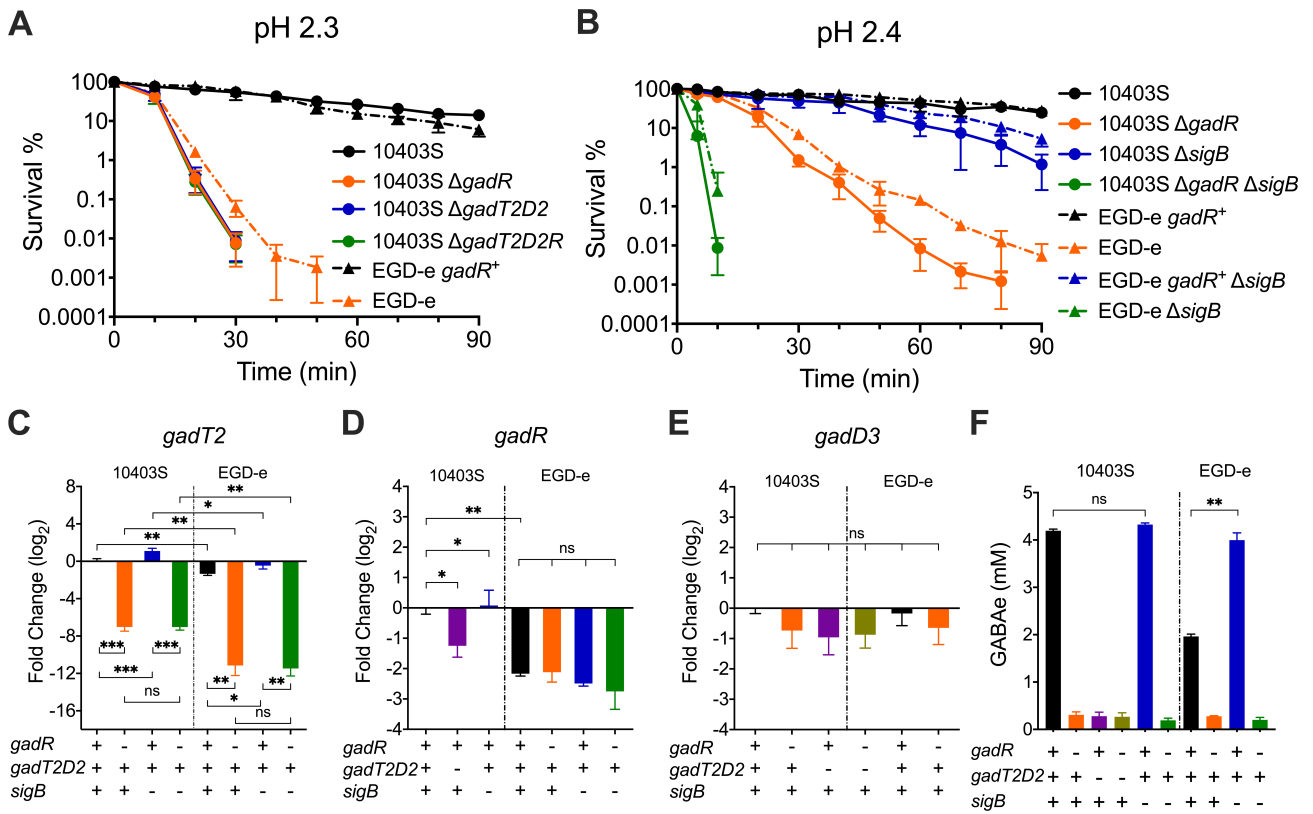
FIG 3 GadR modulates acid resistance by activating GadT2D2 expression independently of SigB. Cultures of *L. monocytogenes* WT or mutant strains were grown to the stationary phase and challenged at pH 2.3 (A) or pH 2.4 (B). EGD-e carries a frameshift in the *gadR* gene, whereas the ORF was restored to full-length by homologous recombination in the strain EGD-e *gadR*+. Transcription of *gadT2* (C), *gadR* (D), and *gadD3* (E) in these strains after overnight incubation for 18 h was calculated relative to the WT strain 10403S. The ability of these strains to secrete GABA into the extracellular environment is shown (F). Three independent experiments were performed with technical triplicates (A, B, and F) or duplicates (C, D, and E). Statistically significant differences were determined by paired *t* test (two-tailed) (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

7). While *gadT2*, *gadD1*, and *gadD3* were all acid-inducible with a peak at pH 5, only the induction of *gadT2* was GadR-dependent (Fig. 4A; Fig. S2). *gadR* transcription was unchanged regardless of the pH, likely indicating that the *gadT2* induction might involve post-transcriptional regulation of *gadR* (Fig. 4B). pH 5 activates *gadT2* transcription to the greatest extent and this activation appeared to be rapid and continuous (Fig. 4C). The transcription of the SigB-dependent *gadD3* and *lmo0913* was also induced, however, to a lesser extent (Fig. 4C). These data demonstrated that GadR was responsible for the activation of *gadT2* transcription in response to mildly acidic stress.

To test the involvement of GadR in the ATR, the ability of strains 10403S and EGD-e to survive at pH 2.3 with or without GadR was measured before (non-AD) and after pH 5 adaption for 60 min (AD 60). All strains from the exponential phase without pH 5 adaptation exhibited low levels of acid resistance. Strains 10403S WT and EGD-e *gadR*+ developed a strong ATR after adaptation (Fig. 4D and E), while strains 10403S Δ*gadR* and EGD-e WT (*gadR*⁻) exhibited greatly reduced levels of acid resistance after pH 5 treatment (Fig. 4D). Since EGD-e is the most widely studied strain, the relative contributions of *gadR* and *sigB* to ATR were examined using strain EGD-e WT (*gadR*⁻) and its isogenic mutants (*gadR*+, Δ*sigB*, *gadR*+ Δ*sigB*). Because of the extreme acid-sensitive phenotype of strain *gadR*⁻ Δ*sigB*, the ability to survive at pH 2.4 (rather than pH 2.3) was measured to compare the ATR. The ATR was only slightly reduced following *sigB* deletion in strain EGD-e *gadR*+, but it was essentially absent when *sigB* was deleted in strain EGD-e (*gadR*⁻) (Fig. 4E). In accordance with these observations, *gadT2* was strongly induced by pH 5 adaption for 15 min (AD 15) in both genetic backgrounds independently of SigB (Fig. 4F).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Reference/source
Strains	
Escherichia coli One Shot TOP10	Invitrogen
L. monocytogenes 1381	Wu et al. (34)
L. monocytogenes 1381R1	Wu et al. (34)
L. monocytogenes 1381 pIMK3	This study
L. monocytogenes 1381 pIMK3::*gadR*	This study
L. monocytogenes 1381R1 pIMK3	This study
L. monocytogenes 1381R1 pIMK3::*gadR*	This study
L. monocytogenes 10403S	Boor
L. monocytogenes 10403S Δ*gadR*	This study
L. monocytogenes 10403S Δ*gadT2D2*	This study
L. monocytogenes 10403S Δ*gadT2D2R*	This study
L. monocytogenes 10403S Δ*sigB*	Boor
L. monocytogenes 10403S Δ*gadR* Δ*sigB*	This study
L. monocytogenes EGD-e	Piveteau
L. monocytogenes EGD-e *gadR*⁺	This study
L. monocytogenes EGD-e Δ*sigB*	Marinho et al. (38)
L. monocytogenes EGD-e *gadR*⁺ Δ*sigB*	This study
L. monoctyogenes EGD-e *gadR*⁺ P*gadT2*_V1-V10	This study
Plasmids	
pIMK3; IPTG-controlled gene expression P*help::lacOid*; Kan^r	Monk et al. (37)
pMAD; Ery^r; Amp^r	Arnaud et al. (39)
pIMK3::*gadR* (pJW2); Kan^r	This study
pMAD *gadR*+ (pJW3); Ery^r; Amp^r	This study
pMAD Δ*gadR* (pJW15); Ery^r; Amp^r	This study
pMAD Δ*gadT2D2* (pJW16); Ery^r; Amp^r	This study
pMAD Δ*gadT2D2R* (pJW17); Ery^r; Amp^r	This study
pMAD Δ*sigB* (COB926); Ery^r; Amp^r	Marinho et al. (38)
pEX-K168-EGD-e_*gadR*_reversion; Kan^r	Eurofins Genomics
pEX-A128-10403S_*gadR*_deletion; Amp^r	Eurofins Genomics

gadR transcription appeared to be slightly downregulated following adaptation in strain 10403S; however, it remained unchanged in the EGD-e genetic background (Fig. 4G), further suggesting that GadR activation by mild acidification was likely achieved through post-transcriptional regulation. Consistent with previous phenotypic and transcriptional observations, Glu/GABA antiporter activity was only present and induced in the *gadR*⁺ strains (Fig. 4H), following an acid challenge at pH 3.25 for 1 h, conditions which are not lethal to strains carrying either *gadR* or *sigB* (Fig. S1). Interestingly, the ability to secrete GABA was notably higher in the absence of *sigB* in EGD-e strains (Fig. 3F and 4H), although these differences were not reflected in the transcriptional or acid-resistance measurements (Fig. 3B and 5E). Taken together, these results suggested that exposure to pH 5 induces a strong GadR-dependent ATR in *L. monocytogenes*.

Two 18 bp-palindromes in PgadT2 are essential for GadR-dependent ATR

Although *gadT2D2* alone confers GadR-mediated acid resistance, it remained unknown whether GadR controls the expression of genes that do not directly contribute to acid resistance. To test this, the global gene transcription during exponential growth with or without a 15 min pH 5 treatment was compared between strains EGD-e WT (*gadR*⁻) and *gadR*⁺. No gene was significantly differentially regulated without acid adaption between these two strains (Fig. S3). In contrast, 382 genes were upregulated and 374 genes were downregulated in response to pH 5 treatment in strain EGD-e *gadR*⁺ (Fig. 5A; Table S2), but only the acid induction of the *gadT2D2* operon and an adjacent gene *lmo2361* (encodes Rrf2 family transcriptional regulator) appeared to be dependent on GadR (Fig.
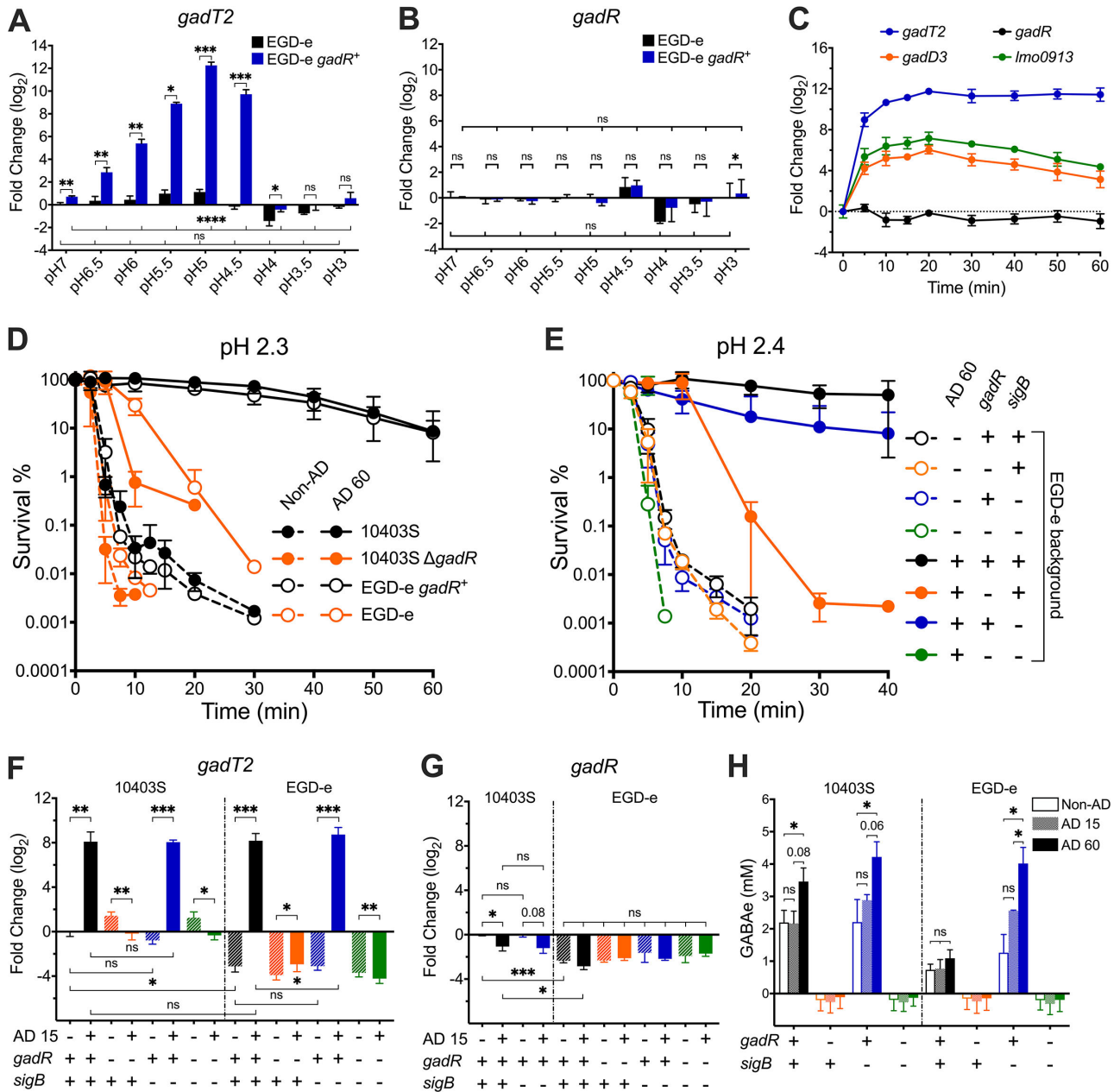
FIG 4 Adaptive acid resistance is GadR-dependent. The transcript levels of *gadT2* (A) and *gadR* (B) in exponential phase cultures of EGD-e WT (*gadR*⁻) or *gadR*⁺ strains with or without a 15 min exposure to pH 3.0–6.5 are shown, expressed relative to untreated EGD-e WT stain. (C) The differential transcription of *gadT2*, *gadR*, *gadD3*, and *lmo0913* in response to pH 5.0 exposure was monitored throughout a 60-min period in strain EGD-e *gadR*⁺. Cultures of *L. monocytogenes* WT or mutant strains grown to exponential phase with (AD 60) or without (non-AD) a 60 min pH 5.0 adaptation were challenged in pH 2.3 (D) or pH 2.4 (E). Transcription of *gadT2* (F) and *gadR* (G) in exponential phase cultures of *L. monocytogenes* WT or mutant strains with or without 15 min exposure to pH 5.0 (AD 15) are calculated and shown relative to the untreated 10403S WT strain. (F) The GABA exported into the medium was measured using exponential phase cultures either untreated (non-AD) or exposed to pH 5.0 for 15 min (AD 15) or 60 min (AD 60). Three independent experiments were performed with technical triplicates (D, E, and H) or duplicates (A, B, C, F, and G). Statistically significant differences between measurements were determined using a paired *t* test (two-tailed) (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). Statistically significant differences across a group of samples were determined by one-way ANOVA (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

5B). Moreover, *gadT2D2* were co-transcribed as a transcriptional unit and they were the most upregulated genes (11.7 and 9.3 log₂ fold changes, respectively) in response to pH 5 treatment across the genome (Fig. 5A; Table S2), supporting a prominent role for GadR
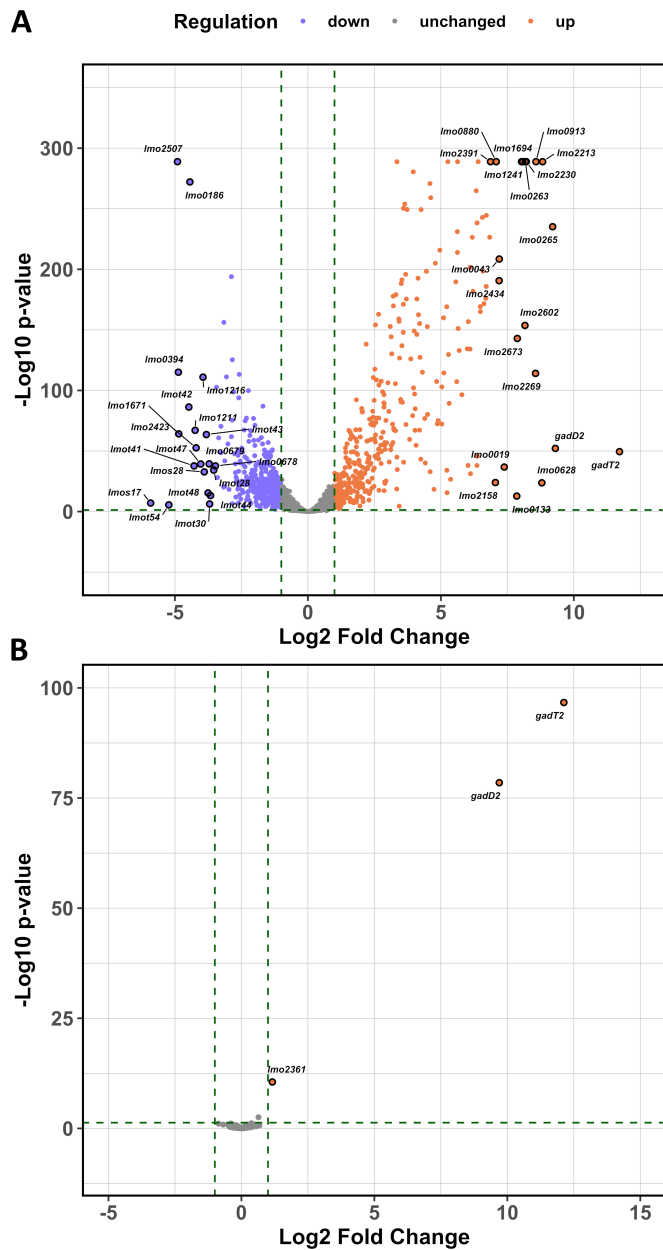
FIG 5 GadR is a dedicated regulator of the *gadT2D2* operon during acid adaptation. (A) Global gene transcription following a 15 min pH 5.0 treatment was measured using RNA-seq, with values expressed relative to the untreated exponential phase culture of strain EGD-e *gadR*⁺. The 20 most upregulated/downregulated genes are labeled. (B) GadR-dependent genes were detected by comparing global gene transcription of strain EGD-e *gadR*⁺ to the WT EGD-e strain (*gadR*⁻) after 15 min at pH 5.0. Genes showing differential transcription are labeled. Three independent sequencing reactions were carried out. Genes with differential transcription greater than twofold and with *P*-value < 0.05 were considered significantly differentially regulated (marked by dotted lines on the Volcano plots).

in the ATR. These data demonstrated that in response to mild acid stress GadR exclusively influences the transcription initiated at the *lmo2361-gadT2* intergenic region.

To identify the sequence motif(s) required for GadR-mediated transcriptional regulation, P*gadT2* (defined as the sequence between the coding sequences of *gadT2* and the upstream adjacent gene) was carefully examined. Since the genes upstream from *gadT2* were not conserved across the *Listeria sensu stricto* clade (Fig. 2), P*gadT2* sequence alignment was performed for *Listeria sensu stricto* spp. in order to search for

the conserved region, which is likely responsible for GadR-mediated *gadT2* regulation (Fig. 6A). The transcription start site (TSS) of *gadT2* was mapped at ~145 bp upstream of start codon using the RNA-seq data, a prediction that was also supported by previous results (40). One notable feature that was conserved in all sequences was a pair of imperfect palindromic sequences that shared sequence similarity within the conserved region of P*gadT2* and upstream of TSS (Fig. 6A and B). To test whether these palindromic sequences influence GadR-mediated *gadT2* transcription, a panel of mutants (designated V1–V10) (Table 1) with various mutations in P*gadT2* was constructed (Fig. 6A and B) and tested for *gadT2* transcription as well as acid resistance at exponential phase with or without pH 5 treatment. Deletion of the conserved region in P*gadT2* (V1, V2, and V3) abolished the GadR-mediated ATR (Fig. 6). Disruption of either one of these two putative GadR-boxes (V4, V5, and V6) or the spacer region (V7) results in the absence of GadR-mediated ATR (Fig. 6). While a Δ1 bp deletion in the spacer between the two palindromic sequences (V8) was tolerable for GadR-mediated ATR, a 1 bp insertion in spacer region (V9) results in moderately reduced *gadT2* induction and absence of GadR-mediated adaptive acid resistance (Fig. 6). The results also suggested that the sequence to the 5′ of these palindromes is responsible for *lmo2361* transcription (Fig. S4). Further examination of P*gadT2* revealed a putative peptide of 23 aa, proceeded by a consensus ribosome binding site (RBS) (GGAGG) (Fig. 6A and B), although deletion of this RBS (V10) did not significantly affect the GadR-mediated ATR (Fig. 6). Taken together, these data suggest that the pair of 18-bp palindromes in P*gadT2* are likely to serve as GadR-binding boxes that are essential for the GadR-mediated ATR in *L. monocytogenes*.
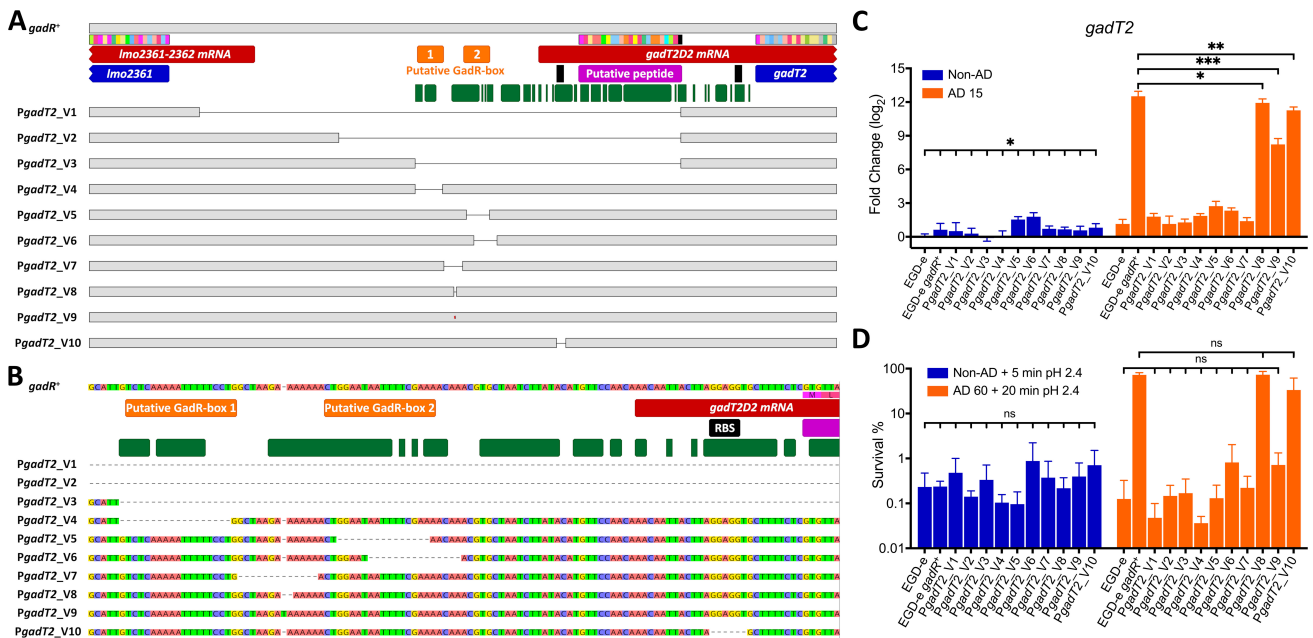

FIG 6 The promoter region of *gadT2* (P*gadT2*) has two 18-bp palindromes required for GadR-mediated activation of *gadT2D2* and induction of acid resistance. The intergenic sequence between the *lmo2361* and *gadT2* ORFs is depicted (A), with the nucleotide sequences shown for the 120 bp region targeted for mutagenesis (B). The sequences are annotated as follows: coding sequences are shown in blue; transcripts deduced from RNA-seq analysis are shown in red; putative short translation frames are shown in pink; the putative GadR-boxes are shown in orange; predicted RBSs are shown in black; the 100% conserved bases across *Listeria sensu strico* spp. genomes that have *gadT2D2-gadR* gene cluster (*n* = 13, Fig. 1) are annotated with green boxes underneath. The mutants, labeled P*gadT2*_V1 to P*gadT2*_V10, were constructed in an EGD-e *gadR*⁺ background and their positions are indicated (A and B). (C) The transcription of *gadT2* was measured for the panel of P*gadT2* mutants during the exponential phase either with (AD 15) or without (non-AD) a 15 min pH 5.0 treatment and expressed relative to the untreated WT EGD-e strain (*gadR*⁻). (D) The ability of these strains to survive at pH 2.4 was determined using exponential phase cultures either with (AD 60) or without (non-AD) a 60 min pH 5 treatment. Three independent experiments were performed with technical triplicates (C) or duplicates (D). Statistically significant differences between conditions were determined by paired *t* test (two-tailed) (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). Statistically significant differences across a group of samples were determined by one-way ANOVA (ns, not significant; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

DISCUSSION

In this study, a hitherto unknown RofA-like regulator, GadR, was predicted by comparative genomics to contribute to acid resistance in *L. monocytogenes*. We have provided phenotypic, transcriptional, and biochemical evidence that GadR has a major impact on the adaptive acid resistance of *L. monocytogenes* by activating *gadT2D2* expression in response to a mild acid stimulus. Through transcriptomic analyses, we showed that GadR is a dedicated regulator of *gadT2D2* and identified two putative GadR-binding boxes that are essential for GadR-mediated acid resistance. The data presented here also help to explain the previously reported differences in GAD activity (23, 28) and acid resistance (34, 41) between strains of *L. monocytogenes*. The results suggest that adaptive acid resistance in this pathogen is largely controlled by GadR and SigB, the regulator of the general stress response. Overall, the study elucidates a key regulatory mechanism for adaptive acid resistance and helps to clarify the previously unresolved questions.

The conservation of *gadR-gadT2D2* in *Listeria sensu stricto* spp. (Fig. 1) points to the possibility that this acid-resistance mechanism is important for the fitness of these species in the gastrointestinal (GI) tract since they are commonly isolated from feces (42). However, the occurrence of loss-of-function mutation in *gadR* in strains EGD-e and 1381 raises the question of how common loss-of-function mutations in *gadR* are in *L. monocytogenes*. An inspection of the rate of PMSC occurrence in the *gadR* open reading frame per 100 bp in 40,080 sequenced *L. monocytogenes* genomes reveals that it is exceptionally high (Fig. S5). The PMSC rate in *gadR* is comparable to that of the *inlA* invasion gene, which has been reported to be highly susceptible to mutations resulting in truncation of the protein (43) and is an order of magnitude higher than the PMSC rates in *gadT2* and *gadD2* (Fig. S5). This finding suggests that allelic variation in *gadR* could be a major determinant of differences in acid resistance between *L. monocytogenes* strains. It is interesting to speculate about the evolutionary pressures that might lead to the selection of mutations in *gadR* despite its importance in acid resistance. One possibility is that the GadT2D2-mediated reaction might be detrimental/dispensable under conditions where the availability of glutamate is limited or where maintenance of a high intracellular pool of glutamate is critical. Glutamate is present at a high concentration in the cytoplasm of *L. monocytogenes* where it contributes to osmoregulation and acts as a counter-ion for potassium (44). It is conceivable, therefore, that under prolonged conditions of osmotic stress, it might be advantageous to dispense with GadT2D2 by inactivating the main regulator. Alternatively, environments outside the mammalian host that rarely experience acidification might allow genetic drift of the *gadR* sequence, in the absence of positive selection for its function. Indeed, 8.4% of LII genomes encode truncated versions of GadR, while this number is only 1.1% for LI genomes and even lower in the hypervirulent CCs: 0.3% in CC1, 1.1% in CC2, 0.2% in CC4 and CC6 (45). Notably, a considerable percentage of strains from the phylogenetic groups that are prevalent in food is predicted to carry loss-of-function mutations in *gadR*, e.g., CC18 (86.3%), CC7 (31.4%), and CC9 (19.5%) (data not shown).

GadR shares significant sequence similarity with RofA, which was identified in *Streptococcus pyogenes* nearly three decades ago as a positive regulator of protein F (46). RofA is the founding member of a group of regulators called RALPs (RofA-like Proteins) that are found exclusively in Group A streptococci and that are a subset of the Mga superfamily of regulators (47, 48). Like the RALPs and Mga, GadR has two putative helix-turn-helix (HTH) DNA binding domains in the N-terminal half of the protein (Uniprot accession numbers: HTH_Mga, PF08280, and Mga, PF05043). Both HTH domains of Mga are necessary for DNA binding and activation of the Mga regulon (49). The presence of the two putative HTH domains in GadR suggests their involvement in binding to one or both of the predicted palindromic GadR-boxes upstream of *gadT2*. The predicted GadR-boxes span from −80 to −32 bp relative to the TSS of *gadT2,* and GadR-box 2 likely overlaps with −35 box of the *gadT2* promoter, which is among the reported features of Mga superfamily transcription factors (50, 51). This finding suggests that when the GadR-box 2 is occupied by GadR, the *gadT2D2* operon would

be repressed, whereas occupancy of the GadR-box 1, following an acid stimulus, would lead to derepression of the operon. Whether GadR dimerization occurs remains to be established, although it is interesting to note that the GadR-mediated transcriptional activation of *gadT2* by acid is sensitive to the spacing between the two GadR-boxes; deletion of the spacer or insertion of 1 bp from it significantly reduces *gadT2* transcription following acid shock (Fig. 6B and C; V7 and V9, respectively). Based on our results, the likely scenario is that *gadR* is constitutively expressed regardless of the extracellular pH (Fig. 4) and that some post-transcriptional modification is induced by mild acid stress that facilitates/alters its interaction with the regulatory region. Two phosphotransferase regulatory domains (PRDs) and a phosphotransferase system enzyme IIB-like domain are predicted at the C-terminal of GadR, similar to Mga and RofA, and these regulators were recently classified as PRD-containing virulence regulators (52). It is possible that the PRDs in GadR are involved in acid stress sensing and regulation of the activity of GadR. Further biochemical characterization of the GadR protein is underway to investigate this possibility.

While the GadR-mediated expression of GadT2D2 is shown to be a major factor in the development of an ATR, the global transcriptome analyses following pH 5.0 treatment reveal the complexity of the cellular response to acid. This acid stimulus was shown previously to trigger SigB activation (53) and indeed, ~36% of upregulated genes ($n = 140$) were SigB-dependent or proceeded by putative SigB promoter sequence (Table S2). Moreover, the SigB regulon accounts for ~86% ($n = 60$) of the 70 most upregulated genes (Table S2), including genes involved in known acid stress response (e.g., arginine/agmatine deiminase) and virulence-associated mechanisms (bile resistance, glutathione biosynthesis, and internalin expression) (20, 54–58). These data further demonstrate the importance of SigB in acid adaption. We have recently demonstrated that trace metal (Mn^{2+} and Zn^{2+}) homeostasis under acid stress is critical for an adequate stress response (35). In accordance with this, the expression of Mn^{2+} importers (*lmo1424* and *lmo1847-1849*) and a putative Zn^{2+} exporter (*lmo2231*) was induced, while the expression of putative Zn^{2+} importers was repressed (*lmo1445-1447* and *lmo1671*) (Table S2). Several upregulated genes are involved in carbohydrate uptake and metabolism (Table S2) indicating a general shift in carbon metabolism during growth in acidic conditions. Expression of multiple transcriptional regulators (e.g., *lmo2241*, *lmo2551*, *lmo0815*, and *lmo2494*) was also affected, and these might be partially responsible for the SigB-independent differentially regulated genes (Table S2).

The identification of GadR as the principal regulator of the GadT2D2 system and the characterization of global transcriptomic response to acid stress exposure help to understand the overall physiological response to mild acid stress in *L. monocytogenes*. Exposure to mild acid stress serves as a signal to the rapidly growing cells to prepare for a harsher environment. The most prominent actions involve rapid GadR- and SigB-mediated transcriptional response. GadR specifically promotes high expression of GadT2D2, which serves to protect against potentially lethal acid stress by helping to neutralize intracellular pH through glutamate decarboxylation. The total GAD activity is possibly supported by SigB-mediated *gadD3* expression, while other proton-consuming mechanisms (e.g., arginine/agmatine deiminase) might also contribute to the overall acid resistance (20). SigB as the general stress response regulator promotes an array of mechanisms that contribute to intracellular pH maintenance (Table S2) (19, 30). Our data suggest that there is an additive (rather than synergistic) effect in acid resistance between GadR and SigB. Besides intracellular pH maintenance, the cells also exquisitely manage metal homeostasis and accelerate carbon source utilization under acid stress, presumably to avoid metal intoxication and to acquire energy. The acid exposure also serves as a signal for host entry for the bacterium to express apparatus that are essential for GI tract survival, intestinal epithelial adhesion, and virulence factor activation (19) as supported by our transcriptomic data (Table S2).

Overall, this study sheds new light on the regulatory mechanisms that underpin the acid stress response of this important food-borne pathogen. It further highlights the

central role that glutamate decarboxylation plays in adaptive acid resistance. Further studies are underway to address some of the most important outstanding questions. Chief among these is the nature of the signal detected by GadR in response to acidification. It could be either acid pH itself or some secondary effect of reduced pH on the physiology of the cell. It seems likely that some post-transcriptional modification of *gadR* is required to activate it, but further work will be needed to clarify this. It will be interesting to learn whether GadR plays a role in colonizing the mammalian host, in particular, if it contributes to surviving the transition through the acidic conditions in the stomach. If it proves to be critical for this early stage of the infectious cycle, it might make a good diagnostic target for identifying strains of concern in food-processing environments. Once the regulation of GadR is fully elucidated, it might also be a useful molecular target to help reduce the survival of this pathogen in acidic environments.

MATERIALS AND METHODS

Strains and culturing conditions

L. monocytogenes and *Escherichia coli* TOP10 strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) (LAB M LAB048) at 37° C with agitation at 150 rpm unless otherwise specified. For stationary phase culture, an isolated *L. monocytogenes* colony was inoculated to 5 mL BHI broth in 50 mL centrifuge tubes and incubated for 18 h. To prepare exponential phase culture, overnight culture of *L. monocytogenes* was washed twice with fresh BHI broth and inoculated to 5 mL BHI broth in 50 mL centrifuge tubes to achieve initial $OD_{600nm} = 0.05$ and incubated for ~3 h until mid-exponential phase ($OD_{600nm} = 0.4$). *E. coli* strains were grown in Luria-Bertani (Sigma). The following antibiotics were added to the medium where specified: kanamycin 75 µg · mL^{-1} (^{kan}), ampicillin 100 µg · mL^{-1} (^{amp}), chloramphenicol 10 µg · mL^{-1} (^{chl}), and erythromycin 2 µg · mL^{-1} (^{ery}).

Molecular techniques

Plasmids and primers used in this study are listed in Tables 1 and 2; Table S1. To complement *gadR* in strains 1381 and 1381R1, the full-length *gadR* coding sequence was cloned (Phusion, ThermoFisher) from closely related CC2 strain 1380. The PCR product and expression vector pIMK3 (37) were digested (FastDigest, ThermoFisher) and ligated (T4 DNA ligase, Roche). Three microliters of ligation mixture was used in the thermo-shock transformation of *E. coli* TOP10 chemically competent cells, and the cells were plated on LB^{kan} plates after recovery and incubated overnight at 37°C. A transformant carrying the correct insert in the plasmid was grown overnight in LB^{kan} to propagate the plasmid for purification. Both purified plasmid (pJW2) and empty vector (pIMK3) were electroporated into electrocompetent cells of *L. monocytogenes* strains 1381 and 1381R1 as previously described (19, 37). To construct EGD-e *gadR*$^+$ and 10403S Δ*gadR*, two pMAD derivatives were constructed each with a synthesized (Eurofins) 600-bp insert containing 300 bp upstream and 300 bp downstream of the mutation site with restriction sites introduced on both ends. For EGD-e *gadR*$^+$, the *gadR* nonsense mutation in strain EGD-e was reverted in the insert (*374L) and several silent mutations were also introduced to facilitate the discrimination of mutant from WT using PCR (19). The constructed plasmids were transferred to electrocompetent *L. monocytogenes* EGD-e or 10403S cells and spread onto BHI^{ery} plates as previously described (19, 37). The mutation was achieved by a two-step integration (19, 59). The mutant and WT were discriminated by PCR. For introducing the *sigB* deletion, the previously constructed pMAD Δ*sigB* was transferred to strains 10430S Δ*gadR* and EGD-e *gadR*$^+$ (38). To introduce Δ*gadT2D2R* and Δ*gadT2D2* in strain 10403S and to create mutant panel EGD-e *gadR*$^+$ P*gadT2*_V1-V10, plasmids used for mutagenesis were constructed by splicing by overlap extension (SOE). Deletion of the entire P*gadT2* sequence was first introduced to EGD-e *gadR*$^+$ to obtain EGD-e

gadR[+] P*gadT2*_V1. Various lengths of P*gadT2* were then complemented to EGD-e *gadR*[+] P*gadT2*_V1 by two-step integration to obtain EGD-e *gadR*[+] P*gadT2*_V2-V10.

Transcriptional analysis

Transcriptional analysis was performed as previously described with minor adjustments (19). Briefly, for stationary phase transcription analysis, RNA samples were taken directly from overnight culture. In acid adaption analysis, 5 M HCl was added to exponential phase culture (OD_{600nm} = 0.4) to acidify the media to pH 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, and 3.0 (the volumes of 5 M HCl required were pre-determined). RNA samples were taken for transcription analysis after 15 min of exposure to acid stress. Alternatively, the exponentially growing culture was acidified to pH 5 and incubated at 37°C. RNA samples were taken at 0, 5, 10, 15, 20, 30, 45, and 60 min for transcription analysis. To extract RNA, 1 mL of culture was mixed with 5 mL RNALater (Sigma) and incubated at ambient temperature (~18°C) for RNA preservation. All subsequent RNA extraction and cDNA synthesis steps were carried out as previously described (34). Cells were then recovered by centrifugation and resuspended in the RNA Lysis buffer RLT (Qiagen RNeasy minikit). The mixture was transferred to lysis matrix B tubes for mechanical lysis (40 s at 6 m $\cdot$ s^{-1}, twice). The rest of the RNA extraction procedures followed the manufacturer's instructions. The resulting RNA samples were treated with DNase (Turbo, Invitrogen). The quantity and quality of RNA samples were examined using Nanodrop before reverse transcription (SuperScript III, Invitrogen) was carried out (13 µL reaction, ~0.5 µg RNA applied). qPCR (LightCycler 480 SYBR Green I Master, Roche) was performed with 100× diluted cDNA samples in a Roche LightCycler 480 system (34). All primers for gene expression analysis were designed to anneal to regions that were conserved across strains to be analyzed (Table 2). Three independent experiments were carried out each with two technical repeats. Relative gene transcriptions were calculated using Q-gene (60) with 16S as the reference gene.

Acid survival experiments

The ability of *L. monocytogenes* strains to survive in lethal acidic conditions was tested as previously described with minor adjustments (61). To examine the acid resistance at the stationary phase, 100 µL stationary phase culture was mixed with 900 µL BHI pH 2.3 or pH 2.4 in 1.5 mL microcentrifuge tubes and incubated at 37°C statically. Samples were taken at indicated time points, serial diluted (10×, in phosphate-buffered saline), and 10 µL of cell suspensions from each dilution was then spotted onto BHI agar to quantify viable cells. Acid resistance was determined as the percentage of survival at each time point relative to the starting cell count. Three independent experiments were carried out, each with triplicates. To examine the GadR-mediated adaptive acid stress response, 100 µL exponential phase culture with/without acid adaption for 60 min was mixed with 900 µL BHI pH 2.3 or pH 2.4 in 1.5 mL microcentrifuge tubes. The samples were incubated and analyzed following the same procedures as described for stationary phase acid resistance determination.

GABA assay

The abilities of strains 10403S and EGD-e and their derivatives to produce extracellular GABA at the stationary phase and exponential phase with/without acid adaption were examined using a previously established method with minor adjustments (62). For stationary phase culture, 4 mL overnight culture was resuspended in 2 mL BHI^{chl} pH 3 and incubated for 1 h at 37°C. For adaptive acid stress response, 2 mL culture was taken from exponential phase cultures that were exposed under pH 5 stress for 0, 15, and 60 min, then resuspended in 200 µL BHI^{chl} pH 3.25, and incubated for 1 h at 37°C. Following this incubation, the samples were spun down (10,000 *g·* 5 min) and the supernatant was collected for analysis. To quantify the GABA in the supernatant collected, 5 µL sample was added to 95 µL freshly prepared reaction master mix (80 mM Tris buffer, 750 mM

TABLE 2 Primers used in this study

Primer name	Primer sequence (5'–3')
Cloning	
EGD-e_gadR+_F	GCTTAGGGATCCTCGACTAATTTTAG
EGD-e_gadR+_R	GACATGGAATTCCGGAATAATAG
10403S_ΔgadR_F	ATATGGATCCGTATACTATGATACCA
10403S_ΔgadR_R	ATATGCGTCGACCCACTTACCAAT
ΔgadTD2_up_PstI_F	ATATCTGCAGGTGATGGCGAAAAATCCGAA
ΔgadTD2_up_EcoRI_R	ATATGAATTCAATGCGTTTGCTGCGAATAG
ΔgadTD2_down_BamHI_F	ATATGGATCCATTTCAGGTGGAACAGGAGC
ΔgadTD2_down_PstI_R	ATATCTGCAGCGATAATCAACAATCCGGCG
ΔgadRTD2_down_BamHI_F	ATATGGATCCCTGGACTCAAAATCCTGTGC
ΔgadRTD2_down_PstI_R	ATATCTGCAGACCCTCTCCATAAAATTGCAAC
RT-qPCR	
16S_RT_F	TGGGGAGCAAACAGGATTAG
16S_RT_R	TAAGGTTCTTCGCGTTGCTT
lmo2362_RT_F	ATCCAACATTTGCCACTTCC
lmo2362_RT_R	AAGAAGATTGCGGCAAAACC
lmo2365_RT_F	ACTTCTCGGGTGACGGT
lmo2365_RT_R	CTCCTCCACATTCGTAACAAAA
lmo2434_RT_F	CTGAGGAAGAAAGCACGAGT
lmo2434_RT_R	TTTTTCTCGAGCGTTTCTGC
lmo0447_RT_F	TACCGGTGTTTGGCTCTTTT
lmo0447_RT_R	CATGATTTGCTCAGCTTCCG
lmo0913_RT_F	CCTGATTGGGCAAAAATGGA
lmo0913_RT_R	ATCTTCTTGCTTCTTCCGCA
lmo2361_RT_F	GACGATGTTGTACACCCAGA
lmo2361_RT_R	TCTTCAGGGTCTTTAGCAAGC
lmo2230_RT_F	TGGGCGAAAAGACTTTCACT
lmo2230_RT_R	TGGAAATTTTGGTGCAGTTTCA

sodium sulfate, 10 mM dithiothreitol, 1.4 mM NADP+, 2 mM α-ketoglutarate, and 0.1 g · L^{-1} GABase) and incubated at 37°C for 1 h in a temperature-controlled plate reader with $OD340_{nm}$ measured every 60 s for 3 h. Standard curves were made by analyzing 1–10 mM GABA solution (made with BHI pH 3 or pH 3.25). OD_{340nm} measured at 1 h was inspected for GABAe calculation. Three independent experiments were carried out, each with duplicates.

Transcriptomic analysis

RNA samples for transcriptomic analysis were extracted as mentioned above and sent to Novogene for RNA sequencing and subsequent bioinformatic analysis following a standard procedure. Briefly, for library construction, ribosomal RNA was removed from total RNA with the Illumina Ribo-Zero Plus rRNA Depletion Kit. After fragmentation, the first strand of cDNA was synthesized using random hexamer primers. During the second strand cDNA synthesis, dUTPs were replaced with dTTPs in the reaction buffer. The directional library was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, PCR amplification, and purification with AMPure XP beads. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required. The sequencing was performed in the Illumina NovaSeq6000 using a sequencing strategy based on paired-end reads with a sequencing length of 150 bp per read (PE150). The raw reads were mapped to reference genome EGD-e, and log2 fold changes were calculated

by comparing the normalized read counts between two given groups. Raw sequencing data are available from NCBI SRA under project number: PRJNA947476.

Statistics

All statistical analyses were performed in Prism 8.

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AUTHOR CONTRIBUTIONS

Jialun Wu, Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing, Formal analysis, Software, Validation | Olivia McAuliffe, Data curation, Funding acquisition, Project administration, Supervision, Writing – review and editing | Conor P. O'Byrne, Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental data (mBio01716-23-S001.docx). Figures S1-S5; Tables S1 and S2.

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