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Characterization of the Intracellular Glutamate Decarboxylase System: Analysis of Its Function, Transcription, and Role in the Acid Resistance of Various Strains of *Listeria monocytogenes*

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The glutamate decarboxylase (GAD) system is important for the acid resistance of *Listeria monocytogenes*. We previously showed that under acidic conditions, glutamate (Glt)/γ-aminobutyrate (GABA) antiport is impaired in minimal media but not in rich ones, like brain heart infusion. Here we demonstrate that this behavior is more complex and it is subject to strain and medium variation. Despite the impaired Glt/GABA antiport, cells accumulate intracellular GABA ($GABA_i$) as a standard response against acid in any medium, and this occurs in all strains tested. Since these systems can occur independently of one another, we refer to them as the extracellular (GAD_e) and intracellular (GAD_i) systems. We show here that GAD_i contributes to acid resistance since in a Δ*gadD1D2* mutant, reduced $GABA_i$ accumulation coincided with a 3.2-log-unit reduction in survival at pH 3.0 compared to that of wild-type strain LO28. Among 20 different strains, the GAD_i system was found to remove 23.11% ± 18.87% of the protons removed by the overall GAD system. Furthermore, the GAD_i system is activated at milder pH values (4.5 to 5.0) than the GAD_e system (pH 4.0 to 4.5), suggesting that GAD_i is the more responsive of the two and the first line of defense against acid. Through functional genomics, we found a major role for GadD2 in the function of GAD_i, while that of GadD1 was minor. Furthermore, the transcription of the *gad* genes in three common reference strains (10403S, LO28, and EGD-e) during an acid challenge correlated well with their relative acid sensitivity. No transcriptional upregulation of the *gadT2D2* operon, which is the most important component of the GAD system, was observed, while *gadD3* transcription was the highest among all *gad* genes in all strains. In this study, we present a revised model for the function of the GAD system and highlight the important role of GAD_i in the acid resistance of *L. monocytogenes*.

The glutamate decarboxylase (GAD) system is an important system of acid resistance in various Gram-negative and Gram-positive bacteria (8, 10). It is the most efficient system of acid resistance in *Escherichia coli* (4) and probably in *Listeria monocytogenes* (7). According to the current model for the function of the GAD system, an extracellular glutamate (Glt_e) is imported by an antiporter in exchange for an intracellular γ-aminobutyrate ($GABA_i$). Each molecule of Glt is then decarboxylated by a decarboxylase to produce a molecule of $GABA_i$ in a process that consumes a proton which is incorporated in the $GABA_i$ molecule (Fig. 1). Subsequently, the $GABA_i$ is exported by the antiporter in exchange for another Glt molecule, which starts a new cycle, which will remove another proton from the intracellular milieu (22).

The GAD system plays an essential role in the acid resistance of the bacterial food-borne pathogen *L. monocytogenes* (6, 7). It promotes the growth of this bacterium under mild acidic conditions or survival under severe acidic conditions, which can occur in certain foods (9). Furthermore, it promotes passage through the stomach, enabling it to reach the intestine, where it can invade the intestinal epithelial cells and initiate a potentially fatal disease called listeriosis (7). A basic prerequisite for the function of the system is the presence of Glt, which is contained in all foods and living organisms. During the decarboxylation of Glt, one proton from the intracellular milieu is incorporated in the backbone of the Glt molecule in the place of the carboxyl group to form GABA (Fig. 1). This proton, which is attached with a highly stable bond, cannot be subject to ionization, and therefore, it cannot be released to the intracellular milieu. Subsequently, the GABA molecule that carries the removed proton is either exported by the antiporter as extracellular GABA ($GABA_e$) or remains inside the cell ($GABA_i$) as has been shown previously (12).

The GAD system in most *L. monocytogenes* strains is encoded by a total of five genes. Two of these genes (*gadT1*, *gadT2*) encode antiporters, while *gadD1* and *gadD2* encode Glt decarboxylases. In general, the *gadD1T1* operon is absent in serotype 4 strains (9). The fifth gene (*gadD3*) encodes a putative glutamate decarboxylase, but its role has not been demonstrated experimentally. No role for GadD3 in acid tolerance has been established, although a *gadD3* insertion mutant has been shown to be defective for intracellular growth (11). Recently, the construction of a *gadD3* deletion mutant has been reported by Begley et al. (2). However, in this work it was demonstrated that unlike GadD1, GadD3 does not play a role in nisin resistance, but no role in acid resistance was investigated. All five genes are organized in three separate genetic loci: *gadD1T1*, *gadT2D2*, and *gadD3* (9). The *gadT2D2* locus plays an important role in survival under extreme acidic conditions (7, 9), while the *gadD1T1* locus is reported to enhance growth under mild acidic conditions (9).

We have shown previously that the GAD system can utilize intracellular Glt (Glt_i) to produce $GABA_i$ independently of the antiport (12). Due to the independent activity of these two processes, we propose for the first time the division of the GAD system

Received 25 January 2012 **Accepted** 4 March 2012
Published ahead of print 9 March 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.00227-12

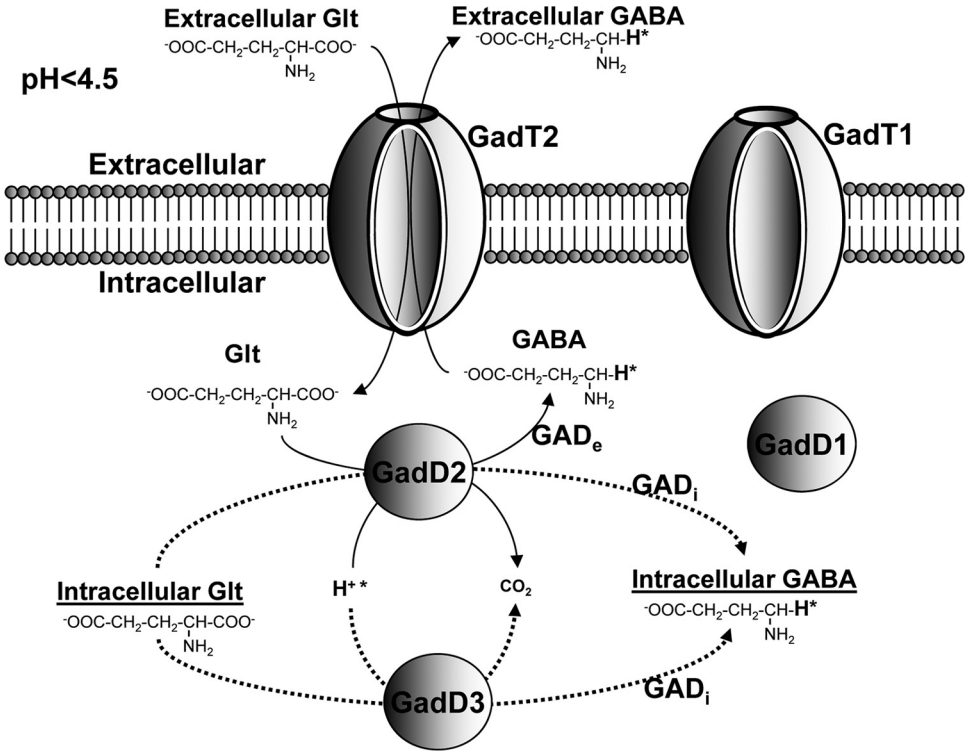

FIG 1 Model for the function of the GAD system under severe acid conditions (pH $<$ 4.5). The GadT2 antiporter imports extracellular Glt, which is decarboxylated by GadD2 to GABA with the concurrent consumption of a proton ($H^{+}*$). GABA is then exported by the GadT2 with the simultaneous import of Glt. The above-described process is carried out by the GAD_e, which is depicted by bold lines. Intracellular Glt is decarboxylated by GadD3 and GadD2, resulting in the accumulation of $GABA_i$. The latter process is carried out by GAD_i, which is depicted by dotted lines. The contribution of GadD1 and GadT1 in both the GAD_e and GAD_i processes is minor according to the results presented here. GadD3 has previously been suggested by various authors to be a Glt decarboxylase, but further work is required to prove this.

into extracellular (GAD_e) and intracellular (GAD_i) components. We also investigated the significance of GAD_i in acid resistance and which genes contribute to it. Furthermore, we studied the activity of GAD_i over a range of pH values and the differences in the transcription of the *gad* genes of three reference strains during an acid challenge. The contribution of GAD_i in the overall GAD-dependent removal of protons was also investigated in a variety of food and clinical isolates.

The present study advances our understanding of the function of the GAD system in *L. monocytogenes* and its role in acid resistance. Furthermore, by refining the existing model for the GAD system, the data presented here also have implications for the understanding of the acid resistance of other important foodborne pathogens (e.g., *E. coli* and *Shigella flexneri*) or commensals (e.g., lactic acid bacteria) that also possess the GAD system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. An array of strains including three reference strains and various food and clinical isolates was used in this study (Table 1). All strains were stored at −80°C in 15% (vol/vol) glycerol. Prior to experiments, stock cultures were streaked onto brain heart infusion (BHI) agar (LAB M, Lancashire, United Kingdom) and incubated at 37°C overnight. A single colony from this medium was transferred to 2 ml of sterile BHI (LAB M, Lancashire, United Kingdom), tryptone soy broth with 0.6% yeast extract (TSBY), or defined medium (DM) broth prepared according to the method of Amezaga et al. (1) with or without 10 mM Glt (Sigma-Aldrich, Steinheim, Germany) and incubated overnight at 37°C with shaking (160 rpm). Subsequently, a portion of these overnight cultures served as the inoculum (3% [vol/vol]; initial optical density at 600 nm [OD_{600}], 0.06) to prepare the cultures that were used in the experiments. Cultures were prepared in 250-ml conical flasks containing 20 ml of the same medium as the one used for the inoculum and incubated overnight (~18 h) at 37°C with shaking (160 rpm). Subsequently, these overnight cultures were used for all acid challenges and assays described below.

Survival under acidic conditions. Acid survival experiments were performed in DM and BHI. The use of different media and different strains resulted in great differences in acid resistance. Therefore, to achieve cell death at a measurable rate, different pH values needed to be applied in each medium. In these experiments, the pH of the overnight cultures was adjusted to 2.7 (BHI; see Fig. 2B), 3.2 (DM without Glt [DM (−Glt)]; see Fig. 3B), or pH 3.0 [DM (−Glt) or BHI; see Fig. 4], depending on the medium or the strain challenged with the addition of 3 M HCl. Samples were obtained prior to the pH adjustment and thereafter at regular time intervals, and 10-fold serial dilutions were prepared from those samples and plated onto BHI agar in triplicate. These plates were incubated at 37°C overnight, and subsequently, colonies were counted to assess survival under lethal acidic conditions.

GABase assay. A commercial preparation known as GABase was used to determine the $GABA_i$ and $GABA_e$ concentrations. $GABA_i$ was quantified as described by O'Byrne et al. (21), while $GABA_e$ was quantified according to the method of Tsukatani et al. (24), as modified by Karatzas et al. (12). Values for $GABA_i$ are estimations of the concentration in the cell obtained following calculations taking into account the concentration of cells and their hypothetical volume, as shown previously (12, 21). Overnight cultures had their pH, optical density, and cell concentrations estimated. Subsequently, the pH of the cultures was adjusted to 4.0 to quan-

TABLE 1 Strains used in this study

Isolate	Source	Presence of *gadD1T1*[a]	Serotype	% of H^+ removed by GAD_i compared to GAD in TSBY (pH 4)	Reference or source
102	Human clinical isolate	*	1/2a	15.31	This study
103	Human clinical isolate		4	15.66	This study
104	Human clinical isolate	*	1/2c	17.89	This study
294	Fish		ND	21.32	This study
295	Fish		ND	18.62	This study
299	Fish		ND	20.13	This study
302	Fish		ND	18.90	This study
437	Chicken salad sandwich		4b	19.38	This study
438	Human clinical isolate		4b	19.84	This study
439	Chicken salad sandwich		4b	18.28	This study
440	Ham and coleslaw sandwich		4b	26.03	This study
441	Human clinical isolate		4b	25.87	This study
442	Human clinical isolate		4b	13.04	This study
443	Human clinical isolate		4b	22.54	This study
444	Swine	*	1/2	11.79	This study
445	Human clinical isolate		4b	25.92	This study
446	Human clinical isolate		4b	17.09	This study
10403S	Lab strain, human clinical isolate	*	1/2a	6.21	12
EGD-e	Lab strain, rabbit isolate	*	1/2a	100.00	20
LO28	Lab strain, clinical isolate	*	1/2c	28.98	7
LO28 Δ*gadD1*	Mutant		1/2c	ND	7
LO28 Δ*gadD2*	Mutant	*	1/2c	ND	7
LO28 Δ*gadD1D2*	Mutant		1/2c	ND	7

[a] * indicates the presence of *gadD1T1*.

tify the levels of $GABA_i$ and $GABA_e$, with the exception of the experiments whose results are presented in Fig. 5 and Fig. 2A, where cultures were adjusted to various pH values to quantify the $GABA_i$ and/or $GABA_e$. Although survival was tested under lethal conditions, $GABA_e$ and $GABA_i$ were assessed at the nonlethal pH of 4 to avoid any interference of cell death in the $GABA_i$ quantification. This value of pH 4 was specifically selected to create conditions as close to lethal as possible, without resulting in cell death, which could compromise the interpretation of the GABase results. The course of the GABase reaction was monitored by measurement of the absorbance at 340 nm every 1 min for 3 h at 37°C using a Sunrise spectrophotometer (Tecan, Männedorf, Switzerland) operated by Magellan software (Tecan, Männedorf, Switzerland). All reagents used for the GABase assay were obtained from Sigma-Aldrich (Steinheim, Germany).

Real-time RT-PCR determination of *gad* gene transcription. Transcription of the *gad* genes in response to acidification was quantified as previously described by Karatzas et al. (12) following real-time reverse transcription-PCR (RT-PCR). The primers used previously (12) were designed as such to recognize the corresponding sequences in all three reference strains in which they were used. Efficiencies of the primer pairs 16SF-16SR, gadT1F-gadT1R, gadD1F-gadD1R, gadT2F-gadT2R, gadD2F-gadD2R, and gadD3F-gadD3R were 2.27, 1.94, 2.12, 2.07, 2.09, and 2.03, respectively, and these values, which were all close to 2, were used for efficiency correction in the quantification step. In all cases, overnight cultures grown for ~18 h in DM were acidified at pH 4.0 with 3 M HCl to create conditions as close to lethal as possible without killing the cells. Samples were taken before acidification (0 min) and at 12 and 30 min postacidification. Relative expression was calculated as a ratio between expression of each of the target genes (*gadD1*, *gadT1*, *gadD2*, *gadT2*, and *gadD3*) and the expression of the 16S rRNA gene, which served as the reference gene in each cDNA sample. Calculations were carried out following the advanced relative quantification settings of the LightCycler 480 software program, with PCR efficiency correction as described previously (12). Relative expression of each gene was calculated by comparison of its expression relative to that of the 16S rRNA gene in each strain and under each condition and expressed as a percentage of the maximal level detected for that transcript in all strains and under all conditions (see Fig. 6). Furthermore, to allow comparisons between the expression of the different *gad* genes within the same strain, we present the expression of all genes per strain (see Fig. S1 in the supplemental material). To allow comparisons between strains, all transcription data are expressed in this case as a percentage of the maximal level detected for any gene in any strain.

Statistical analysis of results. In all sets of experiments except RT-PCR, mean values were calculated from experiments performed in triplicate, and standard deviations were also determined and are depicted with error bars on the graphs. In addition, in each separate experiment, values were calculated as an average from three technical replicates.

For each RT-PCR experiment, measurements were performed on three independent biological samples, with three technical replicates performed on each. The data were normalized to those for the 16S rRNA using the advanced relative quantification with PCR efficiency correction feature of the Roche LightCycler 480 software. Tukey's multiple-comparison test was then performed to define if the expression of each gene from a specific strain was statistically significantly different from that of the other strains at each time point. Tukey's multiple-comparison test was used because multiple comparisons between three different values were required for each gene and time point (see Fig. 6). *P* values were calculated and deemed statistically significant (*) when *P* was <0.05. Furthermore, to assess the change in the expression of each gene in each strain, the expression at each time point was compared to that at time zero and is shown as fold change. To estimate the statistical significance of this change, Student's *t* test comparing the expression at a certain time and that at time zero was performed, and if *P* was <0.05, it was deemed statistically significant and indicated (†).

Tukey's multiple-comparison test was also used to identify a significantly different expression of a specific gene in a strain at a specific time point, and if a statistically significant difference was found ($P < 0.05$), it was marked (*) (see Fig. S1 in the supplemental material). The fold change in the expression of each gene in each strain compared to that at time zero is also depicted only if it was >2.00 or <0.50 and is indicated (†) if it was statistically significant, as estimated by Student's *t* test ($P < 0.05$).

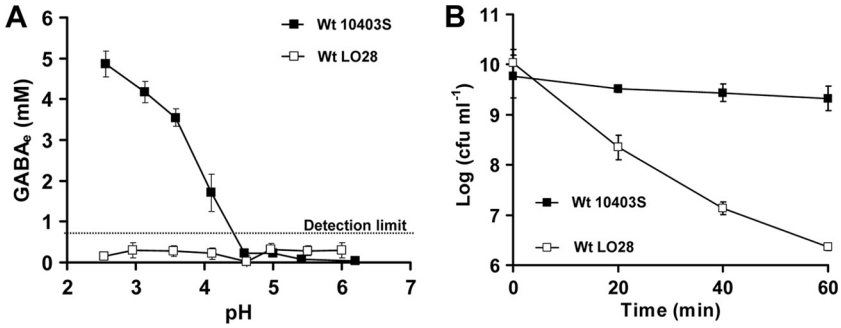

FIG 2 Strain LO28 does not use the GAD$_e$ system in BHI. (A) Concentration of GABA$_e$ measured at 80 min after acidification of the cultures to various pH values. The detection limit for GABA$_e$ quantification in BHI was 0.8 mM. (B) Acid resistance of stationary-phase cultures of wild-type (Wt) strains LO28 and 10403S following acid challenge at pH 2.7. All cultures were grown to stationary phase overnight at 37°C, and viability of cells was determined prior to acid challenge and every 20 min thereafter. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation.

Presence of *gad* genes in strains. The presence of all five *gad* genes in all strains used in this study was assessed by the use of PCR. The sets of primers used for each of the *gad* genes were the ones used for the RT-PCR previously described by Karatzas et al. (12).

RESULTS

GABA$_e$ export is strain and medium dependent. We showed previously that under acidic conditions, Glt/GABA antiport is impaired in minimal media but not in rich ones, like BHI. The ability of *L. monocytogenes* LO28 and 10403S to export GABA$_e$ in response to acidification (pH 4) was measured in BHI. Both strains were cultured to stationary phase in BHI at 37°C, and then the medium pH was adjusted to a range of pH values from 6.3 to 2.5. GABA$_e$ levels in the culture supernatant were recorded following 80 min of acidification to allow sufficient GABA$_e$ export. Over the whole range of pH values, LO28 exported undetectable levels of GABA$_e$ (below the 0.8 mM GABA detection limit; Fig. 2A). In contrast, 10403S exported GABA$_e$ once the medium pH dropped below 4.5, with the highest level recorded when the pH was dropped to 2.5 (Fig. 2A). To assess the acid survival of these cultures, we treated them at pH 2.7. We selected a value between pH 2.5 and 3.0 because the first was too severe, causing rapid death, and the last was too mild. At pH 2.7, 10403S was found to survive well, whereas LO28 lost viability rapidly (Fig. 2B). Interestingly, we found that GABA$_e$ export in response to acidification was absent from LO28 only when the cells were cultured in some growth media but not in others. For example, we confirmed that when we cultured LO28 to stationary phase in TSBY, it was able to produce GABA$_e$ in response to acidification, as it has been shown previously (7). These data highlight that the Glt/GABA antiport is strain and medium dependent. Furthermore, as explained in more detail in the Discussion, the results presented above support the idea of a division of the GAD system into the intracellular decarboxylase system (GAD$_i$) transforming Glt$_i$ to GABA$_i$ and the extracellular- or antiport-dependent decarboxylase system (GAD$_e$) transforming Glt$_e$ to GABA$_e$ through the Glt/GABA antiport (Fig. 1).

Accumulation of GABA$_i$ in different *L. monocytogenes* reference strains correlates with acid tolerance. In a recent study, we have shown that although *L. monocytogenes* 10403S was not able to produce any GABA$_e$ through GAD$_e$ in DM with Glt, it was able to accumulate substantial pools of GABA$_i$ through GAD$_i$ following acidification (12). This suggests that GABA$_i$ accumulation could be a response against acid and it might play a role in survival under these conditions. Therefore, we investigated whether GABA$_i$ accumulation could correlate with acid survival under conditions that do not permit Glt/GABA antiport [DM (−Glt)]. The capacity of three different reference strains to produce GABA$_i$ in response to acidification of the medium was determined. Cultures of 10403S, LO28, and EGD-e were grown to stationary phase (~18 h) in DM and then adjusted to pH 4.0, after which the GABA$_i$ levels were measured at regular time intervals for a period of 1 h. Large differences in GABA$_i$ production were observed between the three strains. After 1 h at pH 4.0, *L. monocytogenes* 10403S had produced >3-fold more GABA$_i$ than LO28 and >10-fold more GABA$_i$ than EGD-e (Fig. 3A). We confirmed that under these conditions no GABA$_e$ was detected for any of the three strains. When acid survival rates were measured at pH 3.2 following growth under identical conditions, 10403S was found to be the most acid tolerant, while EGD-e was the least tolerant (Fig. 3B). Thus, the rates of viability loss at a lethal pH were inversely related to the capacity of these strains to produce GABA$_i$ in response to acidification at a nonlethal pH (Fig. 3), suggesting that higher GABA$_i$ accumulation coincided with higher acid resistance.

GAD$_i$ contributes to survival under acidic conditions. *L. monocytogenes* wild-type (LO28) cells and isogenic mutant derivatives lacking either the GadD1 or GadD2 decarboxylase, or both, were grown in DM (−Glt) or in BHI to stationary phase. In both media, LO28 is not able to perform Glt/GABA antiport and uses only Glt$_i$. When cultures were challenged under lethal acidic conditions in DM (−Glt) at pH 3.0, the mutants lacking the GadD2 decarboxylase were more sensitive, resulting in a 1.75-log-cycle difference in log reduction compared to the wild type (Fig. 4A). Under these conditions, no detectable GABA$_e$ but only GABA$_i$ was produced by LO28. The mutants lacking GadD2 were found to accumulate reduced levels of GABA$_i$ (which decreased by ~40%), whereas mutants lacking the GadD1 system produced levels similar to those observed in the wild type (Fig. 4B). Thus, a good correlation between the capacity of GAD$_i$ to accumulate GABA$_i$ and the ability to survive an acid challenge existed. When cells were acid challenged in BHI at pH 3.0, Δ*gadD1*, Δ*gadD2*, and Δ*gadD1D2* cells showed 0.95- and 1.95-log-cycle differences and a significant 3.26-log-cycle difference in log reduction compared to the wild type, respectively, after 60 min (Fig. 4C). In this case, a good correlation between GABA$_i$ accumulation at pH 4 (Fig. 4D)

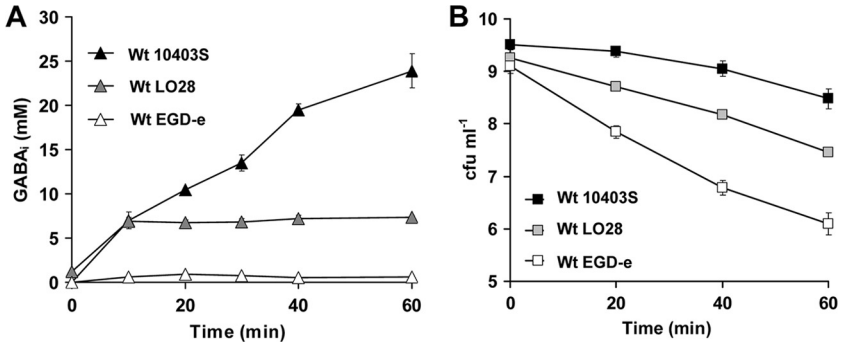

FIG 3 Survival of strains 10403S, LO28, and EGD-e in DM in the absence of any extracellular Glt corresponds with the accumulation of $GABA_i$. Cells were grown overnight in DM (−Glt) until stationary phase (~18 h) at 37°C with shaking. Subsequently, cultures were acid challenged at pH 4, where $GABA_i$ was measured (A), or acid challenged at pH 3.2, where viability was determined at regular time intervals (B). Acidification of cultures was achieved with the addition of 3 M HCl. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation.

and survival existed. These results show that the GAD system utilizing solely intracellular pools of Glt (GAD_i) contributes to the acid resistance of *L. monocytogenes*.

GAD_i contributes significantly to the overall GAD activity of various food and clinical isolates. The GAD_i-dependent production of $GABA_i$ seems to contribute to the acid resistance, and therefore, it should contribute to the proton removal process. According to the current model for the GAD system, each mole of GABA produced carries 1 mole of protons (H^+) which has been removed by the Glt decarboxylation process. Therefore, by measuring the levels of $GABA_i$ and $GABA_e$, we could identify how many protons are removed by the GAD_i and GAD_e, respectively (Fig. 1).

Twenty different strains grown until stationary phase in TSBY were surveyed for their $GABA_i$ and $GABA_e$ production after acid challenge at pH 4 for 60 min. Strains included reference strains, as well as clinical and food isolates (Table 1). Based on the hypothesis that each mole of GABA removes a mole of protons, we calculated the number of millimoles of protons removed by the use of intracellular (GAD_i) or extracellular (GAD_e) Glt, and then we divided

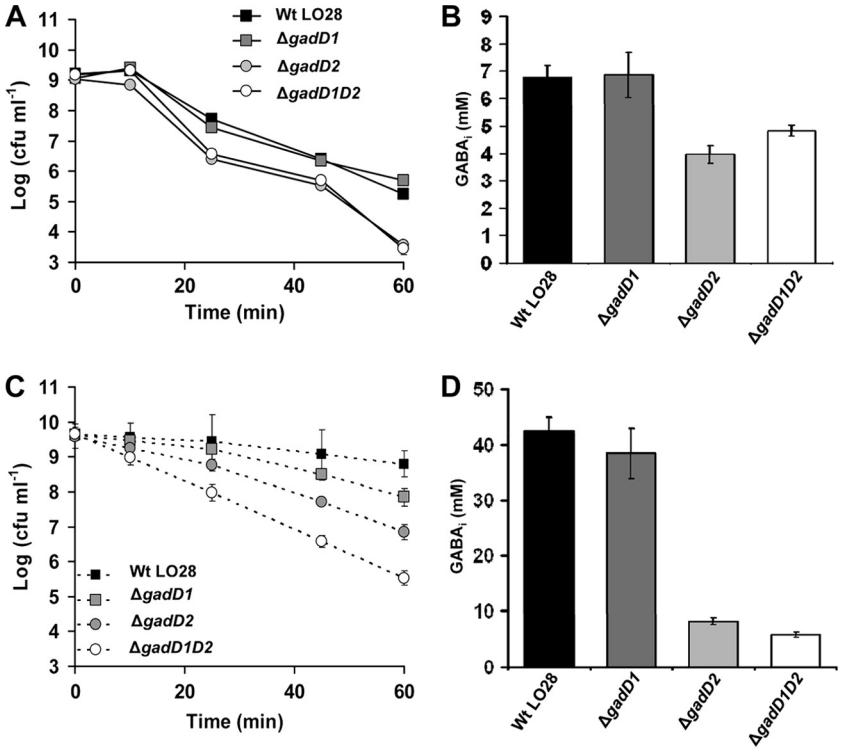

FIG 4 The GAD_i system is able to confer increased acid tolerance in the absence of any extracellular Glt. The GAD system is able to function solely on intracellular Glt, and the strains that accumulate higher $GABA_i$ demonstrate the highest acid tolerance. (A) Acid resistance of wild-type LO28 and its Δ*gadD1*, Δ*gadD2*, and Δ*gadD1D2* isogenic mutants in DM (−Glt) at pH 3. (B) Accumulation of $GABA_i$ in wild-type LO28 and its Δ*gadD1*, Δ*gadD2*, and Δ*gadD1D2* isogenic mutants in DM (−Glt) at pH 4. (C and D) Acid resistance (C) and $GABA_i$ accumulation (D) of wild-type LO28 and its isogenic mutants were measured in BHI at pH 3 and pH 4, respectively. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation.

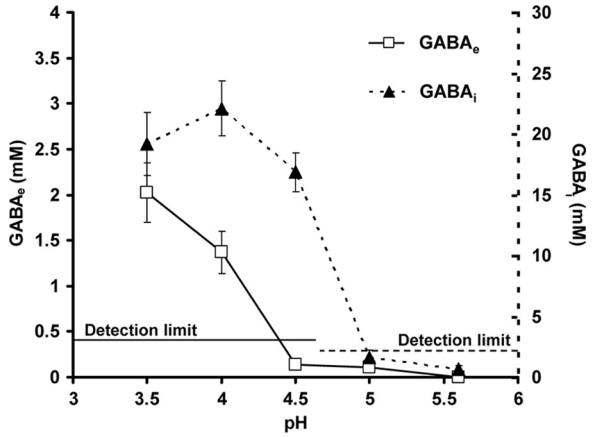

FIG 5 GABA$_e$ versus GABA$_i$ accumulation in *L. monocytogenes* 10403S. Utilization of intracellular Glt initiates at milder pH (4.5) than that of extracellular Glt (pH 4), suggesting that the decarboxylases are active at milder pH values than the antiporters. Cells were grown overnight in TSBY until stationary phase (~18 h) at 37°C with shaking. Cultures were acid challenged at different pH values with the addition of 3 M HCl, and measurements of GABA$_e$ and GABA$_i$ took place following 2 h at the corresponding pH value. In parallel, viability was tested under these conditions, and it was confirmed that cell death did not occur for the length of time that the experiment took place. The detection limits for GABA$_e$ and GABA$_i$ were 0.4 and 2.2 mM, respectively. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation.

these values by the OD_{600} of each strain's culture at the moment of the GABA measurements to allow comparisons. Subsequently, the number of millimoles of protons removed by GAD$_i$ was divided by the number of millimoles of protons removed by the overall GAD system (GAD$_i$ and GAD$_e$). Overall, an average 23.11% ± 18.87% of the total number of millimoles of protons removed by the GAD system was removed by the GAD$_i$. All strains removed protons through the GAD$_i$, while in a unique fashion, the EGD-e strain did not remove any protons through the GAD$_e$. In EGD-e, which is the most-studied reference strain of *L. monocytogenes*, all protons removed by the GAD system were removed through the GAD$_i$. This and all previous work in DM suggest that a standard level of GAD$_i$ activity always exists in all strains under acidic conditions, while the activity of GAD$_e$ might be variable, depending on the environment and the strain (Table 1). Furthermore, the absence of *gadD1T1* (all other genes were present in all strains) did not affect the proportion of protons removed by GAD$_i$ compared to the number of protons removed by the overall GAD system.

In *L. monocytogenes* 10403S, GABA$_i$ accumulation occurs before GABA$_e$ is detected. If GABA$_i$ production is critical to acid survival, then it might be expected to occur as a primary response to acidification of the culture medium. Alternatively, if the export of GABA is more important in acid survival, then this might be expected to be the primary response. Therefore, we investigated the levels of both GABA$_i$ and GABA$_e$ over a range of culture pH values (pH 5.6 to 3.5) in a growth medium that supported both GABA$_i$ and GABA$_e$ production (TSBY). The results revealed that the GAD$_i$ began to accumulate GABA$_i$ 0.5 pH unit before GABA$_e$ was detected in the culture medium; significant GABA$_i$ levels were recorded at a medium pH of 4.5, whereas no GABA$_e$ was detected at this pH (Fig. 5). It was clear that GAD$_i$ activity occurs as the primary response to acidification of the culture medium. Only when the pH had reached 4.0 was GABA$_e$ detected in the medium. Thus, the data show that GABA$_i$ accumulation occurs in advance of Glt/GABA antiport, which suggests that GAD$_i$ is likely to be a critical factor in determining the ability of cells to survive during a transition into an acidic environment. The data further suggest that GAD$_e$ exports GABA via the antiporters only when a critical pH value is reached.

Strain-to-strain differences in *gad* gene transcription. In order to understand the basis for strain-to-strain differences in GABA$_i$ production, real-time RT-PCR was used to measure the transcription of all five *gad* genes following acidification to pH 4.0 in three different strains of *L. monocytogenes*: 10403S, LO28, and EGD-e (Fig. 6). Cells were grown in DM (−Glt), conditions that support GABA$_i$ but not GABA$_e$ production. Furthermore, we have previously demonstrated that in this medium the GAD system showed the most rapid and prominent response compared to the response in BHI, where transcription remained mainly unchanged or even reduced (12). The data revealed that there was considerable strain-to-strain variation in the transcriptional responses of the *gad* genes to acidic pH. To allow a better overview of these differences, we have plotted the data per gene (Fig. 6) and per strain (see Fig. S1 in the supplemental material). In general, transcription was higher the more acid sensitive that the strain was (Fig. 6; see Fig. S1 in the supplemental material). More specifically, following 30 min at pH 4, EGD-e had the highest levels of transcription for all *gad* genes, 10403S had the lowest, and LO28 maintained levels intermediate between those of the other two strains (Fig. 6). Furthermore, following acid treatment for 30 min, transcription of *gadD3* was the highest in all strains (see Fig. S1 in the supplemental material). In EGD-e there was a statistically significant increase in the transcription of *gadD1* by 13.53-fold and *gadT1* by 31.50-fold after 30 and 12 min, respectively, at pH 4, the most prevalent detectable upregulation after 30 min of acid treatment (Fig. 6). In this strain, acid treatment at pH 4 for 30 min caused a minor 2.78-fold increase for *gadD3*, reaching the highest level of transcription for any *gad* gene in any strain during the experiment. In contrast, transcription of the *gad* genes remained unaffected in LO28, with the exception of the *gadT2D2* operon, which was downregulated during the acid challenge. Transcription of *gadT2D2* and *gadD3* in LO28 prior to acid challenge was higher than that in the other strains (see Fig. S1 in the supplemental material). Although transcription of *gadD2* in LO28 was the second highest among all genes in all strains under basal conditions, no transcript could be detected for this gene after acid treatment for 30 min. In 10403S, there was a statistically significant upregulation only of *gadD1* and *gadD3*, by 8.13- and 10-fold, respectively (Fig. 6). Even though both *gadD1* and *gadT1* belong to the same operon, in all strains the transcription of *gadT1* was significantly higher than that of *gadD1* (see Fig. S1 in the supplemental material). Another common feature in all strains was the inability to upregulate the *gadT2D2* operon, which is the most important *gad* operon under severe acid challenges. Despite that, there was a 6.26-fold increase of *gadT2* in EGD-e only after 30 min at pH 4 (Fig. 6).

DISCUSSION

The GAD system is the most important mechanism of acid resistance in *Listeria monocytogenes* (7). It utilizes the freely available Glt$_e$ in the environment through Glt/GABA antiport and decarboxylation, removing protons in the process (22). Until recently, the only known prerequisites for the function of the GAD system

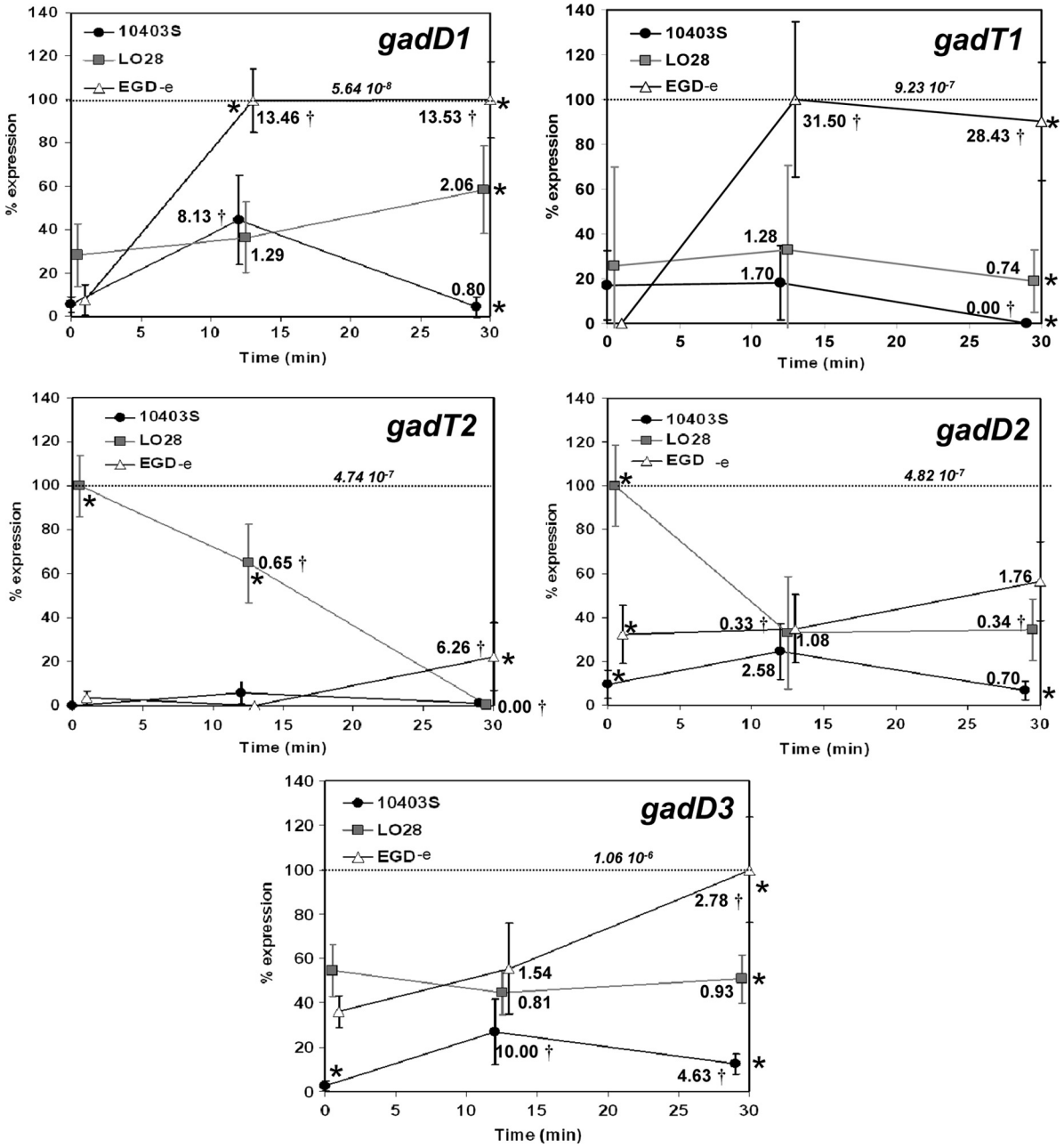

FIG 6 Transcription of each *gad* gene in different reference strains in response to acidification in DM at pH 4. Relative normalized (on the basis of the 16S rRNA gene) expression of all *gad* genes (*gadD1*, *gadT1*, *gadT2*, *gadD2*, and *gadD3*) in 10403S, LO28, and EGD-e in DM before (0 min) and after (12 and 30 min) acidification in pH 4 achieved with 3 M HCl. Expression of each gene was calculated following advanced relative quantification and normalization based on its relative transcription compared to that of the 16S rRNA gene in each strain and time point. For each comparison, the data are expressed as a percentage of the maximal level detected for that transcript in all strains and under all conditions. In order to allow comparisons between the expression of the different *gad* genes, the actual relative normalized (based on the 16S rRNA gene) expression level for each maximum value is also indicated on each graph. The fold change of the expression of each gene in each strain compared to the initial expression at time zero was calculated, and it is indicated close to each marker. A statistically significant change ($P < 0.05$), as estimated by Student's *t* test, is marked (†). A statistical analysis comparing the expression of each gene in each different strain was performed with Tukey's multiple-comparison test, and if P was <0.05, it was deemed statistically significant and indicated on the graphs (*). Error bars represent standard deviations.

in *L. monocytogenes* have been the entrance of cells in stationary phase, acidic conditions, and the presence of Glt_e. Recently, we have shown that despite the presence of all the above-described prerequisites, the Glt/GABA antiport of strain 10403S is impaired in DM but not in rich media like BHI, where the presence of unknown components is indispensable for its use, enhancing acid resistance (12). In this study, we have confirmed that 20 randomly selected clinical and food isolates (Table 1) were unable to use the Glt/GABA antiport in DM (with Glt; data not shown). The Glt/GABA antiport seems to be under complex regulation which is subject to strain variation, as LO28, in contrast to 10403S, is unable to export GABA in BHI (Fig. 2A), resulting in an acid-sensi-

tive phenotype compared to 10403S (Fig. 2B). Interestingly, LO28 is able to use the Glt/GABA antiport in TSBY (Table 1). This strain- and medium-dependent variability of the Glt/GABA antiport might be associated with diverse activation signals present in different niches that each strain explores. Similar to the behavior of 10403S in DM (12), in BHI LO28 cannot use the Glt/GABA antiport but it is able to accumulate $GABA_i$ by the decarboxylation of Glt_i (Fig. 4D).

Since the above-described processes of $GABA_i$ accumulation function independently of the Glt/GABA antiport, we propose the concept of dividing the GAD system into two semi-independent systems, the intracellular (GAD_i) and the extracellular (GAD_e) GAD systems utilizing Glt_i and Glt_e through the Glt/GABA antiport, respectively (Fig. 1). Furthermore, this distinction is necessary since, stoichiometrically, $GABA_i$ and $GABA_e$ derive from equivalent amounts of Glt_i and Glt_e, respectively. The Glt_e imported by GAD_e does not contribute to the $GABA_i$ pools because it is rapidly decarboxylated and exported (Fig. 1).

Despite the variability in GAD_e activity, our work (12) and the results presented here (Fig. 3A and 4B and D) suggest that accumulation of the $GABA_i$ produced by the GAD_i is a standard cellular response against acidic conditions. Although GAD_i activity has previously been suggested to occur in *E. coli* (5) and *S. flexneri* (25), there is no report showing its role in acid resistance. To investigate that, we conducted experiments in DM (−Glt) where the absence of Glt_e in *L. monocytogenes* restricted the use of the GAD_e and limited the GAD system to utilize only Glt_i. Three common reference strains were tested for their ability to accumulate $GABA_i$ at pH 4 and ranked from the highest to the lowest as 10403S > LO28 > EGD-e (Fig. 3A), which corresponded well with their acid resistance at pH 3 (Fig. 3B).

In further experiments, to assess the contribution of the *gad* genes in GAD_i activity and acid resistance, we used mutants with mutations in *gadD1* and *gadD2* and their isogenic wild type, LO28. In TSBY, where GAD_e is functional, GadD1T1 promotes growth under mild acidic conditions, while GadT2D2 promotes survival under severe acid challenge (9). In DM (−Glt), LO28 Δ*gadD1* and wild type showed similar GAD_i activities (Fig. 4B) and acid survival (Fig. 4A), suggesting an insignificant contribution of GadD1 in GAD_i activity and survival under severe acid challenge. However, in BHI, which also restricts GAD_e activity in LO28, the Δ*gadD1* mutant was more sensitive than the wild type by 1 log cycle of viable counts (Fig. 4C), coinciding with lower levels of $GABA_i$, without statistical significance, though, suggesting a minor role for GadD1 in acid resistance under severe acidic conditions. The absence of GadD2 under GAD_e restriction [DM (−Glt)] reduced GAD_i activity (Fig. 4B), which corresponded well with a significant decrease of ~1.75 log cycles in acid resistance (Fig. 4A). In BHI and under GAD_e restriction, the reduction in GAD_i activity was even higher than that in DM (−Glt) (Fig. 4D), resulting in a greater decrease in acid resistance (3.26 log cycles; Fig. 4C). The increased effect in BHI compared to DM (−Glt) could be due to the higher GAD_i activity in this medium, resulting in higher differences in $GABA_i$ between the mutants and the wild type. Therefore, GadD2 and, to a lesser extent, GadD1 contribute to the GAD_i activity which affects intracellular pH homeostasis and acid resistance.

We previously thought that due to the limited levels of Glt_i in comparison to the vast levels of Glt_e, the contribution of GAD_i in acid resistance would be insignificant. However, the results presented above suggest the opposite. Our revised model (Fig. 1) proposes that $GABA_i$ and $GABA_e$ remove stoichiometrically intracellular protons through GAD_i and GAD_e activity, respectively. Through measurements of $GABA_i$ and $GABA_e$ in 20 different strains (TSBY; pH 4) and simple calculations presented in Results, we found that the GAD_i removes an average of 23.11% ± 18.87% of the protons removed by the overall GAD system (Table 1). This contribution of GAD_i to proton removal might be even higher, if we took into account a possible catabolism of $GABA_i$ in *L. monocytogenes*. *In silico* analysis did not reveal any GABA transporters in *L. monocytogenes*, while the reference strains failed to utilize known concentrations of $GABA_e$ preadded in the growth medium (data not shown). However, the possibility of a pathway metabolizing $GABA_i$ cannot be excluded, as $GABA_i$ is one of the most abundant metabolites (~113 mM) under acidic conditions (12). Interestingly, in EGD-e—the most widely studied *L. monocytogenes* strain—the GAD_e system is nonfunctional in all media that we tested and the GAD_i system accounts for all the activity of the GAD system (Table 1). This unique characteristic of EGD-e, together with other peculiarities described previously, supports the idea that its usefulness as a model strain is debatable (23).

Since no Glt is present in DM (−Glt), at least a part of the Glt_i derives from the glutamine present in the medium. It has previously been suggested that *E. coli* might import glutamine via the antiporter GadC (25), but this seems unlikely for *L. monocytogenes*, as no $GABA_e$ was detected in acidified DM (−Glt). Glutamine is probably imported by Lmo0847, a putative glutamine transporter (19), and subsequently converted to Glt_i. Pools of Glt_i could be used instantly by the GAD_i when required. The instant utilization of Glt_i is suggested by the fact that production of $GABA_i$ initiates at milder pH values (4.5 to 5.0) than production of $GABA_e$ (4 to 4.5; Fig. 5). This could be attributed to a different optimal functional pH between the decarboxylases and the antiporters or possible differences in their transcription.

We investigated these possible differences in transcription of the *gad* genes through RT-PCR in three reference strains with high (10403S), medium (LO28), and low (EGD-e) acid resistance during an acid treatment in DM (Fig. 6). DM was the medium of choice, as the *gad* genes in 10403S previously showed a noticeable transcriptional response, unlike in rich media like BHI, where no response was observed (12). Our results show that after 30 min at pH 4, the transcription of every gene was higher the more acid sensitive that the strain was (Fig. 6). This suggests an attempt of the more sensitive strains to recover or maintain a level of GAD activity that would help them survive. However, its effect is questionable since no transcriptional upregulation of *gadT2D2* occurred in any of the strains during the acid challenge (Fig. 6; see Fig. S1 in the supplemental material). Therefore, this behavior that we previously observed in 10403S (12) is common between strains, and it is puzzling because GadT2D2 is the main contributor of acid resistance in this bacterium and yet the cells have to rely on preexisting levels of these proteins produced prior to the acid challenge. Maybe this is related to an attempt to strike a balance between stress resistance and virulence, as it is known that upregulation of acid or stress resistance mechanisms can impair virulence (3, 13–17). Furthermore, no transcriptional upregulation of the *gad* genes occurred in LO28, which could be due to the high transcription of *gadT2D2* and *gadD3* at basal levels (see Fig. S1 in the supplemental material).

The transcription of *lmo2434* or *gadD3*, as it is called in most publications, was the highest among all *gad* genes in all strains during

the whole experiment (see Fig. S1 in the supplemental material), and it showed a transcriptional upregulation in response to acid treatment in EGD-e and 10403S, which suggests a role in acid stress. This gene has previously been suggested to encode a third Glt decarboxylase (9) regulated by σ^B (7, 18, 26), but the inability of Δ*gadD1D2* to export any $GABA_e$ through GAD_e raised doubts about this (7, 9). In this study, we were able to clear these doubts by detecting a significant GAD_i activity in this mutant (Fig. 4B) and thus confirming the existence of a third Glt decarboxylase (possibly GadD3), apart from GadD1 and GadD2. We have repeatedly tried to mutate *gadD3*, without success, but in new evidence showing that this is possible (2), we continue our attempts. However, the deletions in Δ*gadD1D2* are nonpolar (7, 9), and therefore, GadD3 should be able to function synergistically with the antiporters which are intact. This suggests that GadD3 is a part of GAD_i but not of GAD_e, deviating from the model of the Glt decarboxylases cooperating with the antiporters, as described for GadB, the homologue of GadD2 in *E. coli* (4). GadD3 could also be responsible for the initiation of GAD_i at milder pH values than initiation of GAD_e acting on readily available pools of Glt_i (Fig. 1). Although the contribution of the *gadD1T1* operon to acid survival was minor, similar to GadD3, GadD1 was upregulated in EGD-e and 10403S (Fig. 5; see Fig. S1 in the supplemental material), possibly in an attempt to compensate for the low initial transcriptional levels of all Glt decarboxylases. In all strains, the transcription of *gadT1* was significantly higher than that of *gadD1*, as it has been observed previously (26).

The work described above establishes for the first time the concept of GAD_i in a revised model for the function of GAD and demonstrates its important role in the acid resistance of *L. monocytogenes* (Fig. 1). Further study of this revised model could have important implications in the understanding of the acid resistance of various bacteria but also other microorganisms that possess the GAD system and encompass all kingdoms of microbial life (fungi, yeasts, and archaea).

ACKNOWLEDGMENTS

We are grateful to our colleagues and especially to Conor Feehily in Microbiology at NUI Galway for helpful discussions and comments on the manuscript. We also thank David Cullen and David Reddington for technical support, Paul Cotter (Teagasc, Cork, Ireland) and Colin Hill (University College Cork, Cork, Ireland) for providing the *gad* mutants, Martin Cormican (University College Hospital, National University of Ireland, Galway, Ireland) for providing the clinical isolates used in this study, and Cormac Gahan (University College Cork, Cork, Ireland) for useful discussions.

This project was supported by a Science Foundation Ireland Starting Investigator Research Grant (SIRG) awarded to K.-A. G. Karatzas (09/SIRG/B1570). L. Suur was supported by an SFI-UREKA program grant (08/UR/B1350).

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