Molecular Analysis of Glutamate Decarboxylases in *Enterococcus avium*

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*Enterococcus avium* (*E. avium*) is a common bacterium inhabiting the intestines of humans and other animals. Most strains of this species can produce gamma-aminobutyric acid (GABA) via the glutamate decarboxylase (GAD) system, but the presence and genetic organization of their GAD systems are poorly characterized. In this study, our bioinformatics analyses showed that the GAD system in *E. avium* strains was generally encoded by three *gadB* genes (*gadB1*, *gadB2*, and *gadB3*), together with an antiporter gene (*gadC*) and regulator gene (*gadR*), and these genes are organized in a cluster. This finding contrasts with that for other lactic acid bacteria. *E. avium* SDMCC050406, a GABA producer isolated from human feces, was employed to investigate the contribution of the three *gadB* genes to GABA biosynthesis. The results showed that the relative expression level of *gadB3* was higher than those of *gadB1* and *gadB2* in the exponential growth and stationary phases, and this was accompanied by the synchronous transcription of *gadC*. After heterologous expression of the three *gadB* genes in *Escherichia coli* BL21 (DE3), the *K_m* value of the purified GAD3 was 4.26 ± 0.48 mM, a value lower than those of the purified GAD1 and GAD2. Moreover, *gadB3* gene inactivation caused decreased GABA production, accompanied by a reduction in resistance to acid stress. These results indicated that *gadB3* plays a crucial role in GABA biosynthesis and this property endowed the strain with acid tolerance. Our findings provided insights into how *E. avium* strains survive the acidic environments of fermented foods and throughout transit through the stomach and gut while maintaining cell viability.

**Keywords:** gamma-aminobutyric acid (GABA), GAD system, insertion-inactivation, *Enterococcus avium*, acid tolerance, glutamate decarboxylase

**INTRODUCTION**

Gamma-aminobutyric acid (GABA), a non-protein amino acid, has several important physiological effects in human including anti-anxiety effects, anti-hypertension effects, anti-inflammatory effects and growth-promoting effects (Li and Cao, 2010; Dhakal et al., 2012; Shin et al., 2014; Bajic et al., 2020; Yilmaz and Gokmen, 2020). Glutamate decarboxylase (GAD) is a key enzyme in GABA synthesis and is widely distributed among animals, plants, and microorganisms (Li et al., 2010). Lactic acid bacteria (LAB) are important GABA producers and have been isolated from fermented foods enriched with GABA (Shin et al., 2014), and some species are part of the normal intestinal
microbiome (Walter et al., 2000; Hill and Artis, 2010). In this organ, they can convert dietary glutamate to GABA, thereby providing health benefits to the host (Wu and Shah, 2017). Several Enterococcus avium strains have recently been isolated from various fermented foods, particularly East Asian fermented foods, and these strains display a high conversion rate from monosodium glutamate (MSG) to GABA, suggesting that they have the potential to be the starter organisms for GABA-rich functional food production (Tamura et al., 2010; Yang et al., 2016; Lee et al., 2017; Jo et al., 2019). Although a rare pathogen, E. avium is often present as part of the normal microbiota in the gastrointestinal tract of individuals, including infants (Birri et al., 2010; Yang et al., 2016). However, to date, the molecular organization of the GAD system in E. avium remains unclear, and its functional analysis is lacking.

To cope with acid stress, LAB and other bacterial species employ a variety of acid resistance mechanisms, including the acid tolerance response (ATR) and acid resistance (AR) systems (De Biase and Pennacchietti, 2012; Feehily et al., 2013). The ATR system requires pretreatment of log- or stationary-phase (De Biase and Pennacchietti, 2012; Feehily et al., 2013). The acid tolerance response (ATR) and acid resistance (AR) systems employ a variety of acid resistance mechanisms, including the

Determination of Gamma-Aminobutyric Acid Content in the Cultures

The concentration of GABA in the cultures was determined by high-performance liquid chromatography (HPLC) with dansyl chloride (DN5-Cl) (Sangon, China) derivatization method as described (Huang et al., 2006). Briefly, the cell culture supernatants were added at a final concentration of 10% trichloroacetic acid (TCA) to precipitate the protein. The supernatant was diluted with 0.2 M NaHCO₃ solution and derivatized with 0.4% DN5-Cl–acetone solution at 30°C for 1 h. Then, the mixture was filtered through a 0.2-μm membrane filter (Sangon, China) as samples used for HPLC. The chromatographic separation was performed with a column (Waters Xbridge BEH300 C18 4.6 × 150 mm) and detection performed at 254 nm. A gradient elution protocol with A (methanol)/B (tetrahydrofuran/methanol/50 mM pH 6.2 sodium acetate, 5:75:420, by vol.) as mobile phase was carried out at a flow rate of 0.9 ml/min (0 min 80% B, 6 min 80% B, 20 min 50% B, 21 min 0% B, 27 min 0% B, 27.1 min 80% B, 40 min 80% B)
at 28°C. GABA concentration was calculated from the integrated peak area comparing with the standard curve constructed by standard GABA (Sigma, United States) solution.

**Total RNA Extraction and Reverse Transcription PCR Assay**

The growth of *E. avium* SDMCC050406 in MRS or GMRS broth was monitored by optical density (OD) at 600 nm and pH. Total RNAs from cells collected at the early exponential growth phase (2 h, OD<sub>600</sub> = 0.25), exponential growth phase (4 h, OD<sub>600</sub> = 1.00), and stationary phase (8 h, OD<sub>600</sub> = 1.75) were extracted using an RNA Simple total RNA kit (Tiangen, Beijing, China) according to the protocols of the manufacturer. Subsequently, the extracted RNA was reverse transcribed to cDNA using the PrimeScript RT-PCR kit (TaKaRa, Japan). RT-PCR was carried out with SYBR Premix Ex Taq kit (TaKaRa, Japan) in the qTOWER3G system according to the instructions of the manufacturer. The relative expression levels of the five target genes (*gadB1*, *gadB2*, *gadB3*, *gadC*, and *gadR*) were normalized to...
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid                | Characteristics                                      | Sources or references |
|----------------------------------|------------------------------------------------------|-----------------------|
| **Strains**                      |                                                      |                       |
| *E. avium* SDMCC050406           | Wild-type strain isolated from human fecal           | This study            |
| *E. avium* SDMCC050406 A.gadB3   | Wild-type *E. avium* strain isolated from human fecal| This study            |
| *Lb. lactis* MG1363              | Plasmid-free and prophage-cures derivative of *Lb. lactis* NCO 712 | Gasson, 1983         |
| *E. coli* DH5×                   | Cloning host expression host                         | Novagen               |
| *E. coli* BL21 (DE3)             | BL21 containing PET-Duet1-B1                         | This study            |
| *E. coli* BL21/pET-Duet1-B1      | BL21 containing PET-Duet1-B1                         | This study            |
| *E. coli* BL21/pET-22b-B2        | BL21 containing PET-22b-B2                          | This study            |
| *E. coli* BL21/pET-28a-B3        | BL21 containing PET-28a-B3                          | This study            |
| **Plasmids**                     |                                                      |                       |
| pG* host9                        | Erm*, integration vector, thermosensitive replicative plasmid in LAB | Biswas et al., 1993   |
| pG* host9-Gad                    | Erm*, pG* host9 derivative, with the internal fragment of the gadB3 gene | This study            |
| pET-22b                          | Amp*, expression vector                              | Novagen               |
| pET-28a                          | Amp*, expression vector                              | Novagen               |
| pET-Duet1                        | Amp*, expression vector                              | Novagen               |
| pET-22b-B2                       | Amp*, PET-22b derivative, expression GAD            | This study            |
| pET-28a-B3                       | Kan*, pET-28a derivative, expression GAD            | This study            |
| pET-Duet1-B1                     | Amp*, PET-Duet1 derivative, expression GAD1         | This study            |

the constitutive expression of the 16S rRNA housekeeping gene at the same growth phase and were calculated according to the comparative $2^{-\Delta\Delta Ct}$ method with relative expression level of the same gene at the early exponential phase set as 1.0 (Livak and Schmittgen, 2001). The primers used in this study are listed in Table 2. All experiments including culture, RNA extraction, and RT-PCR assays were performed in triplicate independently.

**Heterologous Expression and Purification of Three Glutamate Decarboxylases**

The *gadB1*, *gadB2*, and *gadB3* genes were cloned by PCR amplification using the corresponding primer pairs (*gadB1*-Duet1 F and *gadB1*-Duet1 R for *gadB1*; gadB2-22b F and gadB2-22b R for *gadB2*; gadB3-28a F and gadB3-28a R for *gadB3*). PCR products were digested with the corresponding restriction enzymes and ligated with the vector pET-Duet1, pET-22b, and pET-28a, generating the recombinant plasmid pET-Duet1-B1, pET-22b-B2, and pET-28a-B3, respectively. After transformed into *E. coli* BL21 (DE3), the generated three recombinants were overnight grown at 37°C in LB broth containing 100 µg/ml ampicillin or 30 µg/ml kanamycin. Subsequently, 2 ml overnight cultures were diluted into 100 ml fresh LB broth with the corresponding antibiotics and regrown to an OD$_{600}$ of 0.6, and isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final content of 0.1 mM for induction for 12 h at 16°C, respectively. Cells were harvested and washed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4). Afterward, cells were resuspended in 8 ml binding buffer (20 mM sodium phosphate, 20 mM imidazole, 500 mM NaCl, pH 7.4), disrupted by ultrasonication, and centrifuged to remove the cell debris. The overexpressed GAD proteins were purified from supernatants by Ni-NTA affinity chromatograph. The columns were washed with washing buffer (20 mM sodium phosphate, 40 mM imidazole, 500 mM NaCl, pH 7.4) and the His-tagged proteins were eluted with elution buffer (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4). After that, the proteins were dialyzed with 20 mM sodium phosphate buffer (pH 7.4). The concentration and purity levels of proteins were determined by the NanoDrop 2000/2000c UV–Vis (Thermo Fisher Scientific, United States) at 280 nm with bovine serum albumin as a standard (Supplementary Figure 1). The purified proteins were boiled at 75°C for 5 min as soon as the samples were diluted in the 5 × SDS-loading buffer [250 mM Tris–HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 50% glycerin, and 5% 2-hydroxy-1-ethanethiol], and then they were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) acrylamide gel.

**Enzymatic Activity Assay**

The GAD activity was determined as described previously (Chang et al., 2017). Briefly, the enzyme reaction was carried out with 450 µl of buffer A [20 mM sodium citrate buffer (pH 4.6), 100 mM MSG, 0.1 mM pyridoxal-5’-phosphate monohydrate (PLP)] and 50 µl of 1 mg/mL purified GAD proteins. After incubation at 45°C for 30 min, the reaction was stopped by adding 500 µl of 0.2 M borate saline buffer (pH 9.0) to ensure that that production of GABA was linear and the consumption of MSG was less than 5%. The GABA content was quantified by HPLC. All the enzymatic reactions were carried out in triplicate. One unit of GAD activity was defined as the GABA amount produced by 1 mg/mL enzyme per min under optimal conditions. For the optimal temperature, the purified GADs were incubated with buffer A for 30 min at various temperatures ranging from 30 to 80°C (pH 4.5). In the same way, the optimal pH was determined with 20 mM sodium citrate buffer at pH 3.0 to 7.0. The kinetic parameters were determined with MSG (1–60 mM) as the substrate under the optimal conditions, respectively. The initial velocity of each MSG concentration was determined by measuring GABA production in the first 10 min of reaction. The kinetic constants were estimated by non-linear regression (enzyme kinetics, Michaelis–Menten) using GraphPad Prism 8.2.1. All experiments were performed in triplicate.

**Inactivation of gadB3 Gene in Enterococcus avium SDMCC050406**

The *gadB3* gene was inactivated by the temperature-sensitive pG* host9* plasmid containing erythromycin selection marker
into the active site (Biswa et al., 1993; Lu et al., 2015; Wang et al., 2016). To construct the integration vector, the 804-bp DNA fragment of the gadB3 gene was PCR amplified from the genomic DNA of E. avium SDMCC050406 with the primer pair Gadlna F3 and Gadlna R4, subsequently inserted into the vector pG + host9. The resulting recombinant plasmid pG + host9-Gad was introduced into Lc. lactis MG1363 by electroporation, and the transformants were selected on the plates containing erythromycin after incubation at 30°C for 24 h. Positive clones were selected, and the recombinant plasmid pG + host9-Gad was isolated and transformed into E. avium SDMCC050406 again by electroporation. The transformants of E. avium SDMCC050406 were grown with erythromycin to an OD600 of 0.5, and the temperature of growth shifted from 30 to 37°C (non-permissive temperature for plasmid replication) to continue the incubation for 1 h. A serially diluted solution of the transformant cultures was plated onto MRS plates with erythromycin at 37°C for 24 h. The gadB3 gene was inactivated by homologous crossing, and the mutant E. avium SDMCC050406ΔgadB3 was further verified by PCR amplification with specialized primers GadEN F1 and pG + host-F.

**Cell Survival Under Acidic Conditions**

Acid tolerance assay was followed as described with modification (Seo et al., 2015; Gong et al., 2019). Briefly, E. avium SDMCC050406 and E. avium SDMCC050406ΔgadB3 were grown in GMRS broth for 12 h when the cells were in the stationary phase and began to synthesize GABA. The cells were collected by centrifugation and washed twice with 50 mM potassium phosphate buffer (pH 7.0), followed by suspension in 50 mM potassium phosphate buffer (pH 7.0) to an OD600 of 1.0. The cells were harvested by centrifugation again and resuspended in MRS broth (pH 4.0, 3.5, and 3.0) with or without 10 mM MSG. Incubation samples were collected at 2-h intervals over 12 h and serially diluted 10-fold on the MRS agar plates to monitor bacterial survival. All experiments were performed in triplicate.

**Statistical Analysis**

All experiments were performed in triplicate. Statistical analysis was performed using unpaired two-tailed Student’s t-tests. p-values of <0.05 were considered statistically significant.

**RESULTS**

**Organization of the GAD System in Enterococcus avium**

Eighteen whole genomic sequences from E. avium strains (as of May 10, 2020) were retrieved from the GenBank database. From them, 53 GAD-encoding amino acid sequences were downloaded from the sequenced genomes of E. avium genomes. Sequence alignments suggested that the GADs fell into three independent GAD groups. GAD1 (458 amino acids; aa), GAD2 (466 aa), and GAD3 (466 aa) are encoded by gadB1, gadB2, and gadB3 genes, respectively, (data not shown). The maximum-likelihood phylogenetic tree based on the amino acid sequences of the GADs from E. avium and other species shows that a distinct relationship exists among the three GADs from E. avium (Figure 1A). The multiple sequence alignment for these GADs also showed that GAD1, GAD2, and GAD3 differ (Figure 1B). The PLP-consensus motif was located by aligning the GAD amino acid sequences and from data available at the National Center for Biotechnology Information (NCBI) [CDD Conserved Protein Domain Family: Information (NCBI) [CDD Conserved Protein Domain Family: Table 2] The primers used in this study.

| Primer name    | Primer sequence (5′–3′)                      | Restriction sites | Ligated vector |
|---------------|----------------------------------------------|-------------------|----------------|
| gadB1 F       | AACCCTGACACACGCGAGAAC                     | BamHI            | pET-Duet1      |
| gadB1 R       | TCCTTCAAGTGCGAACAGCA                      | XhoI             | pET-Duet1      |
| gadB2 F       | CATGTTACAGTGCGAACAGAG                     | NdeI             | pET-22b        |
| gadB2 R       | GATCTCGAAGATCGCGAACAGAG                   | XhoI             | pET-22b        |
| gadB3 F       | TCGGATCCACTTGGGCTGCTGCGGCC                    | NcoI             | pET-28a        |
| gadB3 R       | CTGCCTGAAAGATCGCCGGACAA                    | XhoI             | pET-28a        |
| gadC F        | TCAATGTGGCTTGCTGAC                        | EcoRI            | pG+ host9      |
| gadC R        | ATACCGCACCACAAATCTGGT                      | HindIII          | pG+ host9      |
| 16S F         | GTGACTGATGGATGGACCGC                       |                  |                |
| 16S R         | ATTCGAGGAAATGTTCCCTCTTT                    |                  |                |
| gadB3-Duet1 F | CGCCGATCCGAGATCGCATACAGAAGTGATTAGAAGACGCTCAGGGTGTGTGTGTGTT     | BamHI            | pET-Duet1      |
| gadB3-Duet1 R | GGTGATGGAGATCGGGAGAATCGAAATG               | XhoI             | pET-Duet1      |
| gadB2-22b F   | CGCGATCCGAGATCGGGAGAATCGAAATG               | NdeI             | pET-22b        |
| gadB2-22b R   | CGCGATCCGAGATCGGGAGAATCGAAATG               | XhoI             | pET-22b        |
| gadB3-28a F   | CATGCTGAGATCGGCTGATGGAATCGAAATG            | NcoI             | pET-28a        |
| gadB3-28a R   | CATGCTGAGATCGGCTGATGGAATCGAAATG            | XhoI             | pET-28a        |
| Gadlna F3     | CGGAATCTGGTACATAACAGTGC                    | EcoRI            | pG+ host9      |
| Gadlna R4     | CCAAAGCTTAAATCTCCCTGGCGAAAAA               | HindIII          | pG+ host9      |
| GadEN F1      | GGCGGAGCTAAGACGCGTGGGA                      |                  |                |
| GadEN F2      | ACTATGGGCGAGATTGGT                        |                  |                |
DOPA_deC_like; CDD Conserved Protein Domain Family: Glu-decarb-GAD (see text footnote 1) and previous reports (De Biase and Pennacchietti, 2012; Yogeswara et al., 2020). The PLP-binding domains and active site residues in the three E. avium GADs can be seen to be highly conserved, implying that all of them possess potential decarboxylation activity (Figure 1B).

The genetic organization analysis showed that the gadB3 gene is located in a three-gene cluster containing the glutamate/GABA antiporter-encoding gadC gene and the GadR transcriptional regulator-encoding gadR gene. Thus, we surmised that the three genes were probably part of the same operon (Figure 1C). Conversely, gadB1 and gadB2 were found to be located in different genomic regions and were not part of an operon. An app gene, which encodes an amino acid permease, Na/Pi cotransporter, or extra glutamate/GABA antiporter, was found next to the gadB1 gene.

Transcription Levels of the Genes of the Glutamate Decarboxylase System

Glutamate Decarboxylase is a key enzyme catalyzing the conversion of glutamate to GABA. To analyze the functional roles played by the three gadB genes in GABA biosynthesis, a GABA producer (E. avium SDMCC050406) was used to determine the active expression of GABA during the growth of this bacterium in MR5 or GMRS broth, respectively. After incubation for 6 h, the bacterial density in GMRS was significantly higher than that in the MR5 broth, and this growth was accompanied by an increased pH (Figure 2A). The relative expression levels of gadB2 and gadB3 increased along with bacterial growth, achieving ~2.00- to ~7.86-fold during the stationary growth phase, respectively, (Figure 2B). With increased gadB2 and gadB3 expression levels, the relative transcription levels of gadC and gadR were also heightened, particularly that of gadC, whose improvement was significant. On the other hand, gadB1 expression decreased with the growth phase, dropping ~0.58-fold during the stationary growth phase. These results indicated that the three GAD-encoding genes (gadB1, gadB2, and gadB3) were transcribed during the growth phase, and gadB3 was predominant among them (Figure 2B and Supplementary Figure 2). Furthermore, we confirmed the inductibility of the GAD system by acidity in E. avium SDMCC050406 (Supplementary Figure 3). Upon acid treatment, the transcription levels of the three GAD genes increased significantly.

Biochemical Properties and Kinetic Parameters of the Three Glutamate Decarboxylases

To comparatively analyze the biochemical properties of the three GADs, the gadB1, gadB2, and gadB3 genes, whose sizes were 1.377, 1.401, and 1.401 bp, respectively, were cloned from the E. avium SDMCC050406 genome. The deduced amino acid sequences were aligned with those from the aforementioned independent E. avium GAD groups. After expression in E. coli BL21 (DE3), all three purified GAD proteins were obtained.

The UV–visible spectra of the three purified GADs are shown in Supplementary Figure 1. There were only peaks at 280 nm and no obvious PLP peaks at 340 nm. Three GADs were all sized approximately about 55 kDa on SDS-PAGE (Figure 3A), and this agreed with the molecular weights deduced from the amino acid sequences. Interestingly, a minor band lower than 55 kDa by SDS-PAGE was observed in all three GADs but only when samples were boiled at temperatures above 75°C, which is in agreement with the reported anomalous mobilities of proteins on SDS-PAGE (Kurien and Scofield, 2012). Moreover, the biochemical properties of the three GADs differed at various temperatures and pH conditions (Figures 3B,C). The optimal temperature was 50°C for GAD1, 55°C for GAD2, and 60°C for GAD3. The optimal pH was 5.5 for GAD1 and 5.0 for GAD2 and GAD3. Under optimal conditions, K_m and V_max were 12.72 ± 1.47 mM and 0.20 ± 0.01 mM/min for GAD1, 8.17 ± 0.99 mM and 0.31 ± 0.01 mM/min for GAD2, and 4.26 ± 0.48 mM and 0.17 ± 0.01 mM/min for GAD3, respectively. Based on these K_m and V_max values, the k_cat/K_m was 28.83 ± 4.87 mM−1 s−1 for GAD1, 69.56 ± 3.22 mM−1 s−1 for GAD2, and 73.16 ± 3.75 mM−1 s−1 for GAD3. Therefore, although GAD3 had the highest MSG preference, its catalytic efficiency was only marginally higher than GAD2 and approximately 2.6 times that of GAD1 (Table 3).

Inactivation of the gadB3 Gene and Acid Tolerance Resistance

Because gadB3 displayed the highest relative expression level of the three genes and GAD3 displayed the highest preference for MSG, the gadB3 gene was inactivated in E. avium SDMCC050406 using the temperature-sensitive pG′ host9 plasmid to investigate the contribution played by the gadB3 gene in GABA biosynthesis (Figure 4A). The results yielded the mutant E. avium SDMCC050406ΔgadB3 (Figure 4B). To compare the GABA production levels, E. avium SDMCC050406 and SDMCC050406ΔgadB3 were grown in GMRS broth. GABA production in the wild-type SDMCC050406 strain was detected after 12 h of incubation (Figure 4C), the level of which gradually increased along with its growth. When cultured for 120 h, the GABA content in SDMCC050406 reached 1.851 ± 0.205 g/L, whereas only 0.091 ± 0.013 g/L of GABA was detected in SDMCC050406ΔgadB3 (Figure 4C), indicating that the gadB3 gene plays a main role in GABA biosynthesis.

Normal GABA production can act to increase bacterial tolerance to acid stress. To further confirm the function of GAD3, as encoded by gadB3, the cell survival of E. avium SDMCC050406ΔgadB3 was compared with that of the wild-type SDMCC050406 strain after 12 h of incubation. While the viability of the two strains did not statistically differ at pH 4.0 (data not shown), there was a significant difference at pH 3.5 and 3.0. When subjected to acid stress at pH 3.0, the cell counts for both strains decreased by 4–5 orders of magnitude after 2 h of treatment, which was not dependent on MSG (Supplementary Figure 4). The results of the pH 3.5 test clearly illustrated the role of gadB3 in acid tolerance (Figure 4D). The viable cell count for the wild-type SDMCC050406 strain was obviously higher than that of
the mutant SDMCC050406ΔgadB3, both with or without MSG after pH 3.5 treatment, indicating that SDMCC050406ΔgadB3 cells were more sensitive to acid stress than SDMCC050406 cells (Figure 4D). Therefore, the GAD3 encoded by the gadB3 gene contributed to bacterial resistance against acidity in *E. avium*.

**DISCUSSION**

The GAD system plays important roles in GABA biosynthesis and acid tolerance (De Biase et al., 1999; Cotter et al., 2005; De Biase and Pennacchietti, 2012; Feehily et al., 2014; Gong et al., 2020). Recently, most studies have focused on the distribution and biophysiological function of the GAD system in food-grade lactic acid bacteria (e.g., *L. brevis*, *L. plantarum*, *L. reuteri*, *Lc. lactis*, and *S. thermophilus*), but the GAD system in *Enterococcus* sp., which is highly abundant in the intestinal tract, has rarely been reported. Therefore, this is the first report on the molecular organization of the GAD system and its functional analysis in *E. avium*.

We investigated the gene organization of the GAD system in *E. avium* by bioinformatics analysis (Figure 1C). *E. avium* contains three distinct GAD genes, namely, *gadB1*, *gadB2*, and *gadB3*. These genes clearly differ from those of other GABA-producing species except *L. monocytogenes* (Feehily et al., 2014). Although the organization of the genes of the GAD system in *E. avium* is more similar to that of *L. monocytogenes*, their relationships and amino acid sequences are distinct (Figures 1A, B). The NCBI/Blast database alignments show that GAD1 amino acid sequence shares 83.78% identity with that of *Lactobacillus curvatus*, GAD2 shares 75.32% identity with that of *Lc. lactis*, and GAD3 shares 90.13% identity with that of *Lc. lactis*. Therefore, the genetic organization of the GAD system in *E. avium* is extremely different from the system of other LAB strains. The distinctiveness of the amino acid sequences of the three GADs implies that their enzymological properties may differ.

The PLP peak at 340 nm (at neutral pH) or 420 nm (at acidic pH) is observed in the spectrum of GADs from *E. coli* and *Brucella microti* (Pennacchietti et al., 2009; Grassini et al., 2015). On the other hand, there was no PLP peak observed in the spectrum of GADs from *E. avium* SDMCC050406. This unexpected and unusual phenomenon may be caused by the presence of the His-tag at the N-terminal end for GAD1 or at the C-terminal end for GAD2 and GAD3 (based on the cloning strategy), which can negatively affect the overall assembly of GAD and its ability to retain PLP (Gut et al., 2006; Grassini et al., 2015). At neutral-alkaline pH, the PLP interacts with a C-terminal His residue and forms the substituted aldehyde, which exhibits a characteristic absorption peak at 340 nm (Pennacchietti et al., 2009). Therefore, the presence of the His-tag is likely the cause of the absence of PLP in the three purified GADs. Heterologously expressed GADs from several *Enterococcus* species differ in the conditions required for their optimal activity. Maximal GAD activity was observed at pH 5.0–5.5 and 50–60°C for *E. avium* SDMCC050406 (Figure 3B), pH 5.5 and 45°C for *E. avium* M5, pH 4.8 and 50°C for *Enterococcus faecium* GDMMCC60203, and pH 4.6 and 45°C for *Enterococcus raffinosus* TCCCI1660 (Chang et al., 2017; Lee et al., 2017; Yang et al., 2020). These pH and temperature values are not the optimal conditions for bacterial growth; therefore, GABA biosynthesis might be affected when these GABA producers are incubated under normal conditions (Wu and Shah, 2017). In addition, although *E. avium* SDMCC050406 GAD3 has a lower $K_m$ than GAD1 and
GAD2 under optimal conditions, $K_m$ has an intermediate value between that of *E. avium* M5 (3.26 ± 0.21 mM) and *E. raffinosus* TGCC11660 (5.26 µM) (Chang et al., 2017; Lee et al., 2017). These different enzymatic properties might be related to the varied amino acid sequences and conformational structures of these GADs (Wu and Shah, 2017).

In the present study, although *gadB* gene transcription was found to begin during the exponential growth phase (Figure 2B), GABA production was initially detected during the stationary phase (Figure 4C). This indicates that the enzymatic activities of the GADs limit GABA biosynthesis. The optimal growth temperature for *E. avium* SDMCC050406 is 37°C; however, GAD2 and GAD3 exhibit more than 40% enzyme activity at 37°C, whereas GAD1 is less than 20% (Figure 3B). During the stationary growth phase of *E. avium* SDMCC050406, the pH dropped to 4.3 (Figure 2A). GAD2 and GAD3 possess
TABLE 3 | Comparison of properties of three GADs purified from E. avium SDMCC050406.

|          | Predicted molecular weight (kDa) | Optimal temperature (°C) | Optimal pH | $V_{\text{max}}$ (mM/min) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|----------|----------------------------------|--------------------------|------------|-----------------|-----------|----------------------------------|
| GAD1     | 55                               | 50                       | 5.5        | 0.20 ± 0.01     | 12.72 ± 1.47 | 28.83 ± 4.87                    |
| GAD2     | 55                               | 55                       | 5.0        | 0.31 ± 0.01     | 8.17 ± 0.48  | 69.56 ± 3.22                    |
| GAD3     | 55                               | 60                       | 5.0        | 0.17 ± 0.01     | 4.26 ± 0.48  | 73.16 ± 3.75                    |

FIGURE 4 | Effect of gadB3 gene in GABA biosynthesis and acid tolerance. (A) Scheme for gadB3 gene inactivation in E. avium SDMCC050406. (B) Agarose gel electrophoresis of DNAs, lane M1, DL10,000 DNA Marker (TaKaRa, Japan), size range (10,000, 7,000, 4,000, 2,000, 1,000, 500, and 250 bp); lane 1, the product of pG$^{+}$host9-Gad digested by EcoRI and Hind III (TaKaRa, Japan); lane M2, DL5,000 DNA Marker (TaKaRa, Japan), size range (5,000, 3,000, 2,000, 1,500, 1,000, 750, 500, 250, and 100 bp); lane 2 to lane 4, PCR products with the primer pair GadEN F1 and pG$^{+}$host-F using the template DNA extracted from three E. avium SDMCC050406$^{+}$gadB3 clones. (C) GABA production of E. avium SDMCC050406 (black column) and E. avium SDMCC050406$^{+}$gadB3 (gray column) cultured in GMRS. Asterisk represented the statistical significance of GABA. (D) Viable cell counts of E. avium SDMCC050406 (black line) and E. avium SDMCC050406$^{+}$gadB3 (gray line) under acidic condition of pH 3.5. The cells were harvested at 2-h intervals over 12 h. Error bars represented standard errors from three replicate experiments. The lowercase represented the statistical significance of viable counts. *p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001.

Due to the gene locus, high transcriptional levels, and optimal enzymatic parameters, GAD3 encoded by gadB3 was selected to functionally investigate GABA synthesis and acid tolerance in E. avium (Figures 1C, 2B, 3B, 4C). The mutant E. avium SDMCC050406$^{+}$gadB3 strain had lower GABA production and viability in acid conditions. Interestingly, a small amount of GABA (0.091 ± 0.013 g/L) was still produced by SDMCC050406$^{+}$gadB3, and its slight increase in yield along with its prolonged growth suggests that gadB1 and gadB2 genes might functionally substitute for the lack of the gadB3 gene (Figure 4C). Future studies on knock-out (KO) strains for gadB1 and gadB2, or on the KO strain for gadB3 complemented with a more than 60% enzyme activity at pH 4.0, whereas at this pH, GAD1 enzymic activity abruptly decreased and was almost lost (Figure 3B). GAD3 displays the highest preference for MSG, but its catalytic efficiency is only marginally higher than that of GAD2 and approximately twice that of GAD (Table 3). This suggests that GAD2 and GAD3 are the main enzymatic forms involved in the conversion of glutamate to GABA in vivo, and this is particularly true for GAD3.

![Diagram](https://example.com/diagram.png)
plasmid expressing gadB3, will further confirm the contribution of gadB3 in GABA production and acid tolerance. In fact, being this the first report on the molecular manipulation of E. avium, the production of the KO strains for gadB1 and gadB2, as well as the complementation of the KO strain for gadB3, could not be achieved. Nevertheless, our agar gel electrophoresis and sequencing results on gadB1 and gadB2 gene PCR products confirmed that gadB1 and gadB2 were steadily maintained in the insertion plasmid, thus ruling out the possibility of production of double/triple KO strains.

Although E. avium SDMCC050406 produces a low level of GABA (1.851 ± 0.205 g/L, Figure 4C) compared with other the E. avium strains isolated from fermented food and plant leaves (Tamura et al., 2010; Lee et al., 2017), as an intestinal isolate, GABA synthesis in this strain could improve bacterial colonization and bacterial survival in the intestinal tract (Figures 4C,D; Small and Waterman, 1998; Shin et al., 2014; Lyu et al., 2018; Gong et al., 2019). The GAD system, which is one of the most efficient bacterial AR mechanisms in withstanding acid stress (Occhialini et al., 2012; Damiano et al., 2015; Wu et al., 2018; Gong et al., 2019), cannot contribute to acid resistance at pH ≤ 3.0 in E. avium due to the low catalytic activity of the three GADs in this condition (Figure 3B). Therefore, in the present study, inactivating the gadB3 gene directly led to a great decrease in GABA production and bacterial survival under acid stress at pH 3.5 (Figures 4C,D). However, the loss of viability from all the strains at pH 3.0 was not dependent on the MSG, further illustrating the weak roles of the GAD system and other anti-acid mechanisms of E. avium SDMCC050406 in extremely acidic environments (Supplementary Figure 4). Thus, the GAD system in E. avium provides tolerance to acidic environments at pH > 3.0.

In summary, we have detailed the unique distribution of the GAD system genes in E. avium, and the gadB3 gene was experimentally confirmed to be an indispensable factor in GABA biosynthesis. Our findings provide novel insights into the GAD system and GABA biosynthesis in this species.

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**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

XG and JK contributed conception and design of the study. XG, JZ, RZ, and RY performed the experiments. XG, TG, JZ, RZ, and JK wrote and revised the manuscript. All authors read and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.691968/full#supplementary-material
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