



Deciphering Functions of a Putative Histidinol Dehydrogenase in *Acidovorax citrulli* by Phenotypic and Proteomic Analyses

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(Received on March 7, 2025; Revised on March 25, 2025; Accepted on March 27, 2025)

Acidovorax citrulli (*Ac*) is a Gram-negative phytopathogenic bacterium causing bacterial fruit blotch (BFB) on cucurbit crops, specifically in the watermelon industry. However, cultivars of watermelon that are resistant to *Ac* have not been identified. Therefore, virulence factors/mechanisms in *Ac* must be characterized to develop alternative control strategies. Functions of a histidinol dehydrogenase, which is an essential enzyme for histidine biosynthesis, remain elusive in *Ac*. This study aims to elucidate the roles of histidinol dehydrogenase in *Ac* (HisDac) using phenotype assays and proteomic analysis. The virulence of a mutant lacking a histidinol dehydrogenase, *hisDac:Tn5*(EV), was diminished in germinated-seed inoculation and leaf infiltration assays,

and the bacterium was impossible to grow without histidine in minimal media. However, treatment with exogenous histidine completely restored the virulence of the mutant on watermelon and its growth in minimal media, demonstrating that HisDac is required for histidine biosynthesis, which contributes to virulence and growth. The comparative proteomic analysis indicates that HisDac is involved in not only amino acid metabolism but also other biological mechanisms, including cell wall/membrane/envelope functions. This suggests that HisDac may have pleiotropic effects. It was also confirmed that when *Escherichia coli* was incubated with *Ac* strains in water, the population level of *E. coli* increased in the presence of the mutant but not in the presence of the wild-type. This study leads to new insights regarding enzymes related to the production of primary metabolites and provides a promising target to discover an anti-virulence reagent to control BFB.

Keywords : *Acidovorax citrulli*, bacterial fruit blotch, histidinol dehydrogenase

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Handling Editor : Chang-Jin Park

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Acidovorax citrulli (*Ac*) is a Gram-negative, rod-shaped, seed-borne bacterium known to be the causative agent of bacterial fruit blotch (BFB), which affects cucurbit crops, including watermelon (*Citrullus lanatus*), worldwide (Schaad et al., 1978; Willems et al., 1992). BFB was first identified in the United States in 1989 and has since caused significant economic losses in watermelon and melon production, elevating its importance in agriculture (Latin and Rane, 1990). BFB symptoms can occur at all stages of watermelon growth and can appear on any part of the plant (Rahimi-Midani and Choi, 2020). The initial symptoms in

watermelon seedlings infected with *Ac* occur with water-soaked lesions on the cotyledons, followed by gradual wilting (Burdman et al., 2005). Additionally, the surface of fruits infected by *Ac* develops water-soaked lesions, which eventually turn into dark olive-colored lesions, causing the flesh to decay (Latin and Hopkins, 1995). *Ac* strains can be divided into two major groups based on genetic and biochemical analyses (Burdman et al., 2005; Feng et al., 2009; Walcott et al., 2004). These two groups exhibit distinct capabilities due to differences in genetic backgrounds and biological mechanisms. Group I strains show high virulence to various cucurbits, while group II strains exhibit high virulence specifically to watermelon among other cucurbits (Zivanovic and Walcott, 2017). However, to date, cultivars/lines of watermelon with resistance to *Ac* have not been developed, posing challenges in the efficient management of BFB (Burdman and Walcott, 2012). Therefore, to effectively control BFB, it is important to characterize virulence factors and related mechanisms of *Ac*.

In this context, various studies have been conducted to identify the diverse virulence factors and mechanisms of *Ac*. Similar to other Gram-negative plant pathogenic bacteria, type II, III, and VI secretion systems were identified as essential mechanisms for the full virulence of *Ac* (Tian et al., 2015; Zhang et al., 2018). Genes associated with *Ac* type IV pili have also been found to influence not only virulence but also twitching motility and biofilm formation (Bahar et al., 2009). Additionally, quorum sensing, a communication system in bacteria, was identified as the virulence mechanism in *Ac* (Johnson and Walcott, 2013). Recent studies have shown that the putative glycerol-3-phosphate dehydrogenase, pyridoxal phosphate-dependent aminotransferase, and YggS family pyridoxal phosphate-dependent enzymes are essential for *Ac* pathogenicity and can induce changes in various *Ac* phenotypes (Heo et al., 2023a; Kim et al., 2021; Lee et al., 2021). However, the current research results alone are not sufficient to fully understand the virulence mechanisms of *Ac*. Therefore, further research is needed to elucidate virulence-related mechanisms, leading to the development of strategies to efficiently control BFB.

Histidine is one of the 21 proteinogenic amino acids involved in protein synthesis. It is synthesized through a similar multistep pathway in plants and microorganisms, but not in animals (Alifano et al., 1996). The histidine biosynthesis pathway involves a series of 10 enzymatic reactions that transform ATP and 5-phosphoribosyl-1-pyrophosphate substrates into histidine. Histidinol dehydrogenase is a NAD-linked four-electron dehydrogenase required for histidine biosynthesis and catalyzes the conversion of his-

tidinol to histidine in two sequential reactions (Barbosa et al., 2002; Grubmeyer et al., 1989; Teng and Grubmeyer, 1999). In *Xanthomonas oryzae* pv. *oryzicola*, two genes for the histidine biosynthesis pathway have been shown to affect pathogenicity (Su et al., 2018). Additionally, the virulence of *X. oryzae* pv. *oryzae* was reduced when histidinol dehydrogenase was disrupted (Li et al., 2019). However, there are currently no reports on the effect of histidinol dehydrogenase on *Ac*. Additionally, no omics studies have been conducted to understand histidinol dehydrogenase in relation to biological mechanisms in plant pathogenic bacteria.

This report aimed to elucidate the functions of histidinol dehydrogenase in *Ac* strain KACC17005 belonging to group II (Park et al., 2017). First, an *Ac* mutant library was generated. Through Tn5-insertional mutant library screening of *Ac*, we identified one mutant that failed to cause BFB on watermelon. In addition to employing two different virulence tests, phenotypic alteration of the mutant was investigated. To predict and characterize histidinol dehydrogenase in *Ac* (HisDAc) functions in *Ac*, a comparative proteomic analysis, following clusters of orthologous groups (COGs) analysis, was performed.

Materials and Methods

Bacterial strains and growth conditions. The *Ac* group II strain KACC17005, annotated for the full genome sequence (Park et al., 2017), was used as the wild-type strain in this study. *Ac* strains were grown in TSB (tryptic soy broth soybean-casein digested, 30 g/L) and M9 (47.7 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 22 mM KH_2PO_4 , 8.6 mM NaCl, 18.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , and 20 mL of 20% glucose in 1 L) media at 28°C. *Escherichia coli* strain EC100D was used to clone the Tn5-inserted plasmid and identify the Tn5 insertion site. *E. coli* strain DH5 α was used for gene cloning and the construction of a complemented plasmid. *E. coli* strains were grown in Luria Bertani (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl) broth medium at 37°C. Appropriate antibiotics were added to the media for selection at the following final concentrations: rifampicin, 50 $\mu\text{g/mL}$; kanamycin, 50 $\mu\text{g/mL}$; gentamycin, 10 $\mu\text{g/mL}$; and ampicillin, 100 $\mu\text{g/mL}$.

Selection of *hisDAc:Tn5* by screening the Tn5 insertional mutant library and creation of the complemented strain. To select virulence-deficient mutants, the Tn5 mutant library was screened using a previously established protocol (Heo et al., 2023b). After selecting the mutant, the gene, which was disrupted by Tn5, was identified using the

manufacturer's protocol (Lucigen, Middleton, WI, USA). Next, it was confirmed that Tn5 was inserted into a putative histidinol dehydrogenase in *Ac* (*hisDAc*, accession no. ATG95289); thus, the mutant was named as *hisDAc:Tn5*. To create a complemented strain, the open reading frame of the *hisDAc* gene was amplified using *hisDAc*-specific primers (forward: 5'-ctcgagatgaaattaatagctgctcc-3' and reverse 5'-aagcttcgtggtggtggtggcgcttcaaccgcctcctcgca-3'). The amplified DNA fragment was ligated into a pGEM-T easy vector (Promega, Madison, WI, USA) to construct the pGEM-T-*hisDAc* plasmid and confirmed by Sanger sequencing. Next, the cloned gene was excised using restriction enzymes (*Xho*I and *Hind*III) and ligated into pBBR1-MCS5, which contains the LacZ promoter for gene expression (Kovach et al., 1995), generating pMCS5-HisDAc. The generated plasmids were then transferred into *hisDAc:Tn5* by electroporation, creating the complemented strain *hisDAc:Tn5*(HisDAc). To avoid side effects from the pBBR1-MCS5 vector, the empty vector was introduced into the wild type and mutant, generating *Ac*(EV) and *hisDAc:Tn5*(EV), respectively. Supplementary Table 1 lists the bacterial strains and plasmids used in this study.

Comparison of predicted protein structures and amino acid sequences. The putative protein 3D structures of HisDAc and its homolog were predicted by inputting amino acid sequences obtained from the National Center for Biotechnology (NCBI) into the Iterative Threading ASSEMBly Refinement (I-TASSER) program (Roy et al., 2010). The predicted models were then visualized by the PyMOL program (Molecular Graphics System; San Carlos, CA, USA). Additionally, the amino acid sequence similarity of HisDAc and its homologs was compared using the Clustal Omega program (EMBL-EBI, Hinxton, Cambridgeshire, UK).

Virulence test. Two inoculation methods were used to test virulence with watermelon (*Citrullus lanatus* var. *vulgaris* line SBA). Firstly, the germinated-seed inoculation was conducted as described previously (Lee et al., 2022). Specifically, 10 germinated seeds per strain were placed in a petri dish containing 10^6 colony-forming units (CFUs)/mL of the inoculum and incubated for 1 h at 22°C. Afterward, the seeds infected by *Ac* strains were planted and moved to a controlled environmental growth room maintaining 70% relative humidity and a 16/8 h day/night photoperiod. The disease severity of the infected plants was evaluated using a 0-2 scale (0, no symptoms; 1, water-soaked region [spot]; 2, seedling wilt) and calculated as: [(Numbers of no symptom seedlings) $\times$ 0 + (Numbers of seedlings with water-

soaked regions) $\times$ 1 + (Number of wilted seedlings) $\times$ 2]/(Total number of seedlings)] for 7 days. This assay was performed at least three times. Secondly, leaf infiltration was performed following a previously reported protocol (Lee et al., 2022). The first and second true leaves at the four-true-leaf stage were infiltrated by needleless syringes containing approximately 10^5 CFU/mL of the inoculums. Additionally, to test the complementary effect of exogenous histidine in the inoculum, 50 or 100 mM of histidine was added to the bacterial suspension. Next, the infected plants were grown in the growth room for 8 days. Two leaf discs were collected from the leaves for counting CFUs and inoculated using cork borers (0.4 cm in diameter). The CFUs on tryptic soy agar (TSA) medium were then measured from the collected discs 0, 2, 4, 6, and 8 days after inoculation. Three biological replicates were used in at least three independent experiments.

Auxotrophic and growth assay. The growth curves of the *Ac* strains were established in TSB and M9 media with or without 2 mM histidine. *Ac*(EV), *hisDAc:Tn5*(EV), and *hisDAc:Tn5*(HisDAc) were grown on TSA medium and washed once with sterile distilled water (SDW). The washed bacterial cells were then adjusted to an OD_{600nm} of 0.3 (about 10^8 CFU/mL). For the growth analysis of the *Ac* strains in TSB medium, the adjusted bacterial cells were rediluted with TSB medium to 10^5 CFU/mL and incubated for 5 days in a 28°C shaking incubator (220 rpm). The OD values were measured every 12 h using a spectrophotometer. For the growth analysis of the *Ac* strains in M9 media, the bacterial cells were washed three times with sterilized water and adjusted to an OD_{600nm} of 0.5 using sterilized water. The adjusted cells were then diluted to an OD_{600nm} of 0.05 in M9 and M9 media with or without histidine (2 mM) and incubated in a 28°C shaking incubator. Afterward, the OD values of the bacterial suspensions were monitored every 24 h for 8 days. At least three independent experiments with three biological replicates were performed.

A label-free shotgun proteomic analysis. *Ac* and *hisDAc:Tn5* with three biological replicates (six samples total) were used for proteomic analysis. Sample preparation, protein extraction, and quantification were conducted according to a previously published protocol (Heo et al., 2023b). The *Ac* strains were harvested at an OD_{600nm} of 0.6 and the cells were lysed by Ultrasonic Processor (Colo Parmer, Vernon Hills, IL, USA) in a lysis buffer (6 M Guanidine HCl, 10 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.8). After collecting the supernatants, concentrations of the total soluble proteins were determined using

a bicinchoninic acid assay kit (Thermo Fisher Scientific, Rockford, IL, USA). One milligram of the proteins from each sample was then alkylated in the presence of 20 mM dithiothreitol. After precipitation by trichloroacetic acid, the samples were dissolved in ammonium bicarbonate (50 mM, pH 7.8). Peptide generation was conducted with trypsin and the tryptic-digested proteins were then cleaned using Sep-Pak Vac 1 cc tC18 Cartridges (Waters, Milford, MA, USA). Afterward, 1 µg of the peptides was analyzed using split-free nano-LC (EASY-nLC II, Thermo Fisher Scientific, Bremen, Germany) connected to the LTQ Velos Pro instrument (Thermo Fisher Scientific) in the nanospray ion mode. Next, six data-dependent tandem mass spectrometry (ms/ms) scans were conducted in the 300–2,000 m/z mass range to obtain full mass spectra. Dynamic exclusion was permitted with 1 repeat count, 0.5 repeat duration, and 3-min elimination duration. Six of the most intense ions from the full mass scan were then collected and examined within the linear ion trap of the centroid mode.

For protein comparisons, the identification and quantification of proteins and peptides were conducted according to a previously published method (Heo et al., 2023b). First, the derivation spectra obtained from liquid chromatography-tandem mass spectrometry (LC-MS/MS) were verified and quantified using the SEQUEST search algorithm and Thermo proteome discoverer (Thermo Fisher Scientific). The search for spectra information was then performed using the NCBI database for *Ac* strain KACC17005. A target-decoy strategy was used to improve reliability (Elias and Gygi, 2007). In this analysis, two missed cleavages were allowed. The peptides showed a 0.01 false discovery rate and 100 ppm precursor mass exactness with probability scores of at least 20. Next, the identified proteins were reanalyzed with Scaffold 4 (Proteomic software, Portland, OR, USA), which was used for the comparison of differentially abundant proteins in *Ac* and *hisDac:Tn5*. After removing the decoy proteins, the number of detected peptide spectra matches (PSMs) was used for the comparison analysis (Choi et al., 2008). The PSMs of each protein were normalized against the total PSMs of each biological replicate. Subsequently, the average values from three biological replicates of the PSMs were compared to identify proteins that were differentially abundant (more than 2-fold). Identified, differentially abundant proteins were classified using COG analysis (Tatusov et al., 2000). Student's *t*-test ($P < 0.05$) was performed for statistical analysis.

Interspecies co-incubation assay. The interspecies assay was modified from a previous protocol (Fei et al., 2022). *E. coli* DH5α, which carries the pGEM-T easy vector for

counting cell numbers, was used as an indicator. First, the bacterial strains were washed once with SDW. *Ac* strains and DH5α were then adjusted to an OD_{600nm} of 1.5 and 2.0, respectively, in SDW. Next, the *Ac* strains and DH5α were mixed at a ratio of 20:1 and then incubated for 2 and 3 h at 28°C. SDW was used as a negative control. After co-incubation, the mixtures were serially diluted and dropped onto LB medium plates containing ampicillin. Finally, the number of colonies was counted to calculate the survivability of the indicator. At least four independent experiments with three biological replicates were performed in this assay.

Statistical Analysis. To verify the statistically significant difference, the Student's *t*-test and one-way analysis of variance with Turkey HSD^{ab} were employed by the SPSS 12.0K software (SPSS Inc., Chicago, IL, USA). A *P*-value below 0.05 was considered a statistical difference.

Results

HisDac is a histidinol dehydrogenase. By screening a Tn5-insertional library generated using *Ac* strain KACC17005 (group II), one mutant that did not cause BFB was identified. One gene (accession no. ATG95289) disrupted by Tn5 in the mutant was annotated as a histidinol dehydrogenase. The deduced amino acid sequence of ATG95289 showed high homology with those of other Gram-negative bacteria (Fig. 1A). Specifically, ATG95289 exhibited 100%, 66.59%, and 67.51% similarity with histidinol dehydrogenases in group I *Ac* strain AAC00-1 (accession no. ABM31622), *E. coli* (accession no. MRF39866), and *Pseudomonas* sp. SGAir0191 (accession no. AUA34327), respectively. A putative 3D structure of ATG95289 was also very similar to that of MRF39866 in *E. coli* (Fig. 1B and C). From these results, ATG95289 was confirmed to be a histidinol dehydrogenase and was named *hisDac* (histidinol dehydrogenase in *Ac*).

HisDac is indispensable for virulence in *Ac*. To confirm whether HisDac is involved in the virulence of *Ac*, a virulence test of *Ac*(EV), *hisDac:Tn5*(EV), and *hisDac:Tn5*(HisDac) was conducted using germinated-seed inoculation and leaf infiltration. After inoculating the germinated watermelon seeds with *Ac*(EV), *hisDac:Tn5*(EV), and *hisDac:Tn5*(HisDac), the disease severity was checked for 7 days (Fig. 2A). Seedlings infected with *Ac*(EV) generally showed symptoms of spotting and wilting starting 3 days after inoculation (DAI) and the disease severity reached 2 at 7 DAI. However, the plants inoculated with *hisDac:Tn5*(EV) showed little spots

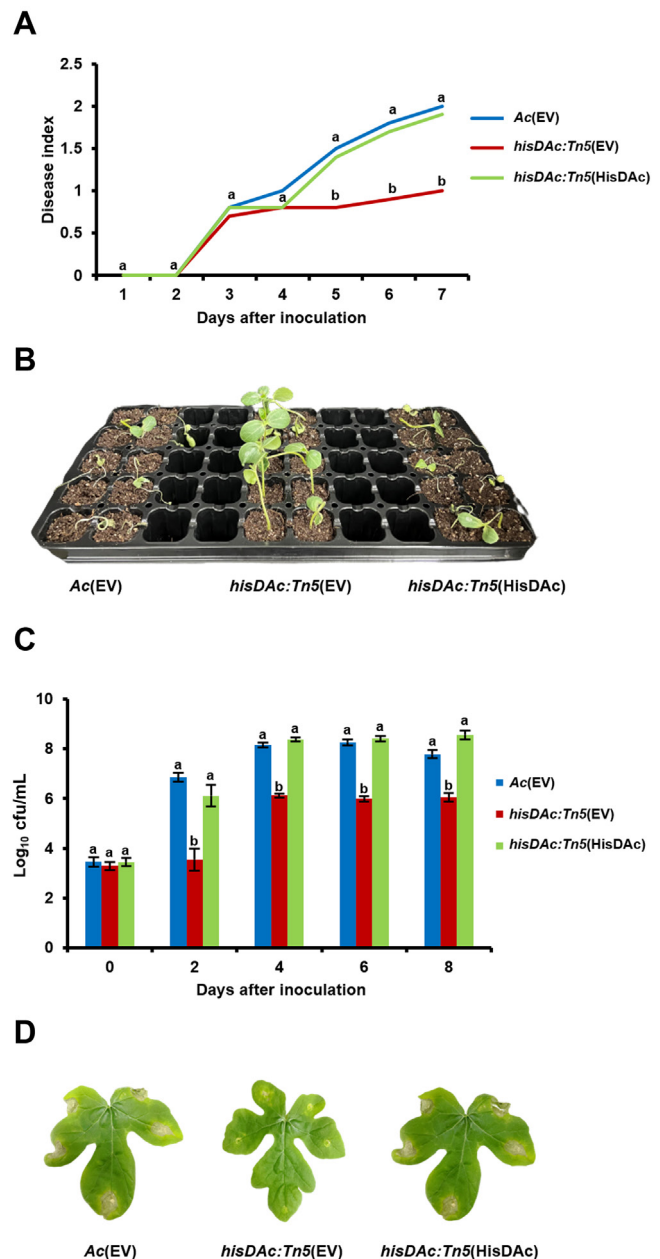


Fig. 2. Virulence assays of *Acidovorax citrulli* (Ac) strains using germinated-seed inoculation and leaf infiltration methods on watermelon. (A) The graph indicates disease severity using the disease index. Disease index: $[(\text{Number of no symptoms}) \times 0 + (\text{Number of spots}) \times 1 + (\text{Number of wilting}) \times 2] / \text{Total number of plants}$. (B) The photograph showing disease symptoms was taken 7 days after seed-germinated inoculation. (C) The bacterial growth of the infected leaves was examined by the colony counting method for 8 days. (D) The photographs from the leaf infiltration were taken 8 days after inoculation. Error bars indicate the standard error of means. The different letters indicate statistical differences from ANOVA ($P < 0.05$) with Turkey HSD^{ab}.

and wilt symptoms compared to the wild-type strain and the disease index remained 1 at 7 DAI. In addition, the complemented strain, *hisDAC:Tn5*(HisDAC), restored its virulence to that of the wild-type strain (Fig. 2A and B), indicating that there is no polar effect by the insertion of Tn5. In addition, the leaf infiltration analysis also showed similar results to those in the germinated-seed inoculation method. It was confirmed that the bacterial population on the leaves inoculated with *hisDAC:Tn5*(EV) was significantly lower than that of the leaves infected with both *Ac*(EV) and *hisDAC:Tn5*(HisDAC) during the observation period (Fig. 2C). Leaves inoculated with *Ac*(EV) and *hisDAC:Tn5*(HisDAC) displayed the typical necrotic symptoms, whereas those inoculated with the mutant did not (Fig. 2D).

Histidine is essential for *hisDAC:Tn5* growth. Because *hisDAC* encodes a putative histidinol dehydrogenase involved in histidine biosynthesis in bacteria, we carried out an auxotrophic assay regarding histidine requirement. Firstly, the growth curve of *Ac*(EV), *hisDAC:Tn5*(EV), and *hisDAC:Tn5*(HisDAC) in the rich medium, TSB, was investigated and there was no difference in growth between the three strains (Fig. 3A). Additionally, the growth of *Ac*(EV), *hisDAC:Tn5*(EV), and *hisDAC:Tn5*(HisDAC) in M9 medium with or without histidine was investigated for 8 days. The *Ac*(EV) and *hisDAC:Tn5*(HisDAC) populations continuously increased during the observation period, but that of *hisDAC:Tn5*(EV) did not (Fig. 3B). However, the growth of *hisDAC:Tn5*(EV) was dramatically halted without histidine. Next, the growth of the three strains in an M9 medium supplemented with histidine was investigated. The growth of *hisDAC:Tn5*(EV) was restored to the levels of *Ac*(EV) and *hisDAC:Tn5*(HisDAC). Furthermore, there was no statistical difference in the growth of the three strains (Fig. 3C).

Replenishment of histidine restores *hisDAC:Tn5* virulence. To determine whether histidine supplementation restores the virulence of *hisDAC:Tn5*(EV), the virulence of each strain was tested using a bacterial suspension supplemented with histidine. Because the leaf infiltration assay is analyzed by counting CFUs to obtain quantitative data, we performed the leaf infiltration assay rather than the germinated-seed inoculation. First, the inoculum of each strain without histidine was tested. Similar to previous results (Fig. 1C), the leaves infected by *hisDAC:Tn5*(EV) were much lower than those infected by *Ac*(EV) and *hisDAC:Tn5*(HisDAC) during the observation period (Fig. 4A). In addition, the leaves inoculated with

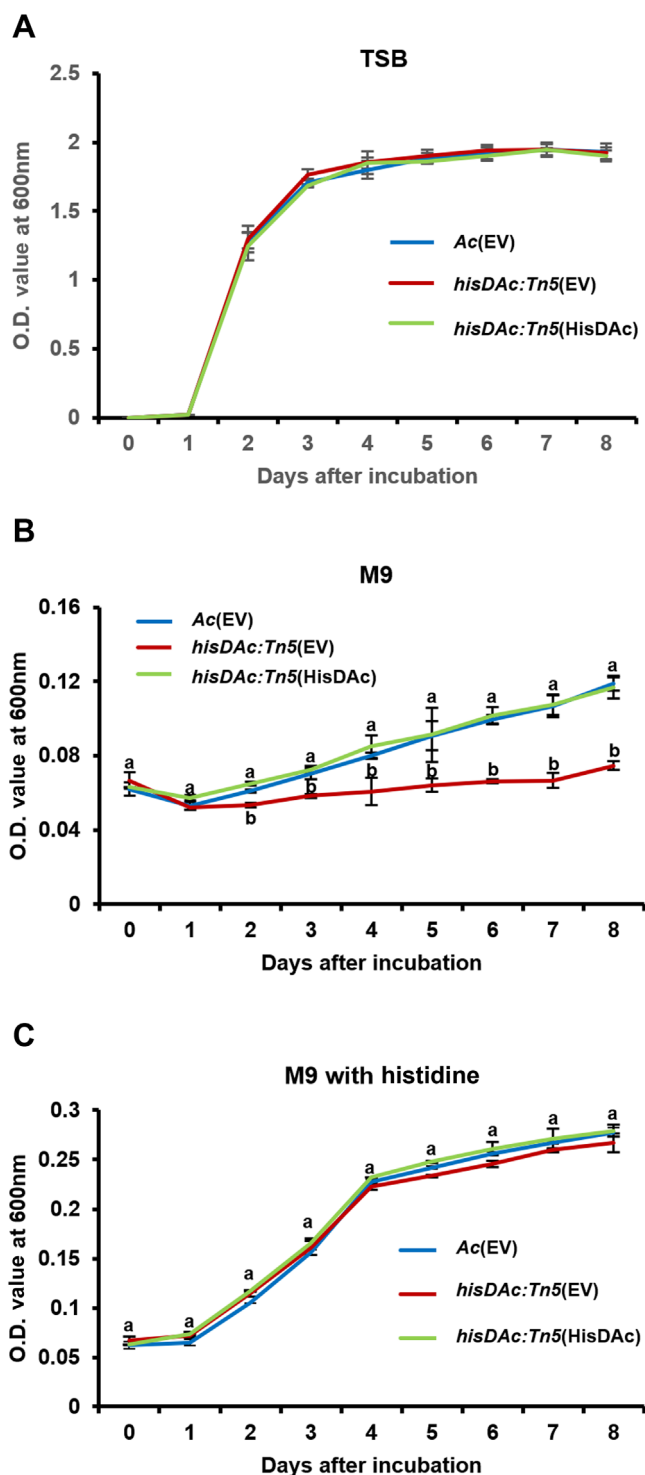


Fig. 3. Histidine auxotrophic assay. The growth of *Acidovorax citrulli* (*Ac*) strains in (A) TSBM9 medium (B) without 2 mM histidine or (C) with 2 mM histidine was determined by measuring OD values at 600 nm using a spectrophotometer. Error bars indicate standard deviations. The different letters indicate statistical differences from ANOVA ($P < 0.05$) with Turkey HSD^{ab}.

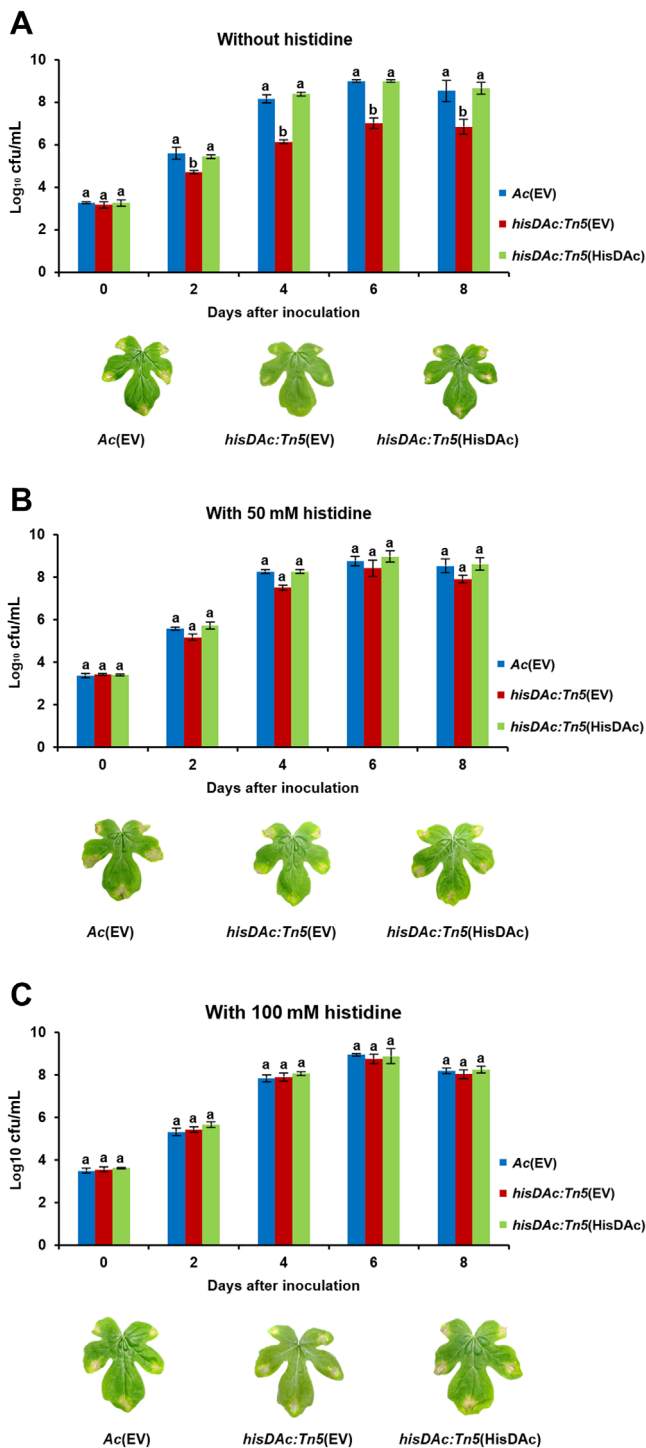


Fig. 4. A complementary effect of exogenous histidine in the inoculums. Bacterial suspensions were prepared with (A) absence of, (B) 50 mM, and (C) 100 mM histidine. In the leaf infiltration assay, bacterial growth was measured by the colony counting method. Error bars indicate the standard error of means. Different letters indicate statistical differences from ANOVA ($P < 0.05$) with Turkey HSD^{ab}. The photographs were taken 8 days after inoculation.

hisDAc:Tn5(EV) showed fewer symptoms compared to those inoculated with *Ac(EV)* and *hisDAc:Tn5(HisDAc)*. When the inoculums were prepared with 50 mM histidine, the virulence of the mutant population was restored (Fig. 4B). Although the mean values of the mutant were slightly lower than those of the wild-type and complemented strains, there were no statistical differences among the three strains. Additionally, the leaves infected by *hisDAc:Tn5(EV)* showed similar symptoms to those inoculated with *Ac(EV)* and *hisDAc:Tn5(HisDAc)*. Moreover, when 100 mM histidine was supplemented into the bacterial inoculums, the CFU values and disease symptoms from the leaves infected by the mutant were fully restored to those of the *Ac(EV)* wild-type and complemented strains (Fig. 4C). These results demonstrate that sufficient supplementation of exogenous histidine can restore *hisDAc:Tn5(EV)* virulence.

Comparative proteomic analysis. HisDAc is essential for histidine biosynthesis. Particularly, bacterial growth in media and plants was recovered by exogenous histidine addition. Kim et al. (2020) reported that a chorismate mutase/prephenate dehydratase is required for phenylalanine biosynthesis and is involved in other cellular mechanisms in *Ac*. To predict mechanisms related to HisDAc, comparative proteomic analysis was performed using the *Ac* and *hisDAc:Tn5* strains. A total of 1,031 and 1,086 proteins were commonly detected by LC-MS/MS in three biological replicates of *Ac* and *hisDAc:Tn5*, respectively (Supplementary Table 2), and the commonly identified proteins were subjected to comparative analysis. The number of proteins detected only in *Ac* and *hisDAc:Tn5* was 63 and 53, respectively, and the number of proteins detected more abundantly (over 2-fold) in *Ac* and *hisDAc:Tn5* was 4 and 1, respectively (Fig. 5A). In addition, the differentially abundant proteins in the comparison between *Ac* and *hisDAc:Tn5* were categorized using COG analysis (Fig. 5B, Supplementary Tables 3 and 4). In most categories, the number of proteins detected in the wild-type strain was higher than those in the mutant. In addition, the number of proteins found in the mutant belonging to groups O (post-translational modification, protein turnover, and chaperones), P (inorganic ion transport and metabolism), and S (function unknown) was significantly higher compared to those in the wild-type strain. In the COG groups, except for group S (function unknown), group M (cell wall/membrane/envelope biogenesis) showed the highest protein number (Fig. 5B). In addition, seven proteins in group M were related to the type VI secretion system (T6SS) (Supplementary Tables 3 and 4).

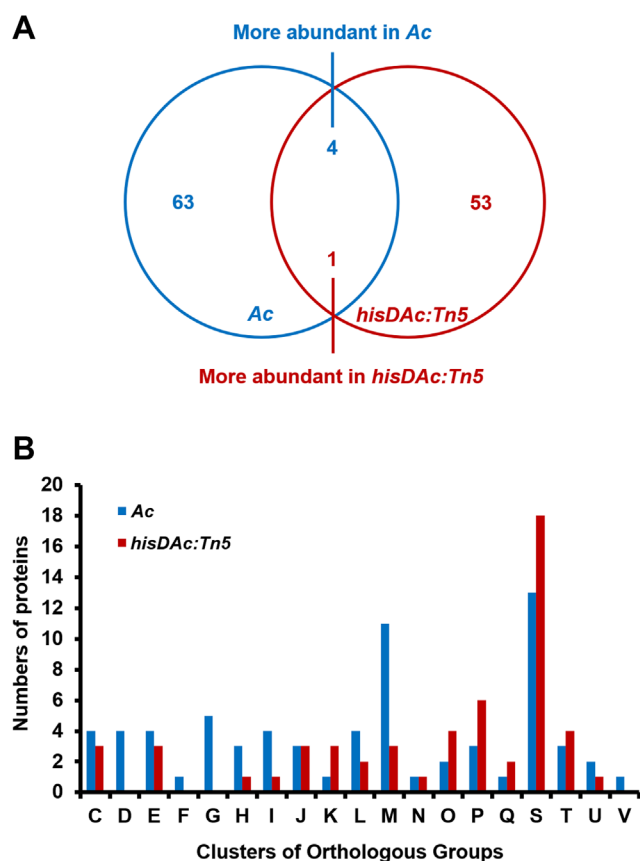


Fig. 5. A label-free shotgun, comparative proteomic analysis between *Acidovorax citrulli* (*Ac*) and *hisDac:Tn5*. (A) Venn diagrams display the number of proteins identified from comparative analysis. A total of 63 and 53 proteins were uniquely detected in wild-type *Ac* and the mutant lacking histidinol dehydrogenase, *hisDac:Tn5*, respectively. A total of 4 and 1 protein were differentially (>2-fold) abundant in *Ac* and *hisDac:Tn5*, respectively. (B) Classification of differentially abundant proteins using clusters of orthologous groups. C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification, protein turnover, and chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; S, function unknown; T, signal transduction; U, intracellular trafficking and secretion; V, defense mechanisms.

***hisDac:Tn5* enhances the population level of *E. coli* during co-incubation.** The proteomic analysis exhibited that diverse proteins with different abundances were classified into the cell wall/membrane/envelope biogenesis group, specifically T6SS. In Gram-negative bacteria,

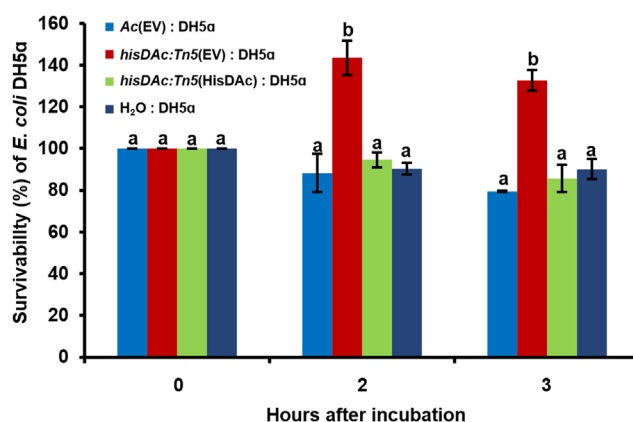


Fig. 6. Co-incubation assay of *Acidovorax citrulli* (*Ac*) strains with *Escherichia coli*. *Ac* strains were co-incubated with *E. coli* DH5a (indicator) at an *Ac*:DH5a ratio of 20:1. After incubation for 2 and 3 h, the number of surviving DH5a cells was counted using the colony counting method. The survivability was calculated based on the number of colonies at 0 h (control) to the number of colonies after 2 and 3 h, respectively. Error bars indicate standard deviations. Different letters indicate statistically significant differences by ANOVA ($P < 0.05$) with Turkey HSD^{ab}.

T6SS is involved in ecological interaction, competition, and fitness (Serapio-Palacios et al., 2022). Therefore, to investigate the effects of HisDac on other bacteria, the competition assay with high cell densities was conducted to ensure that the *Ac* strains would directly contact an indicator bacterium. Because *E. coli* does not possess any effect on *Ac* growth, *E. coli* DH5a was used as an indicator (Fei et al., 2022). *Ac*(EV), *hisDac:Tn5*(EV), and *hisDac:Tn5*(HisDac) were co-incubated with *E. coli* DH5a in water to remove any side effects from nutrients or ions and the survivability of *E. coli* DH5a was then evaluated (Fig. 6). Interestingly, the DH5a population incubated with *hisDac:Tn5*(EV) was much higher than those with *Ac*(EV), *hisDac:Tn5*(HisDac), and water. Additionally, the survivability of DH5a cultured with *hisDac:Tn5*(EV) was 143% after 2-h incubation, and then slightly decreased (132%) after 3-h incubation. However, the survivability of DH5a incubated with *Ac*(EV), *hisDac:Tn5*(HisDac), and water was not statistically different.

Discussion

The biosynthesis of various amino acids, including histidine, contributes to pathogen virulence. For example, mutations in genes involved in several amino acid biosynthetic pathways reduced the virulence of *X. oryzae* pv. *oryzae* and

X. oryzae pv. *oryzicola* (Li et al., 2019; Su et al., 2018). In addition, the histidine biosynthesis pathway plays an important role in pathogenicity not only in bacteria but also in *Aspergillus fumigatus*, an airborne human fungal pathogen (Dietl et al., 2016). Thus, histidinol dehydrogenase, which is essential for histidine production, is important for virulence. In this study, we also demonstrated the essential role of histidinol dehydrogenase involved in histidine biosynthesis for the complete virulence of *Ac* through two different inoculation methods. In addition, through amino acid sequence analysis and protein 3D structure prediction, it was confirmed that the sequence and structure of HisDAC in *Ac* showed high homology and conservation to many other Gram-negative bacteria. However, except for bacterial virulence and histidine biosynthesis, research results on the cellular mechanisms related to histidinol dehydrogenase remain unclear. Our comparative proteomic analysis reveals that HisDAC is associated with not only histidine biosynthesis but also other mechanisms.

Plants, lower eukaryotes, bacteria, and archaea synthesize histidine in a similar, multistep pathway. It should be noted that histidinol dehydrogenase catalyzes two oxidation reactions from L-histidinol to L-histidinaldehyde and from L-histidinaldehyde to histidine in the histidine biosynthetic pathway (Ruszkowski and Dauter, 2017). Using auxotrophic analysis, we confirmed that HisDAC is required for histidine biosynthesis, which is essential for the growth of *Ac*. Although the biochemical activity of HisDAC was not tested in this study, the putative 3D structure of the protein is very similar to that of the previously characterized histidinol dehydrogenase, suggesting that HisDAC may also possess enzymatic activity. Furthermore, in the virulence assay, the mutant's virulence was restored in the presence of histidine. Taken together, HisDAC is indispensable for bacterial growth, which contributes to the virulence of *Ac*. Additionally, using TSB and M9 media supplemented with histidine, we also showed that HisDAC is not required for bacterial multiplication.

The comparative proteomic analysis implies the involvement of HisDAC in various cellular mechanisms, including amino acid metabolism and bacterial cell wall/membrane/envelope. Similar to our results, a histidine auxotroph mutant of the pathogenic bacterium, *Brucella abortus*, displayed an abnormal phenotype associated with cell development (Roba et al., 2022). Furthermore, in the proteomic analysis, several types of T6SS-associated tip VgrG proteins, categorized in group M, were more abundant in the wild-type strain compared to those of the mutant. The T6SS system is well-characterized for mainly regulating

fitness, competition, and interaction with other bacteria in a contact-dependent manner (Basler et al., 2013). Specifically, a previous study reported that VgrG proteins are involved in pathogenicity and adaptation to chemotactic substances of *Pseudomonas plecoglossicida*, a causal agent for white spot disease in croaker (Yang et al., 2023). Surprisingly, in the co-incubation assay, the population of the indicator, *E. coli* DH5 α , increased under co-incubation with *hisDAC:Tn5*(EV). It is postulated that some molecules leaked from the mutant via an abnormality of the bacterial membrane functions, including T6SS, enhanced the population level of the indicator. However, the specific mechanisms that caused the phenomena are still elusive.

This study demonstrates that HisDAC, an enzyme essential for histidine biosynthesis, is linked to other biological mechanisms in *Ac*. These findings offer valuable insights into the role of key enzymes with pleiotropic effects in bacteria. However, the precise molecular mechanisms underlying these pleiotropic functions remain unclear. To address this gap, further research is needed to explore these mechanisms at molecular, biochemical, and histochemical levels. Enzymes involved in the synthesis of primary metabolites, such as histidinol dehydrogenase, have been identified as potential targets for managing bacterial diseases (Khanapur et al., 2017; Monti et al., 2016). Notably, the structural differences between bacterial and plant histidinol dehydrogenases (Barbosa et al., 2002; Ruszkowski and Dauter, 2017), suggest that HisDAC could serve as a promising target for developing agents aimed at disrupting its function to control bacterial disease.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

We thank J. Kim for their technical help at the BT research facility center, Chung-Ang University. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (grant no. RS-2023-00253708). This research was also supported by the Chung-Ang University Graduate Research Scholarship in 2024 (awarded to Suhyun Lee).

Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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