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Chemical or Genetic Alteration of Proton Motive Force Results in Loss of Virulence of *Burkholderia glumae*, the Cause of Rice Bacterial Panicle Blight

Asif Iqbal,[a] Pradip R. Panta,[a] John Ontoy,[b] Jobelle Bruno,[b] Jong Hyun Ham,[b] William T. Doerrler[a]

[a]Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA
[b]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA

ABSTRACT Rice is an important source of food for more than half of the world's population. Bacterial panicle blight (BPB) is a disease of rice characterized by grain discoloration or sheath rot caused mainly by *Burkholderia glumae*. *B. glumae* synthesizes toxoflavin, an essential virulence factor that is required for symptoms of the disease. The products of the *tox* operons, ToxABCDE and ToxFGHI, are responsible for the synthesis and the proton motive force (PMF)-dependent secretion of toxoflavin, respectively. The DedA family is a highly conserved membrane protein family found in most bacterial genomes that likely function as membrane transporters. Our previous work has demonstrated that absence of certain DedA family members results in pleiotropic effects, impacting multiple pathways that are energized by PMF. We have demonstrated that a member of the DedA family from *Burkholderia thailandensis*, named DbcA, is required for the extreme polymyxin resistance observed in this organism. *B. glumae* encodes a homolog of DbcA with 73% amino acid identity to *Burkholderia thailandensis* DbcA. Here, we created and characterized a *B. glumae* Δ*dbcA* strain. In addition to polymyxin sensitivity, the *B. glumae* Δ*dbcA* strain is compromised for virulence in several BPB infection models and secretes only low amounts of toxoflavin (~15% of wild-type levels). Changes in membrane potential in the *B. glumae* Δ*dbcA* strain were reproduced in the wild-type strain by the addition of subinhibitory concentrations of sodium bicarbonate, previously demonstrated to cause disruption of PMF. Sodium bicarbonate inhibited *B. glumae* virulence in rice, suggesting a possible non-toxic chemical intervention for bacterial panicle blight.

IMPORTANCE Bacterial panicle blight (BPB) is a disease of rice characterized by grain discoloration or sheath rot caused mainly by *Burkholderia glumae*. The DedA family is a highly conserved membrane protein family found in most bacterial genomes that likely function as membrane transporters. Here, we constructed a *B. glumae* mutant with a deletion in a DedA family member named *dbcA* and report a loss of virulence in models of BPB. Physiological analysis of the mutant shows that the proton motive force is disrupted, leading to reduction of secretion of the essential virulence factor toxoflavin. The mutant phenotypes are reproduced in the virulent wild-type strain without an effect on growth using sodium bicarbonate, a nontoxic buffer that has been reported to disrupt the PMF. The results presented here suggest that bicarbonate may be an effective antivirulence agent capable of controlling BPB without imposing an undue burden on the environment.

KEYWORDS membrane protein, proton motive force, rice, bacterial panicle blight, antibiotic resistance, plant pathogens, virulence

Rice is a main source of food for more than half of the world's population. It is an important cash crop in the United States, and six states account for over 99% of the U.S. rice output (California, Arkansas, Louisiana, Texas, Mississippi, and Missouri). Bacterial

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Address correspondence to William T. Doerrler, wdoerr@lsu.edu.

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panicle blight (BPB) has become a global disease of rice and is capable of causing losses in production approaching 75% where it strikes. A characteristic grain discoloration or sheath rot is usually an indication of BPB. BPB is caused by *Burkholderia glumae* or, less frequently, by the related *Burkholderia gladioli* (1). Both are hardy soil bacteria and, like other *Burkholderia* species, are highly resistant to antibiotics.

B. glumae synthesizes and secretes an essential virulence factor called toxoflavin that is required for symptoms of BPB. Toxoflavin acts as an electron carrier between NADH and oxygen and can produce hydrogen peroxide, increasing levels of reactive oxygen species and leading to toxicity to the plant (2). The products of the *tox* operons, ToxABCDE and ToxFGHI, are responsible for the synthesis of toxoflavin and the secretion of toxoflavin, respectively (3). Both *toxABCDE* and *toxFGHI* operons are regulated in a quorum sensing (QS)-dependent manner. The *toxFGHI* operon encodes the following two types of membrane transporters: a tripartite efflux pump with similarity to AcrAB of *Escherichia coli* belonging to the resistance-nodulation-division (RND) family of efflux pumps (ToxGHI) and an unrelated transporter belonging to the drug/metabolite transporter (DMT) superfamily (ToxF). Both the DMT and RND family transporters are proton-dependent transporters and thus are dependent upon the membrane proton motive force (PMF) for function.

Our laboratory has a longstanding interest in the conserved DedA membrane protein family. Our data show that processes requiring the PMF, including proton-dependent transporters, are compromised in certain *dedA* family mutants. Our work with the DedA family in *Burkholderia thailandensis* and *Escherichia coli* has shown that they are membrane transporters required for efficient operation of efflux pumps belonging to several families, including the RND family and the major facilitator superfamily (MFS). *E. coli* encodes two members of the DedA family (YqjA and YghB) that carry out partially redundant functions and display roughly 60% amino acid identity. Mutation of *E. coli yqjA* and *yghB* in the same strain results in antibiotic sensitivity (4, 5), temperature sensitivity, and cell division defects (6–8), as well as activation of several envelope stress responses (9). The cell division defect is caused by loss of the PMF-dependent twin arginine transport (TAT) pathway, needed for the export of periplasmic amidases (6). Antibiotic sensitivity is due to loss of activity of PMF-dependent multidrug efflux pumps belonging to the MFS and RND families (4). Expression of *mdfA*, a multidrug resistance protein of the MFS, or artificially restoring the PMF by growth at pH 6.0 can mitigate most of these phenotypes (4, 9). YqjA is required for growth of *E. coli* above pH 8.5 (10), similar to what was reported for MdfA (11) and other membrane transporters (12–14). Both YqjA and YghB possess essential membrane-embedded charged amino acids (4, 15) that are present in proton-dependent transporters belonging to the MFS and RND families of efflux pumps (16–21), suggesting key transport functions of the DedA family.

Burkholderia species, including *B. glumae*, are highly resistant to polymyxin (Pm) with MICs exceeding 500 μg/ml (22). A contributing factor is the expression of aminoarabinose (Ara4N)-transferase (ArnT), which transfers Ara4N to lipopolysaccharide (LPS) lipid A, neutralizing the negative charge of lipid A and weakening Pm binding (23). Pm acts in a manner mechanistically similar to the CAMPS (cationic antimicrobial peptides) of the innate immune response by disrupting negatively charged bacterial membranes, leading to cell lysis and death (24). CAMPs are found in all kingdoms of life where they play roles in immune defense, predation, and competition (25, 26). Plant CAMPS have been isolated from roots, stems, leaves, flowers, and seeds and often have antibacterial or antifungal activity (27). Most CAMPS produced in plants are cysteine-rich and include defensins, thionins, and cyclotides (28).

Mutation of a *Burkholderia thailandensis dedA* gene results in sensitivity to polymyxin, due to inefficient modifications to lipopolysaccharide by the cationic sugar (Ara4N) that is required for Pm resistance (5). We have renamed the gene *dbcA* (*dedA* required for *B*urkholderia *C*AMP resistance) (5, 29, 30). *Burkholderia glumae*, the cause of BPB, encodes a homolog of DbcA that displays 73% amino acid identity to *B. thailandensis* DbcA. In

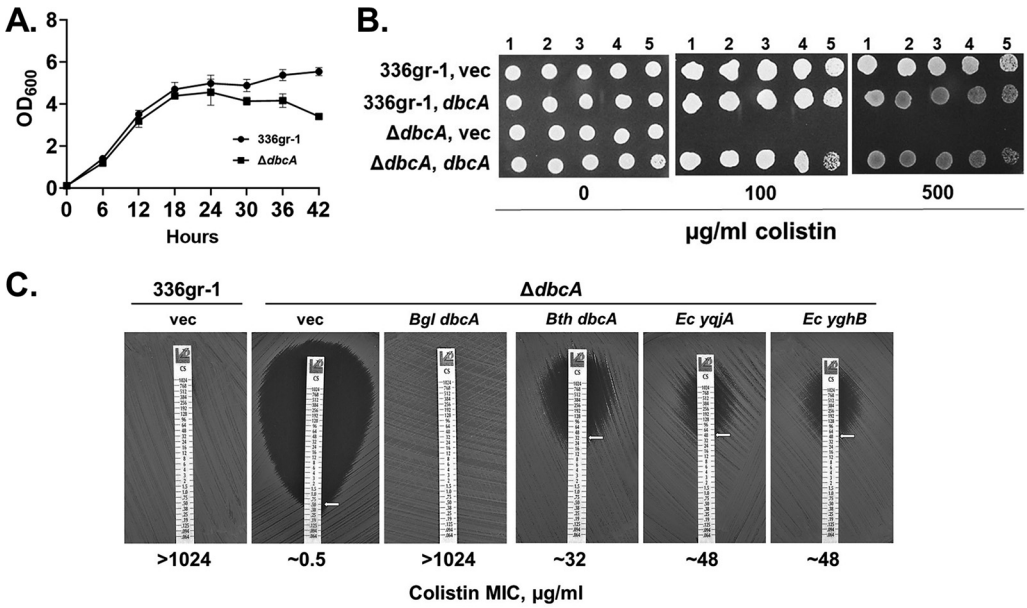

FIG 1 The *B. glumae* Δ*dbcA* strain is sensitive to colistin. (A) Growth curve of *B. glumae* 336gr-1 and Δ*dbcA* strains in LB broth buffered to pH 7.0 with 70 mM Tris. Equal amounts of cells (5×10^7 CFU/ml) were used to inoculate LB broth, and bacterial growth was analyzed at 6-h intervals. (B) Serially $\log_{10}$-diluted cells of *B. glumae* 336gr-1 and Δ*dbcA* strains transformed with control vector pSCrhaB2 (vec) or pSC301 (*dbcA*) were spotted and grown on MH2 agar medium containing 100 μg/ml trimethoprim and the indicated concentration of colistin. The 500 μg/ml colistin plate also contained 0.0004% rhamnose. (C) Colistin E-strips were used to determine the colistin MIC for *B. glumae* 336gr-1 transformed with pBBR1MCS-2 (vec), and the Δ*dbcA* strain transformed with pBBR1MCS-2, pMCS501, pRP101, pRP102, or pRP103 (see Table 1). Bacterial strains were grown in MH2 agar containing 100 μg/ml Kan. Bgl, *B. glumae*; Bth, *B. thailandensis*; Ec, *E. coli*.

this work, we created and characterized a *B. glumae* Δ*dbcA* mutant. In addition to sensitivity to Pm, we demonstrate that the *B. glumae* Δ*dbcA* strain is hyperpolarized, secretes toxoflavin inefficiently, and is avirulent in several plant infection models. The membrane hyperpolarization can be chemically replicated in the wild-type (WT) strain by addition of 5 mM sodium bicarbonate, a nontoxic and inexpensive chemical. Simultaneous application of wild-type *B. glumae* and sodium bicarbonate causes a marked reduction of toxoflavin secretion and virulence in rice, suggesting a possible chemical intervention to prevent bacterial panicle blight.

RESULTS

***B. glumae* possesses a homolog of *B. thailandensis* DbcA.** Previously, we demonstrated that mutation of a *B. thailandensis* DedA family gene, renamed *dbcA* (*ded*A required for *B*urkholderia *c*olistin resistance), results in sensitivity to colistin (5). DbcA is required for PMF-dependent modification of outer membrane lipid A with the cationic sugar aminoarabinose (Ara4N), thus contributing to colistin resistance (29). *B. glumae* possesses a DbcA homolog (GenBank accession number WP_012734729.1) of 231 amino acids with 73% amino acid identity to *B. thailandensis* DbcA. *B. glumae* DbcA also possesses 35% and 29% identity to *E. coli* YqjA and YghB, respectively, which are involved in antibiotic and alkaline pH tolerance (4, 10). *B. glumae* does possess additional genes encoding members of the DedA superfamily, possibly some with redundant functions, similar to most other bacterial species (8, 31). Alignments were performed using Needleman-Wunsch alignment (32, 33).

***B. glumae* DbcA is required for colistin resistance.** To study the function of *B. glumae* DbcA, we constructed a Δ*dbcA* in-frame deletion strain (see Fig. S1 in the supplemental material), which grows similarly to wild type, displaying a slight growth defect in late stationary phase (Fig. 1A). We also constructed a complementing plasmid with *dbcA* expressed behind a rhamnose-inducible promoter (pSC301). We first tested sensitivity of the *B. glumae* Δ*dbcA* strain to colistin and found that it is indeed highly sensitive to the antibiotic, with an MIC of ~0.5 μg/ml (Fig. 1B and C), similar to that of *E. coli* K-12, which

possesses no lipid A modifications contributing to colistin resistance (34). The MIC of the parent *B. glumae* strain was at least 1,000 times greater, consistent with resistance observed in other *Burkholderia* species (22). Complementation with a plasmid copy of *dbcA* completely restores colistin resistance to the Δ*dbcA* strain (Fig. 1B and C). In addition, we observed partial complementation of this phenotype with expression of *B. thailandensis dbcA*, *E. coli yqjA*, and *E. coli yghB* (Fig. 1C), indicative of an evolutionarily conserved function shared by these DedA family proteins.

***B. glumae* DbcA is required for virulence in BPB infection models.** Since *B. glumae* is a plant pathogen and the cause of rice BPB, we were interested in measuring virulence of the wild-type and Δ*dbcA* strains. Virulence of *B. glumae* strains on rice was found to correlate to virulence on onion bulb scales, suggesting that this can be a convenient host system to measure *B. glumae* virulence (35). Onion scales were inoculated with growth medium alone, wild-type *B. glumae*, or the Δ*dbcA* strain (harboring control or complementing vectors) and incubated at 30°C for 72 h. The macerated area visible after inoculation with the wild-type *B. glumae* was found to be significantly larger than that seen with the Δ*dbcA* strain, and the defect was corrected with a plasmid copy of the gene (Fig. 2A and B). This suggests that DbcA may be required for virulence in rice.

We proceeded to measure virulence using mature rice plants. For this assay, rice panicles were sprayed with 5 ml of 5×10^7 CFU/ml suspensions of the wild-type *B. glumae* and Δ*dbcA* strains twice at 2-day intervals, on the panicles at the 20 to 30% flowering stage of rice plant. The panicle blight symptoms were evaluated at 3, 7, and 10 days after the first inoculation by assigning virulence scores on a 0 to 9 scale (36). We observed that the panicles sprayed with wild-type bacteria started showing symptoms within 3 days of exposure compared to the water-treated control, and these symptoms worsened for the duration of the experiment (Fig. 2C). In contrast, panicles exposed to the Δ*dbcA* strain displayed significantly fewer symptoms and fared not much worse than water-treated controls (Fig. 2C). At the conclusion of the experiment, virulence scores were 0, 7, and 1.7 for water, the wild-type *B. glumae* strain, and the Δ*dbcA* strain exposures, respectively (Fig. 2C and D). Rice panicles taken from plants exposed to wild-type *B. glumae* were distinctively discolored while Δ*dbcA* strain-treated panicles were only slightly browned (Fig. 2E). This experiment shows that DbcA function is required for *B. glumae* to cause symptoms of BPB in rice panicles.

***B. glumae* DbcA is required for toxoflavin production.** Toxoflavin is the sole yellow pigment produced by *B. glumae* (37) and is an essential virulence factor, the production of which is controlled by quorum sensing (1). To begin to understand the loss of virulence, we extracted and measured levels of toxoflavin produced by each strain. The *B. glumae* Δ*dbcA* strain is nearly nonpigmented (Fig. 3A), and much less toxoflavin is secreted by the Δ*dbcA* strain into the solid growth medium (Fig. 3B). Toxoflavin concentrations in liquid growth media were also significantly lower during growth for up to at least 42 h (Fig. 3C), and this was restored upon introduction of a complementing plasmid (Fig. 3D). Thin-layer chromatography (TLC) was used to resolve chloroform extracts of strains grown for 30 h. Levels of each secreted phytotoxin reumycin, fervenulin, and toxoflavin were lower in the uncomplemented Δ*dbcA* strain, and levels were restored to wild-type levels with expression from a complementing plasmid (Fig. 3E). These results collectively suggest that *B. glumae* DbcA is required for efficient toxoflavin synthesis and secretion and is consistent with loss of virulence of the Δ*dbcA* strain.

***B. glumae* DbcA is required for normal PMF.** In order to understand the physiological state of *B. glumae* in the absence of *dbcA*, we analyzed the state of the PMF since the *B. thailandensis* Δ*dbcA* strain has altered PMF as do certain *E. coli* DedA family mutants (9, 29). According to chemiosmotic theory (38), the membrane PMF is equal to the sum of the charge difference across the membrane ($\Delta\Psi$) and the pH difference across the membrane (ΔpH). Importantly, when either ΔpH or $\Delta\Psi$ is dissipated chemically or by mutation, bacteria have the ability to increase the other component of the PMF to compensate (39). To examine the PMF in more detail, we measured the $\Delta\Psi$ component of the PMF using the dye JC-1. JC-1 is a membrane permeable dye that exhibits green fluorescence (530 nm) as a monomer but forms aggregates in the

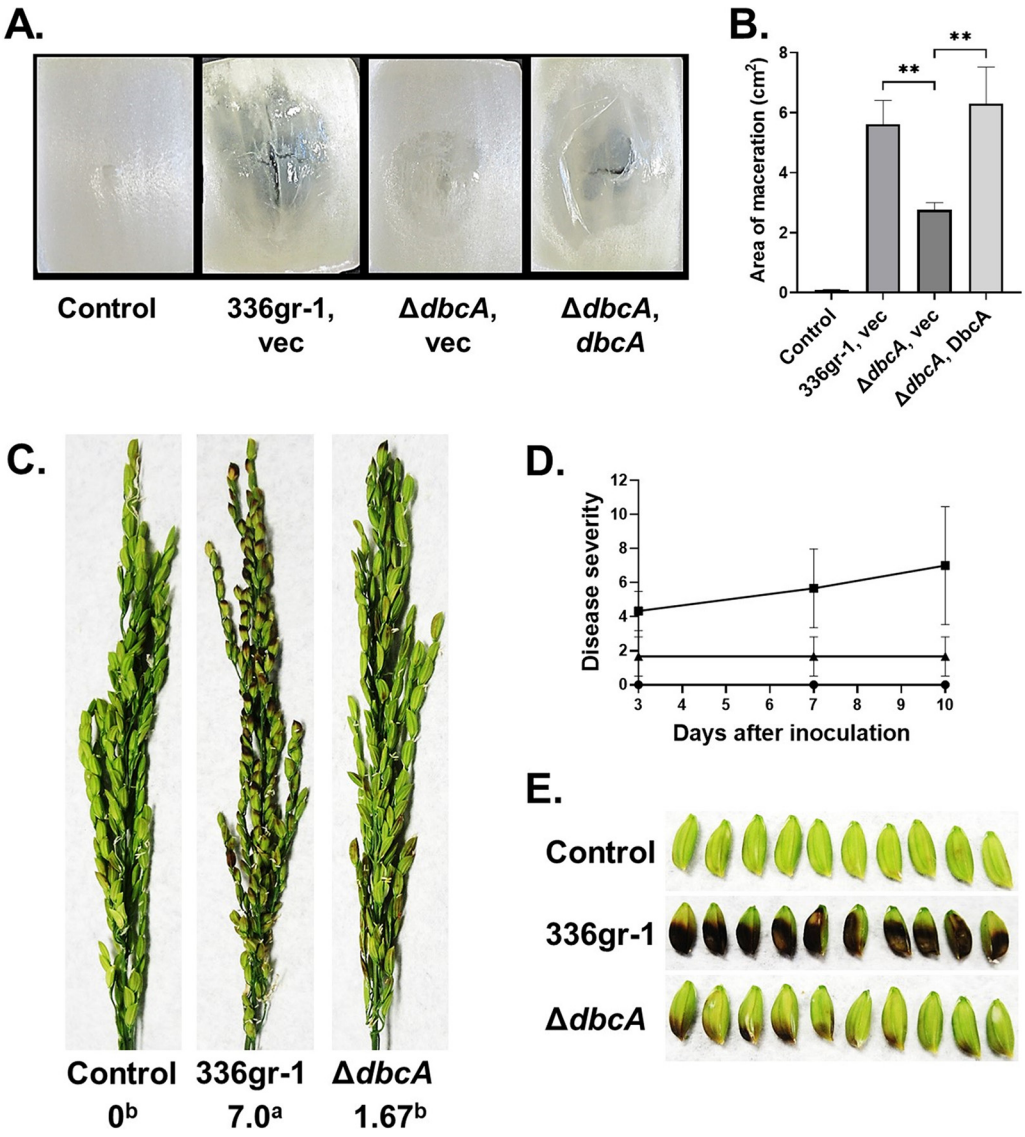

FIG 2 *B. glumae* Δ*dbcA* strain is compromised for virulence in onion slices and rice panicles. (A) The area of maceration on onion slices indicates the virulence phenotype for each bacterial strain. Onion slices were infected with equal amounts of cells from *B. glumae* 336gr-1 transformed with control vector (vec) and the *B. glumae* Δ*dbcA* strain transformed with control vector (vec) and pSC501 (*dbcA*). Onion slices were incubated at 30°C for 72 h in a humid chamber. (B) Area of maceration (cm^2) produced by indicated strains. **, $P < 0.01$. (C) Rice seed discoloration (black necrosis) indicates the virulence phenotype for control (water), *B. glumae* 336gr-1, and the Δ*dbcA* strain. The images were taken 10 days postinoculation, and disease severity score was determined with a 0 to 9 scale. Different letters below the score indicate statistical significance. (D) The line graph shows the disease progress for rice panicles inoculated with water (circles), *B. glumae* 336gr-1 (squares), and the Δ*dbcA* strain (triangles) at 3, 7, and 10 days postinoculation. (E) Detailed view of seed discoloration produced by control, *B. glumae* 336gr-1, and the Δ*dbcA* strain at 10 days postinoculation.

presence of membrane potential, shifting its emission from green to red (595 nm). Therefore, relative membrane potential can be expressed as the ratio of red to green fluorescence (40, 41). Wild-type *B. glumae* and the Δ*dbcA* strain harboring either control or complementing vector were treated with JC-1 dye. Cells treated with proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were included as a control. *B. glumae* strains and the complemented Δ*dbcA* strain exhibited a consistent 595/530 ratio (Fig. 4A). However, the *B. glumae* Δ*dbcA* strain displayed a higher red/green ratio (Fig. 4A), suggesting partial hyperpolarization of the PMF. This result was unexpected, since we previously reported PMF depolarization of the *B. thailandensis* Δ*dbcA*

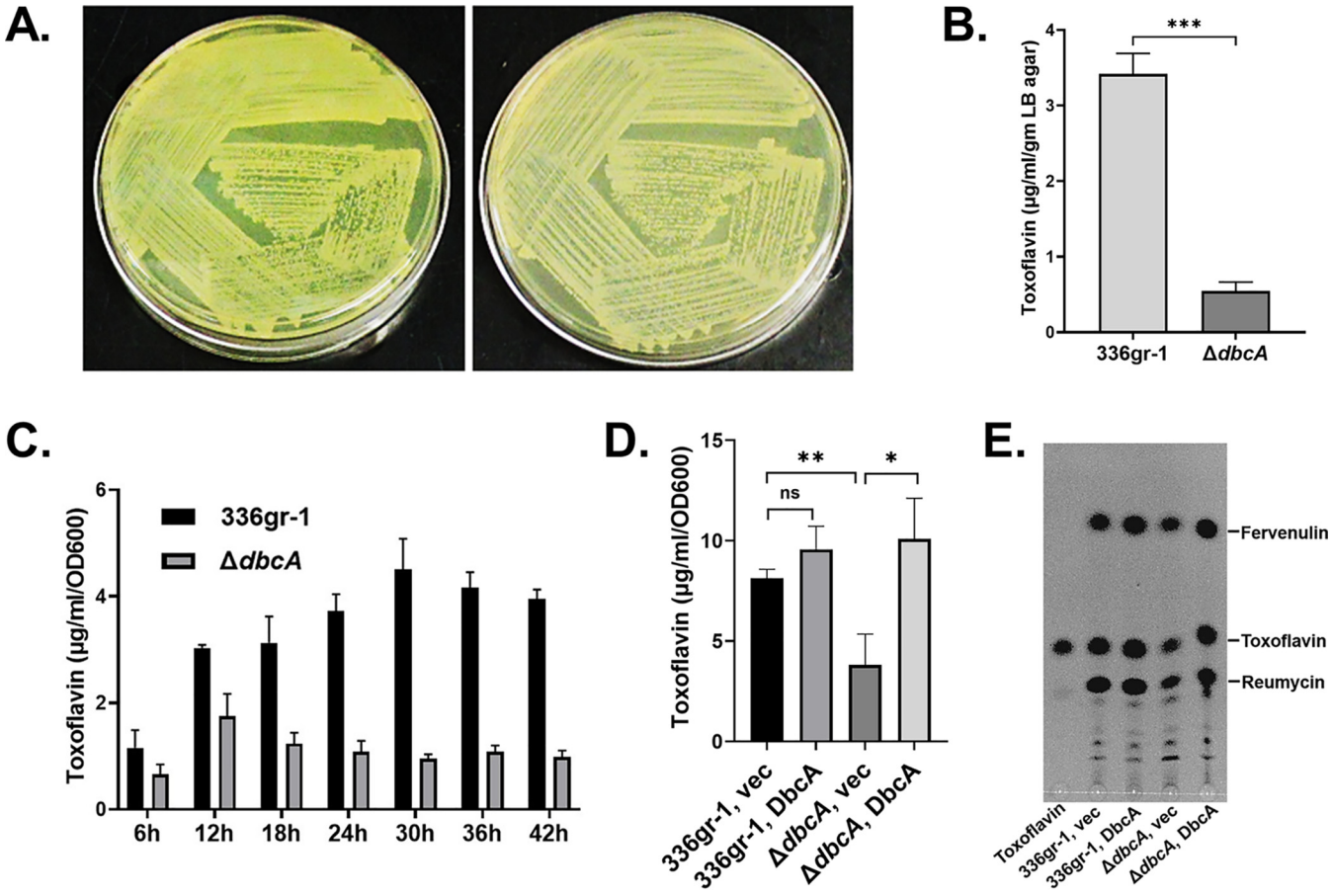

FIG 3 Toxoflavin production is compromised in the *B. glumae* Δ*dbcA* strain. (A) Visual observation of toxoflavin (yellow pigment) produced by *B. glumae* 336gr-1 and the Δ*dbcA* strain on an LB agar plate after growth at 37°C for 72 h. Left and right panels of the figure indicate toxoflavin produced by the *B. glumae* 336gr-1 and Δ*dbcA* strains, respectively. (B) Quantification of toxoflavin production by the *B. glumae* 336gr-1 and Δ*dbcA* strains on LB agar plates. (C) Quantification of toxoflavin production in LB broth medium over time. *B. glumae* 336gr-1 (black bars) and the Δ*dbcA* strain (gray bars) were grown in LB broth medium buffered with 70 mM Tris (pH 7.0) at 37°C with shaking for 42 h. Toxoflavin levels in the culture medium were determined at 6-h intervals. (D) Complementation of toxoflavin production in the *B. glumae* Δ*dbcA* strain. Indicated strains were grown in LB broth medium buffered with 70 mM Tris (pH 7.0) without addition of antibiotic and rhamnose at 37°C with shaking for 30 h. (E) Analysis of toxoflavin produced by *B. glumae* 336gr-1 and the Δ*dbcA* strain after 30 h of growth by TLC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

strain (5). Consistent with this observation, the *B. glumae* Δ*dbcA* strain is resistant to CCCP (Fig. 4B) and tetracycline (see Fig. S2 in the supplemental material), suggesting a dissipation of the ΔpH component of the PMF of *B. glumae* Δ*dbcA* strain (42, 43). These observations suggest that any alteration of the PMF can have profound effects on bacterial physiology.

Sodium bicarbonate ($NaHCO_3$) at physiological concentrations dissipates the ΔpH component of the PMF and can alter the sensitivity of bacteria to commonly used antibiotics (42). $NaHCO_3$ at 5.0 mM has no effect on the growth of wild-type *B. glumae* with slight effects on the Δ*dbcA* strain (Fig. 5A and B), consistent with our previous results with *B. thailandensis* (30). Treatment of *B. glumae* and *B. glumae* Δ*dbcA* strains with 5 mM $NaHCO_3$ results in a significant increase of ΔΨ (Fig. 5C). The effect of $NaHCO_3$ on the ΔΨ of the wild-type strain is similar in magnitude to the effect of the Δ*dbcA* mutation. This was coupled with a significant decrease in toxoflavin production (Fig. 5D and E) and colistin sensitivity (see Fig. S3 in the supplemental material), showing that we can mimic the effect of the Δ*dbcA* mutation on the PMF using low concentrations of $NaHCO_3$. Furthermore, the presence of 5 mM $NaHCO_3$ significantly impacts *B. glumae* virulence in an onion scale model (Fig. 5F and G). Collectively, these results demonstrate that $NaHCO_3$ can disrupt the bacterial PMF in a manner similar to the Δ*dbcA* mutation, and this disruption can impact *B. glumae* toxoflavin secretion and virulence. The additive effect of

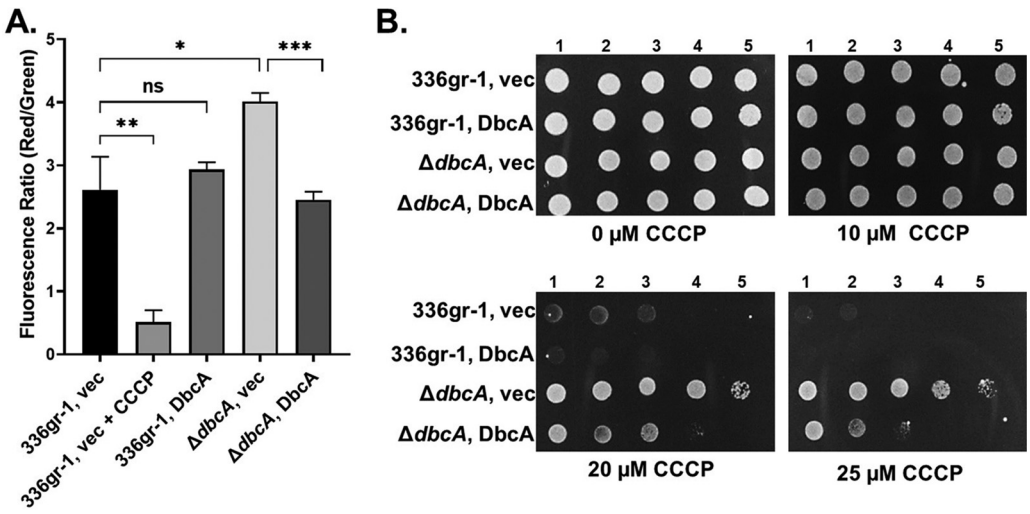

FIG 4 Measurement of membrane potential and CCCP sensitivity. (A) Measurement of membrane potential ($\Delta\Psi$) for *B. glumae* strains. Fluorescence ratio in the graph represents the red (595 nm)/green (530) emission ratio of JC-1 dye. Twenty-five micromolar CCCP added to *B. glumae* 336gr-1 (vec) was used as a control for loss of $\Delta\Psi$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. (B) CCCP sensitivity of *B. glumae* strains. Serially $\log_{10}$-diluted cells of *B. glumae* 336gr-1 and the Δ*dbcA* strain transformed with control vector (vec) and pSC301 (DbcA) were spotted and grown on MH2 agar growth medium containing 100 μg/ml Tmp, no rhamnose, and either 10, 20, or 25 μM CCCP at 37°C for 48 h.

bicarbonate on growth (Fig. 5A) and membrane potential (Fig. 5C) of the Δ*dbcA* strain implies that this is not due to a direct effect of bicarbonate on DbcA.

We then measured the effect of bicarbonate on *B. glumae* virulence using the rice panicle model. Wild-type *B. glumae* was grown and resuspended in the presence or absence of 5 mM $NaHCO_3$ and applied to rice panicles as described for Fig. 2. At day 10 following exposure of panicles, *B. glumae* in water caused a similar pathology as described in Fig. 2. Panicles were discolored and wilted with a virulence score of 7.0 (Fig. 6A and B), while 5 mM $NaHCO_3$ alone caused no harm to panicles. Plants that were treated with the combination of *B. glumae* and $NaHCO_3$ fared much better (virulence score of 2.3) and displayed much less wilting and discoloration (Fig. 6A and B). Panicles from these plants were also less discolored than plants exposed to *B. glumae* delivered in water (Fig. 6C). These results suggest that the common buffer sodium bicarbonate may be capable of reversing virulence of *B. glumae* independently of any effect on growth and suggest a potential intervention strategy for bacterial panicle blight.

DISCUSSION

Rice is a primary source of food for more than half of the world's population. Bacterial panicle blight (BPB), caused by *Burkholderia glumae*, results in extensive damage to rice (*Oryza sativa*) crops in the United States and worldwide. It is an important cash crop in the United States, and six states account for over 99% of the U.S. rice output (California, Arkansas, Louisiana, Texas, Mississippi, and Missouri). The U.S. rice output was about 18.6 billion pounds in 2019 with a value of $2,370,000,000 (USDA). Bacterial panicle blight (BPB) has become a global disease of rice and is capable of causing losses in production approaching 75% where it strikes. *B. glumae* infection is thought to originate from infected seeds. The infection can spread to upper tissues and leaves during plant growth and inoculate emerging panicles during the flowering stage (1, 44). A characteristic grain discoloration or sheath rot is usually an indication of BPB. A survey found it to be present in 60% of Louisiana fields, and yield losses may reach 40% in severely infected fields (45). Based upon annual rice production in the American South, it has been estimated that BPB caused $61 million in damage

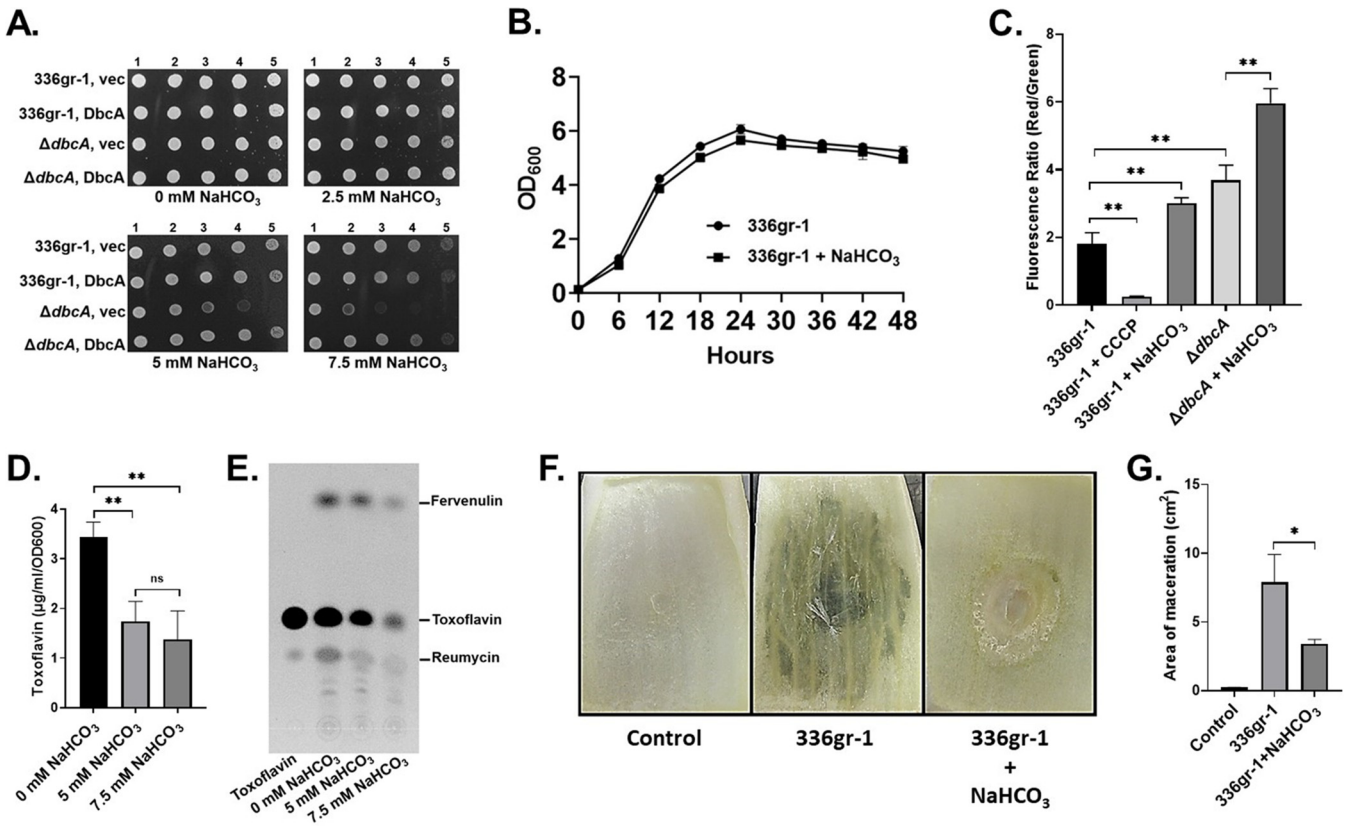

FIG 5 Bicarbonate selectively dissipates *B. glumae* PMF and reduces toxoflavin production and virulence without impacting growth. (A) Sodium bicarbonate ($NaHCO_3$) sensitivity of *B. glumae* strains. Serially $\log_{10}$-diluted cells of *B. glumae* 336gr-1 and the Δ*dbcA* strain transformed with control vector (vec) and pSC301 (DbcA) were spotted and grown on MH2 agar medium containing 100 μg/ml trimethoprim and either 2.5, 5, or 7.5 mM sodium bicarbonate at 37°C for 48 h. (B) Growth curve of *B. glumae* 336gr-1 in LB broth buffered to pH 7.0 with 70 mM BTP with and without 5 mM $NaHCO_3$. (C) Measurement of membrane potential ($\Delta\Psi$). Fluorescence ratio in the graph represents the red (595 nm)/green (530) emission ratio of JC-1 dye. 5 mM $NaHCO_3$ was present when indicated. **, $P < 0.01$. (D) Production of toxoflavin by *B. glumae* 336gr-1 in growth medium containing sodium bicarbonate. LB broth medium was buffered with 70 mM BTP, pH 7.0. Equal numbers of bacterial cells (5×10^7 CFU/ml) were transferred into 250-ml conical flasks containing 25 ml buffered LB broth and either 0, 5, or 7.5 mM $NaHCO_3$ and grown at 37°C with shaking for 30 h. (E) TLC analysis for toxoflavin produced by *B. glumae* 336gr-1 in growth medium containing indicated concentrations of $NaHCO_3$. (F) Onion scales were inoculated with *B. glumae* preincubated with or without 5 mM $NaHCO_3$, which were then incubated at 30°C for 6 days in a humid chamber. (G) Area of maceration (cm^2) produced by indicated strains. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

between 2003 and 2013. While the United States produces only 1.3% of the world's rice, it is traded to 120 countries and accounted for 7.7% of the global rice trade between 2014 and 2016 (USDA). Therefore, rice shortages in the United States can have extensive impacts on global food security (46). BPB is predicted to significantly increase due to climate change (1, 47). While some rice varieties are more resistant than others to BPB, there is no completely resistant rice species, and no chemical treatment is currently recommended in the United States to combat the disease (1).

The DedA superfamily is a highly conserved membrane protein family that remains poorly characterized and for which there is little structural information. There are currently 29,230 individual sequences in the protein database across 7,915 species belonging to the "SNARE-associated PF09335" family of proteins (PFAM 34.0). DedA family proteins have been shown to be required for a number of important functions in various bacterial species, including growth and cell division (6, 7, 48), envelope integrity (9), and resistance to a number of antibiotics and biocides (4), including polymyxin and antimicrobial peptides in a number of different species (5, 49–53). Absence of certain DedA family members results in pleiotropic effects, impacting multiple pathways that are energized by PMF (8). Our lab has recently demonstrated that a *B. thailandensis* DedA family protein is required for resistance to colistin, an antibiotic belonging to the CAMP family (5). We named the *B. thailandensis* DedA protein DbcA (DedA of *Burkholderia* required for CAMP resistance) and note that *B. glumae* possesses a homolog of DbcA with ~73% amino acid identity. Here, we show

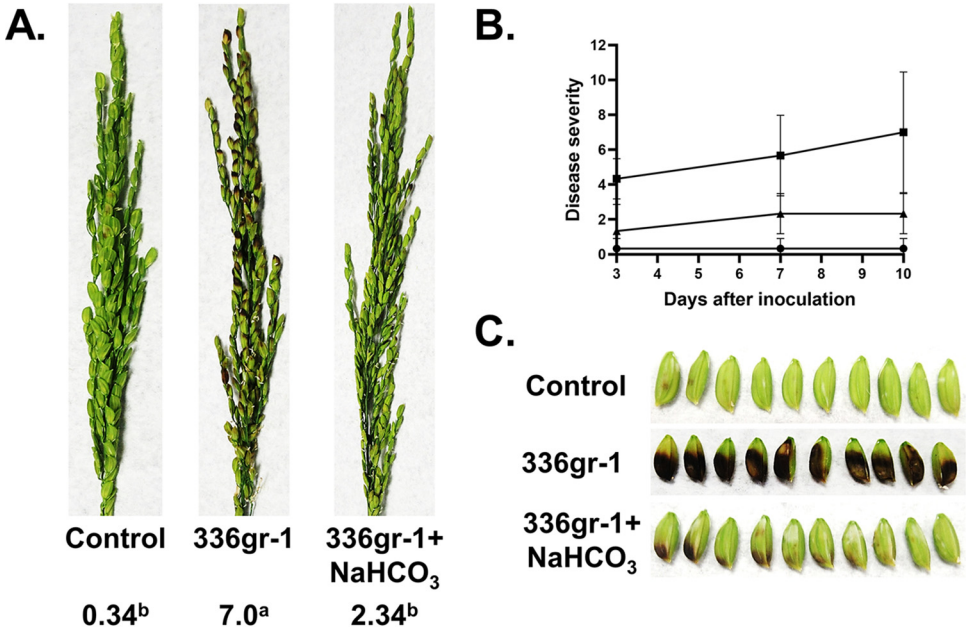

FIG 6 Bicarbonate treatment results in loss of *B. glumae* virulence in rice. (A) Virulence phenotype on rice panicles for control, *B. glumae* 336gr-1 cells with no sodium bicarbonate, and *B. glumae* 336gr-1 cells with 5 mM sodium bicarbonate. For the control, 5 mM sodium bicarbonate was directly sprayed on rice panicles. The images were taken at 10 days postinoculation, and disease severity score was determined with a 0 to 9 scale. (B) The line graph shows the disease progress for rice panicles inoculated with control (circles), *B. glumae* 336gr-1 cells with no sodium bicarbonate (squares), and *B. glumae* 336gr-1 cells with 5 mM sodium bicarbonate (triangles) at 3, 7, and 10 days postinoculation. (C) Detailed view of seed discoloration observed by indicated treatments at 10 days postinoculation.

that *B. glumae* DbcA is required for resistance to colistin, efflux of toxoflavin, and virulence (Fig. 1 to 3).

In addition to a near absence of toxoflavin secretion, the sensitivity of the *B. glumae* Δ*dbcA* strain to polymyxin antibiotics, such as colistin, may be significant in understanding its loss of virulence. Polymyxin is thought to act in a manner similar to the CAMPs of the innate immune system of both plants and animals, by disruption of membranes possessing lipids with exofacial negatively charged headgroups (24). A number of CAMPs have been isolated from different plants tissues, including seeds, stems, leaves, flowers, and roots (27). Certain plant CAMPs do possess antibacterial and antifungal activity and are believed to play important roles in immunity (27, 28). Direct application (54) and overexpression (55) of CAMPs are being explored as strategies for disease control in rice. Whether the *B. glumae* Δ*dbcA* strain is sensitive to specific plant CAMPs is an important question that remains to be answered.

While characterizing the physiological effects of the Δ*dbcA* mutation on *B. glumae*, we observed that the $\Delta\Psi$ component of the PMF was higher in the mutant than the wild type, an unexpected result considering that the opposite was observed with the *B. thailandensis* Δ*dbcA* strain (29). We took this to indicate that the Δ*dbcA* mutation was causing a dissipation of the ΔpH component of the PMF and was increasing $\Delta\Psi$ to compensate for this loss. This further indicates that optimal maintenance of membrane potential is required and any perturbation can result in colistin sensitivity and additional phenotypes. Consistent with hyperpolarization, the Δ*dbcA* strain is also resistant to tetracycline (see Fig. S2 in the supplemental material), which can be caused by dissipation of ΔpH (42, 43). Recently, it was reported that a common buffer, sodium bicarbonate, could potentiate the effects of certain antibiotics specifically by dissipating the cellular ΔpH component of the PMF (42). Therefore, we tested to determine if sodium bicarbonate by itself could reproduce the effects of the Δ*dbcA* mutation on toxoflavin production and virulence. Indeed, this was found to be the case (Fig. 5 and 6) and at concentrations that did not cause a

significant impact on bacterial growth. Importantly, these effects were not due to a trivial effect of bicarbonate on pH, as all growth media were buffered to pH 7.0 for these studies and no change in pH of the media was observed during growth (data not shown).

Sodium bicarbonate has been used for many years as a common and nontoxic household item and is an additive to foods and dental products. While it has been reported to inhibit the growth of fungi (56, 57) and bacteria including *Streptococcus mutans* (58), its antibacterial and antivirulence properties have not been fully exploited to date. In an agricultural setting, bicarbonate can be delivered as an ammonium or potassium salt, rather than sodium, with similar effects on PMF (42). The results presented here clearly suggest that bicarbonate may be an effective antivirulence agent capable of not just controlling BPB, but doing so in a responsible manner without imposing an undue burden on the environment typically seen with common herbicides, fungicides, and insecticides (59, 60).

In summary, we have identified a chemical treatment that mimics the effect of a mutation in a *dedA* family gene of *Burkholderia glumae*, the cause of bacterial panicle blight. Both mutation of *B. glumae dbcA* and exposure to sodium bicarbonate act similarly to dissipate the PMF, resulting in a loss of virulence. Since the DedA family is widespread in bacteria, similar phenotypes may be found in other bacterial species when the PMF is targeted.

MATERIALS AND METHODS

Culture conditions and chemicals. Table 1 lists bacterial strains and plasmids used in this study. *E. coli* strains were grown in lysogeny broth (LB) medium (1% NaCl, 1% tryptone, 0.5% yeast extract) with appropriate antibiotics. Mobilizer strain *E. coli* RHO3 was grown in LB containing 200 μg/ml DAP (2,6-diaminopimelic acid; LL-, DD-, and *meso*-isomers; VWR). *B. glumae* 336gr-1 was grown in either LB or cation-adjusted Mueller-Hinton broth 2 (MH2, pH 7.3; Sigma-Aldrich). LB buffered with 70 mM Tris (pH 7.0 or 7.4) was used for toxoflavin production experiments and growth curves in liquid medium. For toxoflavin production in the presence of sodium bicarbonate, LB medium buffered with 70 mM BTP (bis-Tris propane), pH 7.0, was used. Antibiotics were purchased either from VWR or Sigma-Aldrich and used at the following concentrations: nitrofurantoin (Nit), 100 μg/ml (*B. glumae*); colistin (at the indicated concentrations); kanamycin (Kan), 30 μg/ml (*E. coli*) and 100 μg/ml (*B. glumae* and *B. thailandensis*); gentamicin (Gen), 30 μg/ml; trimethoprim (Tmp), 100 μg/ml; and tetracycline (Tet), 15 μg/ml. All bacterial strains were grown at 37°C unless otherwise indicated. Toxoflavin was purchased from Cayman Chemical. All other chemicals were purchased from VWR. Table 2 lists oligonucleotide primers used in this study (Sigma-Aldrich).

Deletion of *B. glumae* 336gr-1 *dbcA* gene. Homologous recombination was used to knock out *dbcA* (*bglu_1g06460*) from the *B. glumae* genome (61, 62). Q5 DNA polymerase (New England BioLabs) was used for PCR amplification of DNA fragments. The 1,258-bp upstream and 1,395-bp downstream regions of the *dbcA* gene were PCR amplified from *B. glumae* genomic DNA. A trimethoprim resistance (Tmp^r) cassette was PCR amplified from pUC18T-mini-Tn*7*-Tp plasmid (5). The 5′ end of the amplified Tmp^r fragment contained a 35-bp homology with the 3′ end of amplified upstream region of *dbcA*. Similarly, the 3′ end of the amplified Tmp^r fragment contained 35-bp homology with the 5′ end of amplified downstream region of *dbcA*. The Gibson Assembly kit (New England Biolabs) was used to ligate the three amplified fragments. One microliter of the ligated product (3,525 bp) was used as template for PCR amplification. The PCR product was gel purified using QIAquick gel extraction kit (Qiagen), digested with KpnI and HindIII, and ligated into a similarly digested vector, pEX18Gm (63). The plasmid construct, named pEX3525, was transformed into *E. coli* XL1 Blue and sequenced to confirm 100% similarity with reference sequence. Vector pEX3525 was transformed into mobilizer strain *E. coli* RHO3 and introduced into parental *B. glumae* strain by conjugation (64). Recombinant *B. glumae* colonies were selected on LB agar medium containing 100 μg/ml trimethoprim and 30 μg/ml gentamicin. To induce a secondary homologous recombination, the recombinant *B. glumae* colonies were grown in LB medium containing no antibiotic at 30°C with shaking overnight. The 10^{-2} and 10^{-3} diluted overnight cultures were spread on TY (tryptone and yeast extract) agar medium containing 30% sucrose for counter selection (65). To confirm the deletion of *dbcA*, sucrose-resistant colonies were screened for gentamicin sensitivity and trimethoprim resistance. Replacement of the *dbcA* gene with the Tmp^r cassette was confirmed by PCR. The FLP recombination target (FRT) method was used to remove the Tmp^r cassette from the *B. glumae* Δ*dbcA*::Tmp^r strain to obtain Δ*dbcA*::FRT using pFlpTet (66). The genomic DNA was extracted (Easy DNA kit; Invitrogen) from parental WT, Tmp^r, and Tmp^s. *B. glumae* strains were PCR amplified with KO-FW and KO-REV primers to confirm the *dbcA* mutant strains (see Fig. S1 in the supplemental material).

Transformation and complementation analysis. Heat shock was used for transformation of *E. coli* unless otherwise indicated (67). Biparental conjugation was used for transformation of *B. glumae* (64). Colony PCR (OneTaq Hot Start Quick-Load 2× master mix with GC buffer; New England BioLabs) was performed to confirm the exconjugant *B. glumae* colonies harboring appropriate plasmid. *B. glumae dbcA* with a start codon changed from TTG to ATG was PCR amplified from genomic DNA of *B. glumae*

TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Description	Source
Strain		
Escherichia coli		
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1lac* [F′ *proAB lacI*^{q}*Z*ΔM15] Tn*10* (Tet^r)	Stratagene
RHO3	SM10(λ*pir*) Δ*asd*::FRT Δ*aphA*::FRT, Kan^s	64
Burkholderia glumae		
336gr-1	Wild-type *Burkholderia glumae*	65
Δ*dbcA* strain	336gr-1 Δ*dbcA*::Tmp^r	This study
Δ*dbcA*::FRT strain	336gr-1 Δ*dbcA*::Tmp^s	This study
Plasmid		
pUC18T-mini-Tn7T-Tp	Mini-Tn*7* T-based vector containing a trimethoprim resistance cassette; Tmp^r (GenBank accession no. DQ493875)	69
pEX18Gm	*oriT*$^+$ *sacB*$^+$, gene replacement vector with multiple cloning site from pUC18, Gm^r	63
pEX3525	Assembled upstream and downstream fragment of *dbcA* gene interrupted by Tmp^r fragment cloned into pEX18Gm	This study
pFlpTet	Rham-inducible *flp*, TS ori	66
pSCrhaB2	Expression vector; ori$_{pBBR1}$*rhaR*, *rhaS*, P_{rhaB}Tmp^{r}mob$^+$	68
pSC301	pSCrhaB2 expressing *B. glumae dbcA*	This study
pBBR1MCS-2	Expression vector; RK2*mob lacZ*α *E. coli lac* promoter, Kan^r	70
pMCS501	pBBR1MCS-2 expressing *B. glumae dbcA* with *dbcA* promoter	This study
pRP101	pBBR1MCS-2 expressing *B. thailandensis dbcA*	5
pRP102	pBBR1MCS-2 expressing *E. coli yqjA*	5
pRP103	pBBR1MCS-2 expressing *E. coli yghB*	5

and ligated into NdeI and HindIII restriction sites of an expression vector pSCrhaB2 under rhamnose-inducible P_{rhaB} promoter resulting in pSC301 (68). For one experiment (Fig. 1C), the *dbcA* gene with its own promoter sequence was PCR amplified from genomic DNA of *B. glumae* 336gr-1 and ligated into KpnI and HindIII restriction sites of expression vector pBBR1MCS-2 resulting in pMCS501. Transformed *E. coli* and *B. glumae* strains were selected on 100 μg/ml Tmp (pSCrhaB2-based vectors) or 100 μg/ml Kan (pBBR1MCS-2-based vectors). All DNA sequencing was performed at the Louisiana State University (LSU) College of Science Genomic Facility.

TABLE 2 Oligonucleotide primers used in this study

Primer name	Sequence (5′→3′)[a]
K1F	ATTA***GGTACC***AACACGTACTGGTGGATCGAG
K1R	TCCCCAATTCGAGCTCATCAGCGTATCCAAGACGTTTCCTT
K2F	ACGTCTTGGATACGCTGATGAGCTCGAATTGGGGATCTTGAAGTA
K2R	TCCCACCAGCACGATCACCCACTAGTGAGCTCATGCATGAT
K3F	CATGAGCTCACTAGTGGGTGATCGTGCTGGTGGGAGTC
K3R	ATAT***AAGCTT***CTGTTCCACAGCACCTCGTTT
Fw_pEX18Cont.	AGGCAAATTCTGTTTTATCAGACCGC
Rev_pEX18Cont.	GAGCGGATAACAATTTCACACAGGAA
Seq 1	ATACTTCTTCGTCAACGGCCG
Seq 2	AGGCGTGTTGAAGTACAGCTC
Seq 3	GTGGAATCGGTGGAATCGGTA
Seq 4	GCATAGCCTTCAGGAGTGAGT
Fw_Seq 5A	CGATCATGCATGAGCTCACTAGT
Rev_Seq 5A	TCAAGGTCGGCGACTCGATCT
Seq 5	TCGCAATAGACCTGCCAGTAG
Seq 6	AGGATCAGCACGATGGTCAGC
KO-FW	AACCGAAGGAAACGTCTTGGATACG
KO-REV	AGCTACTGGCAGGTCTATTGCGA
FW-pSCdbcA	CGCC***CATATG***ATGGATACGCTGCTTCACTTCGTCAATC
REV-pSCdbcA	CGCG***AAGCTT***TCAGCCGCGCCCGCGCG
Fr-Seq-pSCdbcA	CATCATCACGTTCATCTTTCCCTG
Rv-Seq-pSCdbcA	GCAAATTCTGTTTTATCAGACCGC
pMCSdbcA-FW	ATTA***GGTACC***ATTCGGACATGCGGGAATTATAACGACG
pMCSdbcA-REV	CGCG***AAGCTT***TCAGCCGCGCCCGCGCG
SeqM13-FW	TGTAAAACGACGGCCAGTGAG
SeqM13-REV	TCACACAGGAAACAGCTATGA

[a]Underlined text represents homology sequences. Bolded italic text represents restriction enzyme sites.

Susceptibility of colistin and CCCP. Colistin and CCCP susceptibility assays were performed in MH2 medium. Fresh 1:50 diluted overnight cultures in MH2 medium with appropriate antibiotics were grown to an optical density at 600 nm (OD_{600}) of ~0.6 at 37°C in a shaking incubator. The MH2 agar plates containing appropriate antibiotics were spotted with 5 μl of serially $\log_{10}$-diluted bacterial cells. The plates were incubated at 37°C, and bacterial growth was analyzed after 48 h of incubation. Colistin MIC strips (Liofilchem, Waltham, MA) were used to measure colistin MIC of *B. glumae* strains according to the manufacturer's instructions.

Virulence assays. Onion scales are a convenient host system to measure the virulence of *B. glumae* strains (35). *B. glumae* strains were grown in MH2 agar medium containing 100 μg/ml Tmp at 37°C for 48 h. Bacterial culture grown on MH2 agar plates was removed with sterile loops and resuspended in MH2. The bacterial suspension was adjusted to 5×10^9 CFU/ml. The fresh scales of yellow onion were cut into rectangular slices with a sterile razor blade. A 5-mm square wound in the center of the inner surface of each of the onion scales was formed using a sterile 200-μl micropipette tip. Ten microliters of bacterial suspension (~5×10^7 CFU) was applied to the onion scales. For control onion scales, 10 μl sterile MH2 medium was added. The infected onion scales were incubated at 30°C for 72 h in a humid chamber. The disease severity of each *B. glumae* strain was determined by visual observation and measuring the area of maceration. For onion virulence assay in the presence of sodium bicarbonate, an equal amount of bacterial suspension (5×10^9 CFU/ml) was added into microcentrifuge tubes containing sterile MH2 medium supplemented with either 0 or 5 mM sodium bicarbonate. The tubes were incubated for 1.5 h at room temperature (RT) without shaking. Ten microliters of suspension was directly inoculated into onion scales. For the control, 10 μl of sterile MH2 medium containing 5 mM sodium bicarbonate was used.

For *B. glumae* virulence assays using rice panicles, the LSU College of Agriculture Greenhouse was used to grow rice (*Oryza sativa* cv. Bengal) and perform the assays as described (35). Briefly, *B. glumae* 336gr-1 and the Δ*dbcA* strain were grown in LB agar without antibiotics at 37°C for 48 h. Bacteria were removed with sterile loops and suspended in sterile water at 5×10^7 CFU/ml. Five milliliters of bacterial suspension were directly sprayed, twice at 2-day intervals, on the panicles at the 20 to 30% flowering stage of rice plant. For the control, sterile water was sprayed on the panicles. The panicle blight symptoms were evaluated at 3, 7, and 10 days after first inoculation by assigning a virulence score on a 0 to 9 scale (36). Virulence scores of 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 indicate no symptom, 1% to 10% symptomatic, 11% to 20% symptomatic, 21% to 30% symptomatic, 31% to 40% symptomatic, 41% to 50% symptomatic, 51% to 60% symptomatic, 61% to 70% symptomatic, 71% to 80% symptomatic, and more than 80% symptomatic panicle, respectively. For panicle assays in the presence of sodium bicarbonate, bacteria were grown in LB agar medium containing either 0 or 5 mM sodium bicarbonate at 37°C for 72 h. LB agar was buffered with 70 mM Tris, pH 7.0. Bacteria were removed with sterile loops and suspended in either sterile water or 5 mM sodium bicarbonate at 5×10^7 CFU/ml. Five milliliters of bacterial suspension was directly sprayed, twice at 2-day intervals, on the panicles at the 20 to 30% flowering stage of rice plant. For the control, 5 ml of sodium bicarbonate was sprayed on the panicles. The panicle blight symptoms were evaluated as described in above. All experiments were repeated three time with three replicates each.

Toxoflavin quantification and thin-layer chromatograph. Extraction and quantification of toxoflavin from liquid and solid culture media were conducted as described (65). For toxoflavin quantification from liquid culture medium, *B. glumae* strains were grown shaking at 37°C in a 250-ml conical flask containing 25 ml fresh LB medium buffered with 70 mM Tris, pH 7.0. The culture supernatant was collected by centrifugation and 1 ml of culture supernatant was extracted with 1 ml of chloroform. The extracted chloroform fraction was transferred to a new microcentrifuge tube and dried in a fume hood at RT. The residue in tubes was dissolved in 1 ml 80% methanol. Absorbance was measured at 393 nm against a toxoflavin-free control and normalized to OD_{600}. Toxoflavin concentration was calculated from a standard curve (see Fig. S4 in the supplemental material).

For toxoflavin quantification from solid medium, *B. glumae* strains were grown in LB agar medium without antibiotics at 37°C for 72 h. The bacterial culture on agar plates was completely removed by scraping the culture with a sterile razor blade. Five grams of agar medium was collected from the plate, cut into small pieces, and soaked in 5 ml of chloroform. The agar medium/chloroform fractions were incubated for 30 min at RT in 50-ml Falcon tubes. One milliliter of the extracted chloroform fraction was aliquoted in new microcentrifuge tubes and dried in a fume hood at RT. The residue in tubes was dissolved in 1 ml 80% methanol. The toxoflavin absorbance and concentration was measured as described above.

Thin-layer chromatography (TLC) analysis for toxoflavin produced by *B. glumae* strains was performed as described previously with some modifications (3). Five milliliters of culture supernatant was extracted with 5 ml of chloroform (vol/vol). The extracted chloroform fraction was transferred to new tubes and dried in a rotary evaporator (Vacufuge; Eppendorf) at 30°C. The residue in tubes was dissolved in 30 μl of 80% methanol. Ten microliters of methanol extract was spotted on a TLC plate (TLC silica gel 60; EMD Millipore Corporation). The TLC plate was developed with chloroform/methanol (95:5, vol/vol), dried, and imaged using 365-nm UV light. Purified toxoflavin was purchased from Cayman Chemical.

Membrane potential. JC-1 dye was used to measure membrane potential (9). All *B. glumae* strains were treated with 6 μM JC-1 in permeabilization buffer (10 mM tris, pH 8.0, 1 mM EDTA, 10 mM glucose). Twenty-five micromolar CCCP was used as a control for loss of $\Delta\Psi$. Bacterial cells were incubated at 30°C in the dark without shaking for 1 h. A JASCO FP-6300 spectrofluorometer was used to measure the fluorescence ratio. To measure membrane potential for bicarbonate-treated bacterial cells, 5 mM sodium bicarbonate was directly added to growth media, and strains were grown until their OD_{600} reached ~0.6.

Statistical analysis. Experiments were repeated three times with three biological replicates. Representative images of virulence assays are included in the figures. The data presented in the graphs indicate the mean ± standard deviation (SD) value for three independent replicates of each treatment.

GraphPad Prism 9.0 was used to produce graphs and calculate the statistical significances by unpaired Student's *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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