Involvement of a Quorum-Sensing-Regulated Lipase Secreted by a Clinical Isolate of \textit{Burkholderia glumae} in Severe Disease Symptoms in Rice\textsuperscript{V}

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\textit{Burkholderia glumae} is an emerging rice pathogen in several areas around the world. Closely related \textit{Burkholderia} species are important opportunistic human pathogens for specific groups of patients, such as patients with cystic fibrosis and patients with chronic granulomatous disease. Here we report that the first clinical isolate of \textit{B. glumae}, strain AU6208, has retained its capability to be very pathogenic to rice. As previously reported for rice isolate \textit{B. glumae} BGR1 (and also for the clinical isolate AU6208), TofR or TofR acyl homoserine lactone (AHL) quorum sensing played a pivotal role in rice virulence. We report that AHL quorum sensing in \textit{B. glumae} AU6208 regulates secreted LipA lipase and toxoflavin, the phytotoxin produced by \textit{B. glumae}. \textit{B. glumae} AU6208 lipA mutants were no longer pathogenic to rice, indicating that the lipase is an important virulence factor. It was also established that type strain \textit{B. glumae} ATCC 33617 did not produce toxoflavin and lipase and was non-pathogenic to rice. It was determined that in strain ATCC 33617 the LuxR family quorum-sensing sensor/regulator TofR was inactive. Introducing the tofR gene of \textit{B. glumae} AU6208 in strain ATCC 33617 restored its ability to produce toxoflavin and the LipA lipase. This study extends the role of AHL quorum sensing in rice pathogenicity through the regulation of a lipase which was demonstrated to be a virulence factor. It is the first report of a clinical \textit{B. glumae} isolate retaining strong rice pathogenicity and finally determined that \textit{B. glumae} can undergo phenotypic conversion through a spontaneous mutation in the tofR regulator.

\textit{Burkholderia glumae} is pathogenic to rice (\textit{Oryza sativa}), producing symptoms that include seedling blight, seedling rot, and grain rot; all these symptoms are often considered manifestations of a single disease which is usually called panicle blight (11, 22, 23, 37, 41). Panicle blight is a recurring problem in rice-producing areas in the United States, Japan, and Korea, and its incidence has been increasing in recent years. The disease causes spikelet sterility and discoloration of the developing grains and appears to be a particular problem under conditions of unusually hot weather, warm nights, and high humidity (38).

Very few genetic or molecular studies related to the virulence of this bacterium on rice have been reported. \textit{B. glumae} is known to produce the phytotoxin toxoflavin \{1,6-dimethylpyrimido[5,4-E]-1,2,4-triazine-5,7(1H,6H)-dione\}, a yellow pigment essential for pathogenicity (23, 37). Toxoflavin production by \textit{B. glumae} reduces the growth of both leaves and roots of rice seedlings, induces chlorotic symptoms on rice panicles, and is responsible for causing bacterial wilt in many field crops (22, 37). The biosynthesis of toxoflavin has been elucidated involving the \textit{toxABCD}E operon and is believed to be synthesized in a pathway common to the synthesis of riboflavin (37). Regulation of the \textit{toxABCD}E operon has been studied recently, and it was demonstrated that the operon is regulated by the LysR family regulator ToxR and that toxoflavin acts as a coinducer (23). Regulation of this operon also requires another regulator called ToxI whose expression is regulated by the ToxI or ToxR quorum-sensing (QS) system (23). \textit{B. glumae} is very closely related to the nine \textit{Burkholderia} species belonging to the \textit{Burkholderia cepacia} complex, which are known for their abilities to colonize several environmental niches and to cause chronic infections in humans (10, 27). Members of the \textit{B. cepacia} complex are not considered significant pathogens for the population of healthy humans but are considered a serious threat for specific groups of patients, such as cystic fibrosis patients (10) and patients with chronic granulomatous disease (CGD) (46). One \textit{Burkholderia} species that is also very closely related phylogenetically to the \textit{B. cepacia} complex is \textit{Burkholderia gladioli}; this species is also pathogenic to rice and has also been associated with chronic infections of cystic fibrosis (40).

In this study we report that a strain of \textit{B. glumae}, isolated from an infant with CGD, retained the ability to cause severe disease in rice. We also investigated the roles of two global regulation systems in panicle blight of this \textit{B. glumae} strain. The two systems, QS and the stationary-phase alternative
Bacterial strains, plasmids, and media. The Burkholderia glumae strains and all plasmids used in this study are listed in Table 1. B. glumae ATCC 33617 is the type strain isolated in Japan from rice (www.atcc.org). B. glumae AU6208 was recovered from a lung abscess from an infant newly diagnosed with CGD and repre-

### Table 1. Plasmids and Burkholderia strains used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description or relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. glumae strains</td>
<td>ATCC 33617T</td>
<td>Burkholderia glumae type strain; wild type</td>
</tr>
<tr>
<td></td>
<td>33617TOFI</td>
<td>tof102::Km of ATCC 33617; Km^r</td>
</tr>
<tr>
<td></td>
<td>33617RPOS</td>
<td>rpoS698::Km of ATCC 33617; Km^r</td>
</tr>
<tr>
<td></td>
<td>AU6208</td>
<td>Burkholderia glumae clinical isolate</td>
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<tr>
<td></td>
<td>AU6208TOFI</td>
<td>tof102::Km of AU6208; Km^r</td>
</tr>
<tr>
<td></td>
<td>AU6208TOFI2</td>
<td>tof1::Km of AU6208; Km^r</td>
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<td>AU6208TOFR</td>
<td>tofR::Km of AU6208; Km^r</td>
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<td>rpoS698::Km of AU6208; Km^r</td>
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<tr>
<td></td>
<td>AU6208LIPA</td>
<td>lip4::Km of AU6208; Km^r</td>
</tr>
<tr>
<td></td>
<td>BGR1</td>
<td>Burkholderia glumae isolate from rice; wild type</td>
</tr>
</tbody>
</table>

### Plasmids

- pMOSBlue: Cloning vector; Ap^r
- pBluescript KS: Cloning vector; Ap^r
- pUC4K: Vector Km^r resistance cassette; Ap^r Km^r
- pKNOCK-Km: Conjugative suicide vector
- pOE30: Expression vector
- pRK2013: Km^r Tra^r Mob^+; CoE1 replicon
- pHI1J1: IncP1; Gm^r
- pMP220: Promoter probe vector; IncP; Te^r
- pLAFR3: Wide-host-range cosmid vector; IncP Te^r
- pCOSGlu: pLAFR3 containing ATCC 33617 DNA
- pMPTOFIR: pMP220 containing tofR gene of strain AU6208
- pLG124: pLAFR3 containing tofI and tofR genes of ATCC 33617 in a 6.5-kb XhoI fragment from pCOSGlu
- pBGLU: pBluescript KS containing tofI and tofR genes of ATCC 33617 in a 2.4-kb XhoI fragment from pCOSGlu
- pBGLU2: pBluescript KS containing tofI and tofR genes of ATCC 33617 in a 6.5-kb BglII-XhoI fragment from pCOSGlu
- pLG24::Km: pLGL24 with a Km cassette in tofI
- pMOGL6: pMOSBlue containing 5.5-kb PvuII fragment of B. glumae AU6208 DNA
- pCOSBR-A: pLAFR3 containing ATCC 33617 DNA
- pMPTOFIR: pMP220 BamHI-XbaI fragment from pMOGL6 containing tofI and tofR genes of strain AU6208
- pBPR-A: pBluescript KS containing rpoS of ATCC 333617 in a 6.0-kb SmaI fragment from pCOSGlu
- pCOSBR::Km: pCOSBR-A with a Km cassette in rpoS
- pKmlipA: Internal PCR fragment of B. glumae AU6208 lip4 cloned in pKNOCK-Km

### MATERIALS AND METHODS

Sigma factor RpoS, are known to play critical roles in plant-bacterium interactions (45). QS is a cell-density-dependent regulation system that relies on signal molecules that accumulate in the medium to which bacteria respond at quorum concentration via transcriptional regulation of target genes. N-acyl homoserine lactone (AHL) signal molecules are most commonly used in gram-negative bacteria. They are produced by an AHL synthase enzyme which most commonly belongs to the LuxI family. At quorum concentrations, AHLS then form a complex with a transcriptional regulator belonging to the LuxR family which then affects the transcriptional status of target genes (15). QS-dependent regulation is most beneficial to a community of bacteria, for example for bacteria producing extracellular enzymes and virulence factors (15, 45, 47). AHL QS in several phytopathogenic bacteria regulates virulence factors, for example in extracellular enzyme production in Erwinia carotovora, conjugation in Agrobacterium tumefaciens, and as previously mentioned, toxin production in an environmental isolate of B. glumae. AHL QS also plays a very important role in the human chronic lung infections caused by Pseudomonas aeruginosa and members of the B. cepacia complex in patients with cystic fibrosis (12, 34, 43). The stationary-phase RpoS sigma factor regulates many genes in stationary phase and is considered a master stress response (17, 21, 42). RpoS has also been reported to play important roles in plant-bacterium interactions in both plant pathogenic and plant beneficial bacteria (4, 8, 9, 29). The RpoS and QS regulons can overlap, meaning that the two global regulatory systems can cross-regulate each other and/or regulate a similar set of genes (33, 42). In the clinical B. glumae isolate reported here, AHL QS played a crucial role in rice pathogenesis, whereas the stationary-phase RpoS sigma factor did not. In addition, it was established that a lipase, which was regulated by QS, was involved in rice pathogenicity. We also determined that a non-pathogenic strain of B. glumae had lost its capacity to cause disease in rice, since it contained a nonfunctional AHL QS system. The rice-Burkholderia model could be a valuable system to study Burkholderia pathogenesis.
FIG. 1. (A) Gene map of the 4.7-kb XhoI fragment which contained the AHL quorum-sensing system of *B. glumae* ATCC 33617\(^T\). The *tofI* and *tofR* genes are shown, as is the position of the Km resistance cassette cloned in order to construct *tofI* genomic knockout mutants. The thick line from positions 1 to 3200 denotes region that has been sequenced and deposited in data banks. (B) Gene map of the 5.5-kb *Pvu*II fragment which contained the *rpoS* gene of *B. glumae* ATCC 33617\(^T\). The *rpoS* gene is shown, as is their precise position within the clone. The thick line from positions 1 to 3500 denotes region that has been sequenced and deposited in data banks. (C) Gene map of the 2.4-kb *Smal* fragment which contained the *rpoS* gene of *B. glumae* ATCC 33617\(^T\). The *rpoS* gene is shown, as is the position of the Km resistance cassette cloned in order to construct *rpoS* genomic knockout mutants. The thick line from positions 1 to 2474 denotes region that has been sequenced and deposited in data banks. (D) Thin-layer chromatographic (TLC) analysis of AHLs produced by *B. glumae* strains. Bacterial AHL biosensor *Chromobacterium violaceum* CVO26 was overlaid on the TLC. AHLs from *B. glumae* strains were purified from a culture volume, and the amount loaded on the TLC corresponded to \(5 \times 10^6\) CFU. Lane 1, ATCC 33617; lane 2, 33617TOFI; lane 3, 33617RPOS; lane 4, AU6208; lane 5, AU6208TOFI; lane 6, AU6208RPOS; lane 7, 600 ng of C\(_6\)-AHL and C\(_8\)-AHL standards. See text for all details.

sent the first *B. glumae* strain reported to cause human infection (46). Its identification as *B. glumae* was confirmed by comparison of its whole-cell protein profile to those of several *Burkholderia* reference strains, including representatives of the related species *B. gladioli* and *B. plantarii*. Further, sequence analysis of its complete 16S rRNA gene demonstrated 99.9% similarity with the 16S rRNA gene of *B. glumae* ATCC 33617\(^T\) (46). *Chromobacterium violaceum* CVO26 is a double mini-Tn\(5\) mutant derived from ATCC 31532; this mutant is nonpigmented, and production of the purple pigment can be induced by providing exogenous AHL inducer molecules (28). Escherichia coli DH5\(\alpha\) (36) and *E. coli* HB101 (31) were used. *Burkholderia* strains were routinely grown in KB medium (24) or minimal M9 medium (31) supplemented with citric acid (0.3%) as the carbon source. Antibiotics for *Burkholderia* growth were added to media at the following concentrations: kanamycin, 100 \(\mu\)g/ml; gentamicin, 100 \(\mu\)g/ml; and tetracycline, 40 \(\mu\)g/ml. Antibiotics for *E. coli* growth were added to media at the following concentrations: ampicillin, 100 \(\mu\)g/ml; kanamycin, 100 \(\mu\)g/ml; gentamicin, 20 \(\mu\)g/ml; and tetracycline, 10 \(\mu\)g/ml.

Recombinant DNA techniques, conjugation, and construction of cosmids gene bank. Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, Southern hybridization, and transformation of *E. coli* were performed as described previously (31). Analytical amounts of plasmids were isolated as described previously (6), whereas preparative amounts were purified with QIAGEN columns. Total DNA from *Burkholderia* sp. was isolated by the sarcosyl-propanase lysis method (5). Triparental matings from *E. coli* to *B. glumae* were performed with the helper strain *E. coli* DH5\(\alpha\)(pRK2013) (13). A *B. glumae* ATCC 33617\(^T\) cosmid library was constructed by cloning partially digested EcoRI genomic DNA in pLAFR3 (36), which was then packaged into lambda phages using the Gigapack III XL kit as described by the supplier (Stratagene, La Jolla, CA) and subsequently used to infect *E. coli* HB101 cells.

Purification, detection, and visualization of signal (AHL) molecules. The purification, detection, and visualization of AHL signal molecules from culture supernatants were performed essentially as described previously (1). Synthetic AHLs were purchased from Fluka Chemie AG (Buchs, CH).

Cloning of the *tofI* and *tofR* QS genes of *B. glumae* and inactivation of *tofI* and *tofR*. About 4 \(\times\) 10\(^6\) cells each of *E. coli* HB101 harboring the *B. glumae* ATCC 33617\(^T\) cosmid library, *E. coli*(pRK2013), and 2 \(\times\) 10\(^6\) cells of *C. violaceum* CVO26 were mixed. The suspension was applied to a 0.45-\(\mu\)m filter (Millipore Corp.) on an LB plate. After overnight incubation at 30°C, the cells were resuspended and spread on LB plates containing ampicillin (100 \(\mu\)g/ml), kanamycin (100 \(\mu\)g/ml), streptomycin (100 \(\mu\)g/ml), and tetracycline (20 \(\mu\)g/ml). Strain CVO26 is naturally resistant to ampicillin and streptomycin and is resistant to kanamycin due to the mini-Tn\(5\) present in the chromosome. Tetracycline will result in selection of transconjugants having received the pLAFR3-based cosmid clone. These plates were incubated for 48 h at 30°C, and transconjugants that turned purple were further assayed. One cosmid (pCOSGLU) from the cosmid library could restore purple pigmentation in strain CVO26. Further subcloning experiments confirmed that a 2.4-kb XhoI fragment cloned in pLAFR3 (creating pLGLU24) could restore pigmentation in strain CVO26. The 2.4-kb XhoI fragment was cloned in the corresponding site in pBluescript KS (generating pBGLU2) in order to completely
sequence tofR (Fig. 1A). In order to create a tofI knockout genomic mutant of strain ATCC 33617, a Km resistance gene cassette from pUC4K was cloned in the PvuII site of the tofI gene at position 102 in plasmid clone pGLU24, creating pGL24::Km (Fig. 1A). This latter plasmid was homogenized with the corresponding target regions in pCOSGLU, creating pCOSGLUKm, which was subsequently used in marker exchange experiment using pPH1JI as the incompatible incoming plasmid. This generated a tofI genomic mutant designated B. glumae 33617::TOF1. The fidelity of the marker exchange event was confirmed by Southern analysis (data not shown).

The tofI or tofR locus was localized by Southern hybridization analysis and cloned from B. glumae AU6208 as a 5.5-kb PvuII fragment in pMOSBlue, creating pMOSGLU. The tofI and tofR genes contained this fragment and were sequenced (Fig. 1B). A subclone of this locus was pMPTOFR, which consisted of an EcoRI-KpnI fragment containing tofR from pMOMGLU6 cloned in pMP220. Having established that the tofI gene of strain AU6208 was almost 100% identical at the nucleotide level with that of strain ATCC 33617; plasmid pCOSGLUKm was used to create a gdhl knockout mutant derivative of strain AU6208 as described above. The resulting mutant was designated AU6208::TOF1; this mutant also acquired a nonfunctional tofI gene originating from strain ATCC 33617 (see Results for details).

Cloning and inactivation of the tofR gene of B. glumae. The tofR gene of B. glumae ATCC 33617 was identified by screening the cosmid gene bank using the tofR gene of closely related B. cepacia as a probe (the probe consisted of a 1-kb PstI fragment of PRBS-3 [2]). A cosmid designated pCOSBR-A was identified, and the tofR gene was further localized in a 6-kb Smal fragment (Fig. 1C), which was subsequently cloned in the corresponding site in pBluescript KS, yielding pCOSBR::Km. The latter plasmid was used in a marker exchange technique (see above) in order to create a tofR knockout mutant in strain AU6208 as described previously (2), generating AU6208::TOF2 and AU6208::TOFR, respectively. The fidelity of the marker exchange events were confirmed by Southern analysis (data not shown).

Cloning and inactivation of the rpoS gene of B. glumae. The rpoS gene of B. glumae ATCC 33617 was identified by screening the cosmid gene bank using the rpoS gene of closely related B. cepacia as a probe (the probe consisted of a 1-kb PstI fragment of PRBS-3 [2]). A cosmid designated pCOSBR-A was identified, and the rpoS gene was further localized in a 6-kb Smal fragment (Fig. 1C), which was subsequently cloned in the corresponding site in pBluescript KS, yielding pCOSBR::Km. The cloned rpoS gene from B. glumae 33617 was inactivated by cloning in its EcoRRI site at position 698. A Km resistance cassette from pUC4K (Fig. 1C) in pCOSBR-A, yielding pCOSBR::Km. This latter plasmid was used in a marker exchange technique (see above) in order to introduce site-specific insertions in the rpoS gene of B. glumae ATCC 33617, creating a rpoS knockout mutant designated 33617::RPO1. The fidelity of the marker exchange event was confirmed by Southern analysis (data not shown). The rpoS gene of pCOSBR-Km was also used in the same marker exchange technique in order to create a rpoS knockout mutant in B. glumae AU6208, which was designated B. glumae AU6208::RPO1.

Analysis of B. glumae secreted proteins and inactivation of the lipA gene of B. glumae AU6208. Total secreted proteins were isolated and characterized as follows. Cells from 10-ml overnight cultures were pelleted by centrifugation for 10 min at 8,000 × g. Cells remaining in the supernatant were removed by an additional centrifugation step for 3 min at 15,000 × g. Proteins in the cell-free supernatant were then precipitated with 10% (wt/vol) trichloroacetic acid, dried, and resuspended in sample buffer. After the suspension was boiled for 10 min, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels containing 12% (wt/vol) polyacrylamide.

The selected protein band was identified as follows: the band was cut out from the Coomassie brilliant blue-stained gel (see Fig. 3A) and placed in a siliconized microcentrifuge tube that had been rinsed with water and ethanol. The band was digested with trypsin, and the resulting peptides were extracted with water and 60% acetonitrile-1% trifluoroacetic acid. The fractions were then analyzed by mass spectroscopy (an internal sequence analysis of the protein spots was performed by using an electron spray ionization mass spectrometer [LCQ DECA XP; Thermofinnigan]), and the lipase protein was identified by analysis of the peptides and by using protein data banks.

The B. glumae AU6208 lipase gene encoding the extracellular lipase was in part amplified by PCR in a 541-bp fragment using primers 5′-GAGCGGATCCCTCACATGTTGAG-3′ and 5′-GGCCCAAGTACACTCAAGCACGGG-3′ and cloned as a BamHI-XbaI fragment in pKNOCK-Km (2), generating pKmLipA. This latter plasmid was used as a suicide delivery system in order to create a lipA knockout mutant in strain AU6208 as described previously (2), generating AU6208::LIPA. Similarly, pKmLipA was used to generate a lipA knockout mutant in B. glumae ATCC 33617, generating strain 333716::LIPA. The fidelity of the marker exchange events was confirmed by Southern analysis (data not shown). Lipase enzyme assays were performed as previously described (12).

Rice plant pathogenicity and toxoflavin assays. For panicle blight assays, rice plants (Oryza sativa cv. Miyang 23) were grown in a greenhouse, inoculated at the flowering stage with a bacterial suspension (107 to 108 CFU/ml), and kept in a greenhouse. Disease in the rice plants was evaluated daily for 10 days, using the following scale: 0 for a healthy panicle, 1 for a panicle 0 to 20% discolored, 2 for a panicle 20 to 40% discolored, 3 for a panicle 40 to 60% discolored, 4 for a panicle 60 to 80% discolored, and 5 for a panicle 80 to 100% discolored. Disease severity was determined using the following: disease degree = (number of samples with each rating × rating value)/total number of samples.

For the seed germination assay, B. glumae strains were grown in KB liquid medium at 37°C and seeds of rice (Oryza sativa cv. Baloro) were pregerminated at 37°C in a bacterial suspension of 5 × 105 CFU/ml. Two days later, germinating seeds were transferred to a growth chamber under 30°C ± 6°C and high relative humidity (close to 100%) in petri plates, under 400-V lamps with a 16-h-light–8-h-darkness photoperiod. The disease evaluation was performed 7 days after inoculation, using a severity scale from 1 to 6. The severity scores were as follows: 6 for coleoptile and phumle macerated, not standing upright; 5 for aerial plant part developed but completely discolored or growth limited to less than 1 cm high; 4 for aerial plant part developed but partially discolored on more than 50% of surface; 3 for aerial plant part developed but partially discolored on less than 50% of surface; 2 for completely green seedlings but roots and aerial part less vigorous compared to noninfected control; and 1 for completely green seedlings and as vigorous as the noninfected control. The experiments were repeated three times. Score ratings were analyzed statistically in the SPSS program using the Kruskal-Wallis multiple-comparison test and the Mann-Whitney comparison test. Score ratings were grouped into three classes for visual presentation as follows: class 1; scores 1 and 2; class 2; scores 3 and 4; and class 3; scores 5 and 6.

The phytotoxins and toxoflavin assay have been previously described (21).

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in GenBank/EMBL/DDJB under the following accession numbers: B. glumae ATCC 33617 tofI and tofR genes, AM422469; B. glumae AU6208 tofI and tofR genes, AM22470; and B. glumae ATCC 33617 rpoS gene, AM422471.

RESULTS

Clinical isolate Burkholderia glumae AU6208 is very pathogenic to rice. The ability of the first clinical isolate of B. glumae, strain AU6208, to induce panicle blight was determined by inoculating rice panicles during the flowering stage. Strain AU6208 caused severe symptoms (disease index of 3.9; Fig. 2A), indicating that this strain has retained its ability to cause disease in rice. Similarly, in a seed germination assay, strain AU6208 displayed severe disease symptoms (Fig. 2B). Interestingly, B. glumae ATCC 33617 type strain did not display disease symptoms in either the panicle blight and seed germination assay (data not shown).

Roles of the tofI and tofR QS system and the rpoS stationary-phase sigma factor in B. glumae AU6208 rice pathogenesis. In order to determine whether B. glumae AU6208 produces AHL signal molecules, AHLs were purified and detected from spent supernatant when grown in minimal M9 medium supplemented with 0.2% (wt/vol) glucose as the carbon source. As shown in Fig. 1D, strain AU6208 and type strain ATCC 33617 produced two AHL signal molecules with an aliphatic chain shown in Fig. 1D, strain AU6208 and type strain ATCC 33617 produced two AHL signal molecules with an aliphatic chain. Specifically, pKmlipA was used to generate a lipA knockout mutant in B. glumae ATCC 33617, generating strain 333716::LIPA. The fidelity of the marker exchange events was confirmed by Southern analysis (data not shown). Lipase enzyme assays were performed as previously described (12).

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sequenced (Fig. 1A and B). TofI and TofR of strain AU6208 were highly identical (99% identity) to the AHL QS system TofI and TofR of \( B. \) glumae BGR1 and PlaI and PlaR of \( B. \) plantarii ATCC 43733T and were highly homologous (approximately 75% identity) to CepI and CepR AHL QS system of the species belonging to the \( B. \) cepacia complex (1, 20, 23, 25, 35, 43). The TofI protein of strain ATCC 33617T was also identical to the other \( B. \) glumae TofI proteins; however, the TofR protein of this strain was 110 amino acids longer. The TofR proteins of strains AU6208 and BGR1 are 239 amino acids long, whereas TofR of strain ATCC 33617T was found to be 349 amino acids long. The first 238 amino acids are identical; however, repeated DNA sequencing of both strands revealed that the translational stop codon of the tofR of strain ATCC 33617T was present over 300 bp downstream, resulting in a protein of 349 amino acids.

The tofI AHL synthase of \( B. \) glumae AU6208 was inactivated, generating AU6208TOFI, and this mutant no longer produced the \( C_6 \) and \( C_8 \)-AHL (Fig. 1D), indicating that it was responsible for producing the two AHLs and that TofR, due to its genetic location and identity to CepR (see above), was therefore most probably sensing and responding to \( C_8 \)-AHL. In order to determine whether AHL QS of strain AU6208 played a role in rice pathogenicity, panicle blight and seed germination assays were performed with the AU6208TOFI knockout mutant. The AHL QS deficient mutants displayed significant decreases in the ability to cause panicle blight (disease index of 0.5; Fig. 2A) and also significant decreases in disease symptoms in the seed germination assay (Fig. 2B). It was established that as in \( B. \) glumae strain BGR1 (21) and also in strain AU6208, AHL QS regulates toxoflavin production (Fig. 3C).

The \( B. \) glumae rpoS gene consisted of 1,094 nucleotides (Fig. 1C) encoding a protein of 364 amino acids displaying over 90% identity with other RpoS proteins of \( Burkholderia \) spp. (data not shown), 77% with RpoS of \( Ralstonia \) solanacearum and approximately 50% with RpoS sigma factors from \( Gammaproteobacteria \). The \( B. \) glumae AU6208RPOS mutant was as pathogenic to rice as the wild-type parent strain in both panicle and seed germination assays (Fig. 2A and C).

The TofI-TofR system of \( B. \) glumae AU6208 regulates a lipase which is involved in rice pathogenicity. Once established that the TofI-TofR system was a major player in rice pathogenicity of strain AU6208, we analyzed the profile of secreted proteins of the wild-type strain versus the profile of the AHL QS mutant AU6208TOFI in order to determine whether any

![FIG. 2. Pathogenicity assays of \( B. \) glumae and mutant derivatives. (A) Panicles were inoculated with the wild-type AU6208 strain, with water only, with mutant AU6208TOFI (TOFI), with mutant AU6208LIPA (LIPA), or with mutant AU6208RPOS (RPOS) as described in Materials and Methods. Panicles inoculated with the AHL QS mutant TOFI and LIPA mutants were almost asymptomatic, whereas the parent wild-type clinical isolate AU6208 caused severe symptoms. The photographs were taken 7 days after inoculation, and the numbers below the photographs refer to the disease index as described in Materials and Methods. (B) Typical disease symptoms of seeds inoculated with the AHL QS mutant TOFI and LIPA mutants were almost asymptomatic, whereas the parent wild-type clinical isolate AU6208 caused severe symptoms, as there was no germination. (C) Evaluation scale of pathogenicity assay using the seed germination assay of \( B. \) glumae infections. The disease evaluation was performed 7 days after inoculation, using a 1-to-6 disease severity scale as described in Materials and Methods. Scores were grouped into three classes for visual presentation as follows: class 1, scores 1 and 2; class 2, scores 3 and 4; and class 3, scores 5 and 6. WT, wild type; RPOS, AU6208RPOS; LIPA, AU6208LIPA; TOFI, AU6208TOFI; Water, only distilled sterile water. See the text for all details.](http://aem.asm.org)
secreted proteins were regulated by AHL QS. In this experiment we also included the type strain ATCC 33617 and tofI knockout mutant derivative 33617TOFI. As depicted in Fig. 3A, in ATCC 33617T and tofI mutant derivative 33617TOFI, the secreted protein profiles were identical, whereas in AU6208TOFI, a secreted protein of approximately 30 kDa was no longer present compared to the parent strain, indicating that AHL QS was important for its production. This protein was digested with trypsin and analyzed by mass spectroscopy resulting in the determination of the following peptides: VYVANLSGFQSDDGPNGR, GEQLLAYVK, VNLIGHSQGGLTSR, GSEFADFVQDV, and ASGQNDGLVSR. By performing a BLAST analysis, the peptides were 100% identical to parts of the identified and characterized lipase LipA of \textit{B. glumae} (14) (accession number CAA01279). Apparently this protein was not present in both spent supernatants of \textit{B. glumae} ATCC 33617T and tofI knockout derivative 33617TOFI, indicating that under the conditions tested, this lipase was not produced by this strain (Fig. 3A). In addition, we performed lipase enzyme assays and determined that the tofI mutant of strain AU6208 displayed considerably less lipase enzyme activity than the parent wild-type strain (Fig. 3B). The stationary-phase RpoS sigma factor was found not to be involved in lipA or LipA regulation (Fig. 3B).

A lipA mutant, designated AU6208LIPA, was constructed and tested in a rice panicle blight and seed germination assay. As can be seen in Fig. 2, the lipA mutant has dramatically lost its capacity to cause disease in rice in both pathogenicity assays, making it an important virulence factor.

\textbf{TofR of \textit{B. glumae} ATCC 33617T is nonfunctional.} It was observed that when we tried to complement the \textit{B. glumae} clinical isolate tofI mutant AU6208TOFI for lipase activity by adding 1 \mu M C\textsubscript{8}-AHL to the growth media, we found that this did not result in the restoration of lipase enzyme activity. We did however observe complementation for lipase activity if the tofR gene of strain AU6208 was provided in \textit{trans} in strain.
AU6208TOFI via plasmid pMPTOFR together with exogenously provided 1 μM C6-HSL (Fig. 3B). The reason for this is that AU6208TOFI was constructed via a marker exchange technique using a plasmid incompatibility technique using the tofR or tofI locus having tofR::Km and surrounding genetic loci of strain ATCC 33617T (as described in Materials and Methods). This marker exchange resulted in the transfer to the AU6208 chromosome of the tofI::Km locus as well as the wild-type tofR locus of B. glumae strain ATCC 33617T. The AU6208TOFI strain was also complemented for lipase activity when both the tofI and tofR genes of strain AU6208 were introduced in the mutant with plasmid pMPTOFR (data not shown). As mentioned above, the TofR protein of strain ATCC 33617T is 100 amino acids longer and could be nonfunctional. Importantly, it was also determined that if the tofR gene of strain AU6208 was provided in trans in strain ATCC 33617T via plasmid pMPTOFR, the strain was then also able to regain its ability to produce toxoflavin as well as lipase activity (Fig. 3B and C). It was therefore concluded that TofR of strain ATCC 33617T was nonfunctional and most probably the major reason why the strain did not produce toxoflavin and lipase enzyme.

Having established that the AU6208TOFI mutant constructed above was a tofIR double mutant (tofI::Tn5 and non-functional tofR from strain ATCC 33617T), we constructed tofI and tofR single mutants in order to clarify the role of each component of the AHL QS system. Consequently, tofI and tofR mutants, designated AU6208TOFI and AU6208TOFR, respectively, were constructed as described in Materials and Methods. Interestingly, it was observed that the single tofI mutant displayed considerably slow and very poor growth in rich medium, while the tofR mutant displayed comparable growth compared to the parent strain. AU6208TOFIR was less pathogenic to rice and could be complemented for virulence by introducing in trans pMPTOFR (data not shown); this mutant however was more pathogenic than AU6208TOFI, indicating that both TofR and AHL are necessary for maximum virulence. Strain AU6208TOFI2 (single tofI knockout mutant) could not be tested due to the very poor growth ability and low survival rates of this strain.

DISCUSSION

B. glumae causes disease to rice grains and seedlings and has emerged in the last 10 years as the most important bacterial pathogen of rice in Japan, Korea, Taiwan, and the United States (41). In addition, B. glumae can cause wilting symptoms in sunflower, tomato, sesame, perilla, eggplant, and hot pepper (22). The observation that it can cause disease in nontraditional crops could indicate that B. glumae could be an opportunistic phytopathogen of several plants rather than a primary pathogen of rice. In this report, we present evidence that the B. glumae strain recovered from the first reported case of human infection by this species retains the capability of causing severe disease in rice. This raises the possibility that as B. glumae rice epidemics increase, there could be an increasing hazard of high-risk individuals being infected with B. glumae. B. gladioli is another rice pathogen, which is phylogenetically very close to B. glumae, which is believed to be an opportunistic pathogen in humans, as it has been recovered from clinical specimens (10, 26). At present, it is not known whether these species could become part of the B. cepacia complex thus having the potential of causing chronic infections in high-risk individuals, such as cystic fibrosis patients. The possibility that they can form mixed communities with members of the B. cepacia complex in chronic infections also cannot be excluded.

It was determined that in clinical isolate B. glumae AU6208 and in B. glumae ATCC 33617T, AHL QS is present and very well conserved with the TofI-TofR system of B. glumae BGR1 (23), the Phl-PlaR system of B. plantarii (35), and the CepI-CepR system of members of the B. cepacia complex (12, 43). AHL QS in members of the B. cepacia complex regulate several important processes including those involved in virulence (12, 43). The system in strain AU6208 is playing a pivotal role in rice pathogenesis, since in two different assays, the tofI synthase knockout mutant dramatically lost its pathogenicity towards rice. It was previously also observed that in B. glumae BGR1, AHL QS is involved in regulating the expression of toxoflavin genes indirectly (23); thus, it is very likely that the TofI-TofR system could also be involved in a similar regulatory cascade of toxoflavin regulation in strain AU6208. In strain AU6208, the secreted LipA lipase was determined to be regulated by AHL QS, since the protein was produced in much lower quantities in the AU6208TOFI strain, and lipase activity was significantly reduced. In Burkholderia thailandensis, a non-pathogenic species very closely related to the human pathogen Burkholderia pseudomallei, lipase production was found to be both positively and negatively regulated by AHL QS (39). In B. cepacia complex member Burkholderia cenocepacia, lipase production is dependent on the growth phase, with maximal activity produced in stationary phase. AHL QS mutants of B. cenocepacia produced 40 to 50% less lipase activity than the parent strain at stationary phase (25). For both B. thailandensis and B. cenocepacia however, the genetic determinants encoding lipase enzymes that are regulated by AHL QS have not been determined. Importantly, the B. glumae AU6208 lipA mutant dramatically lost its capability to cause rice pathogenicity in two different assays. The reason most probably being that the LipA lipase plays a crucial role as a cell wall-degrading enzyme; this enzyme in cooperation with other cell wall-degrading enzymes breaks down components of cell walls. Plant cell walls are composed of cellulose and xylan components which provide strength and rigidity as well as acting as a barrier for pathogens. Few lipases have been reported to be virulence factors in microbial phytopathogens; a secreted lipase of fungal pathogen Fusarium graminearum is important in causing virulence to wheat and maize (44). Similarly, in rice BLAST pathogen Xanthomonas oryzae pv. oryzae, a lipase has been reported to be involved in pathogenicity (30). Lipases may be involved in hydrolyzing ester bonds in xylan, thus promoting xylan degradation; alternatively, they could also be involved in the degradation of epicuticular waxes which cover host epidermal cells, providing an efficient barrier. Lipases are ubiquitous, having been found in animals, plants, fungi, and bacteria, and are also being studied for their many potential industrial applications (7). Importantly, we determined that the expression of lipA in B. glumae is AHL QS dependent, and at present, it is not known whether it is direct or indirect via another regulator. The LipA protein of B. glumae is secreted by the type II secretion pathway; thus, the possibility that AHL QS regulates the gene(s) encoding the protein(s) involved in secretion and
not the lipase structural gene cannot be excluded. However, the observation that the secretion profile of the wild type
versus the AHL QS mutant differs only in this protein is an
indication that QS most probably regulates the lipA structural
gene. Therefore, in B. glumae, two pivotal virulence targets for
AHL QS have now been identified (toxoflavin and lipase pro-
duction), and the degree of cross talk and/or synergy merits
attention.

Interestingly, it was established that the type strain B. glumae
ATCC 33617, originally isolated from diseased rice, has lost its
ability to be pathogenic to rice. Under laboratory growth con-
tions, the type strain is not yellow pigmented like strain
AU6208, and it has lost the capability to produce the yellow
phytoxic pigment toxoflavin. It is possible that the phyto-
pathogenic B. glumae strain can undergo phenotypic variation
due to spontaneous mutations which result in morphological
variants that do not express secondary metabolites. These mu-
tations are most likely to occur in global regulatory genes. This
variation or phenotypic conversion is known to occur in many
bacterial species in order to generate population diversity
thought to be important for niche adaptation (32). Interest-
ingly, the ToFR AHL receptor of strain ATCC 33617\textsuperscript{T}
was found to be 110 amino acids longer and was determined to
be not functional. A spontaneous mutation most probably oc-
curred, resulting in a mutated ToFR; thus, AHL QS is not
functional. A spontaneous mutation most probably oc-
curred, resulting in a mutated ToFR; thus, AHL QS is not

In summary, in this report we describe a B. glumae clinical
isolate (i) which has retained the capability of causing severe
virulence to rice (ii) wherein AHL QS is important for its
pathogenicity (iii) in which the lipA lipase is regulated by AHL
QS and is very important for rice pathogenicity and (iv) that
RpoS does not play a role in pathogenesis and regulation of
AHL QS. In addition, B. glumae could undergo phenotypic
conversion through a mutation in tofR, rendering its patho-
genic potential unstable when this species is grown under lab-
oratory conditions. This study highlights that B. glumae could
be an important bacterium to address for studying Burkhold-
eria pathogenesis having the convenient and reliable rice-
pathogen model. Finally, following the increasing importance
of this plant pathogen, understanding the major virulence fac-
tors is an important step towards designing possible control
mechanisms of this important disease of a very important
cereal.

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