Toxoflavin is an Essential Factor for Virulence of *Burkholderia glumae* Causing Rice Seedling Rot Disease

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Abstract

Mutants of *Burkholderia glumae*, the causal agent of rice seedling rot and grain rot diseases, were isolated by transposon (Tn) mutagenesis with Tn4431 harboring a tetracycline (Tc) resistance gene, which was carried on the suicide vector pUCD623. The resulting Tc-resistant colonies were screened for a loss of toxin production as well as for a lack of pathogenicity to rice seedlings. One of the screened mutants, the mutant No. 19, was shown to have a single copy of Tn insertion on the genomic DNA by Southern hybridization test. Restriction enzyme fragments containing Tn4431-flanking sequences from the genomic DNA of mutant No. 19 were cloned and used as probes for colony hybridization of the genomic DNA cosmid library from a wild strain of *B. glumae*. Six recombinant clones showing homology to the probes were isolated and conjugated into mutant No. 19 by triparental mating. The recombinant cosmid pNP147, one of six clones, was able to complement the mutant No. 19 for toxin production and pathogenicity. The toxin, purified from a culture of mutant No. 19 with pNP147, was identified as toxoflavin by NMR and Mass spectra analyses. These results suggest that toxoflavin is a critical factor in disease development of *B. glumae*, since the nontoxigenic mutants were unable to induce seedling rot disease to rice plants.

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INTRODUCTION

*Burkholderia glumae* (synonym: *Pseudomonas glumae*) is a bacterium causing rice grain rot in paddy fields and seedling rot in rice transplant nursery boxes. Seedling rot has increased, in particular, because of the expanded use of nursery boxes, which promote the high temperature and high humidity environment favorable for bacterial infection. Diseased rice plumules or seedlings brown at an early stage and later rot and die. The mechanism of seedling rot or grain rot of rice has been suggested to involve bacterial toxins. Sato *et al.* isolated two toxins from a culture filtrate of *B. glumae*, identified them as toxoflavin and fervenulin, and showed that the toxins induced a chlorotic spot on leaves of rice seedlings at a concentration of about 10 µg/ml. Iiyama *et al.* also confirmed that the phytotoxin produced by *B. glumae* was closely associated with its virulence. These reports suggested that toxin production by *B. glumae* is responsible for the symptoms produced with rice seedling rot and grain rot diseases.

To better understand virulence of *B. glumae*, we used bacterial transposon mutagenesis which is able to destroy the genes encoding toxin-biosynthetic or virulence-related pathways. As a result of transposon Tn4431 mutagenesis, we isolated a nontoxigenic and avirulent mutant of *B. glumae*, and cloned the genes involved in toxin production by complementation of the mutant with a genomic DNA library of the wild strain.

Here, we report the isolation and characterization of Tn4431 insertion mutants of *B. glumae*, and the cloning of genes involved in toxin production. In addition, we demonstrate that the toxin produced by *B. glumae* is identical to toxoflavin and plays an important role as an essential factor in symptom development.

MATERIALS AND METHODS

**Bacterial strains and plasmids** The sources and relevant characteristics of bacterial strains and plasmids used in this study are shown in Table 1. The cosmid pUCD3101, which is derived from pLAFR3 constructed by Staskawicz *et al.*, has a neomycin phos-
phototransferase gene in place of a tetracycline (Tc) resistance gene, and also has an ori locus from pBR322 at an EcoRI site of multicloning sites on the cosmid.

Transposon Tn4431 mutagenesis of B. glumae
The suicide plasmid pUCD623 with a lux cassette was used to generate Tn4431 insertions in B. glumae B446. The donor E. coli HB101 with pUCD623 was allowed to conjugate with B. glumae by incubating them together on Luria-Bertani (LB) agar medium overnight at 30°C. The mixture was spread onto B. glumae-specific selection medium (S-PG)10 supplemented with 25 μg/ml of Tc to isolate the Tn4431 insertion mutants of B. glumae.

Toxin production and pathogenicity assay To assay for toxin production, the Tn4431 insertion mutants of B. glumae were grown on a CaPG agar plate (peptone 5g, glucose 5g, CaCl₂·H₂O 1g, agar 20g in 1000ml decoction of potato 200g, pH 6.8) containing 5 g/ml of Tc for 24hr at 37°C, and assayed by the production of yellow pigments characteristic of the toxin, the appearance of a toxin spot with a yellowish color on TLC, and a fluorescence under UV irradiation4).

Pathogenicity was assayed by the ability of B. glumae to elicit seedling rot of rice or to cause easily visible, yellow-haloed lesions on leaves of Mioga plants (Zingiber mioga Rosc.)1). Rice seeds (Oryza sativa L. cv. Kinmaze) were sterilized with a 2% sodium hypochlorite solution, then germinated in distilled water. Germinated seeds were inoculated by incubating them for 3 hr at 32°C in a cell suspension of 4 × 10⁸ cfu/ml of the wild or mutant strains that was prepared from water-rinsed cells of an overnight culture. After air-drying, inoculated seeds were grown in a growth chamber at 32°C, for 2 weeks, before symptom development was observed. Detached leaves of Mioga plants were inoculated with 2 μl of 1 × 10¹⁰ cfu/ml suspension of either the wild or mutant bacteria by a needle inoculation method. Inoculated leaves were kept on a water-soaked filter paper in a petri dish at 30°C to develop the lesions.

Construction of genomic library Genomic DNA of B. glumae was partially digested with SAI 3A I and size-fractionated by sucrose density gradient centrifugation. DNA fragments ranging from 20 kb to 35 kb in size were collected and ligated into the cosmid vector pUCD615 for Tn4431 insertion mutants and pUCD3101 for the wild strain. Recombinant molecules were packaged in vitro and transfected into E. coli DH5α for construction of a genomic library. General molecular biology techniques followed the methods of Sambrook et al.6).

Southern blots and colony hybridization For Southern blots, genomic or plasmid DNA was digested with restriction enzymes, and the resulting DNA fragments were separated by agarose electrophoresis and blotted onto a Hybond N membrane (Amersham Co.). DNA fragments of interest were detected using a DNA probe labeled with digoxigenin (Boeringer-Mannheim Co.) according to manufacturer’s instructions.

Colony hybridization was used for screening of the cosmid genomic library from the wild strain of B. glumae. The colonies were transferred onto nitrocellulose membranes and hybridized to a DNA probe labeled with digoxigenin (Boeringer-Mannheim Co.) according to manufacturer’s instructions.

Complementation test Triparental mating of a Tn4431 insertion mutant of B. glumae with a clone of E. coli selected by colony hybridization and a strain DH5α of E. coli carrying pRK2013 was carried out by incubating on LB agar for 15 hr at 30°C. The mixture was then plated on S-PG supplemented with 20 μg/ml of Tc and 30 μg/ml of kanamycin (Km) to screen the conjugants. The obtained merodiploid conjugants were subjected to complementation tests for toxin production and pathogenicity.
Extraction and purification of toxin *B. glumae* No. 19-61 was preincubated in the LB medium containing 35 μg/ml of Tc and 50 μg/ml of Km by reciprocally shaking overnight at 28°C. Ten ml of the preculture was added to 1 liter of King's B medium, and cultivated on a rotary shaker for 5 days at 32°C. The culture was centrifuged at 4°C to remove the bacterial cells, and the supernatant was collected and extracted three times with a half volume of chloroform (CHCl₃). The CHCl₃ extracts were combined and concentrated in vacuo using a rotary evaporator at less than 40°C. The residue was dissolved in a small volume of methanol, and then adsorbed to a silica powder. The silica gel was dried in vacuo, poured on a silica gel (Kieselgel 60, 230–400 mesh ASTM, Merck) column and eluted with CHCl₃. Each eluted fraction was checked for growth inhibition of *E. coli* JM109; active fractions were collected and concentrated in vacuo. The residue was further separated by preparative TLC using precoated silica gel plates (Kieselgel 60, Merck) with CHCl₃ : CH₃OH (95 : 5, v/v) as a solvent. A yellowish spot being antibacterial and phytotoxic to Mioga leaf was scraped and eluted with methanol. After concentration, the residue was dissolved in a small amount of ethyl acetate, and then n-hexane was added dropwise until the solution became slightly muddy. Then the solution was kept at -20°C overnight to crystallize the toxin. The crystallized toxin was collected on a glass wool column and used for its structure analysis.

**General instruments for toxin determination**

UV spectra were measured using a spectrophotometer of Beckman DU640 in methanol. HRMS and EIMS spectra were measured using either Hitachi M-80 or JEOL JMS-AX505WA Mass spectrometer. ¹H- and ¹³C-NMR spectra were measured in CDCl₃ solution using JEOL JMN-EX 270, GSX-500 or a-400 spectrometers.

**RESULTS**

Isolation of nonpathogenic mutants by Tn4431 mutagenesis

As a first step toward the disruption of genes involved in pathogenicity, we tried to obtain Tn4431 insertion mutants with biparental mating of *B. glumae* B446 with *E. coli* HB101 carrying the suicide vector pUCD623. From mating, we isolated about 380 colonies of Tc-resistant transconjugants (presumptive Tn4431 mutants). When these presumptive Tn4431 mutants were screened for pathogenicity to rice seedlings, three kinds of nonpathogenic mutants were selected. Further selection of these three mutants was made for toxin production on a CaPG agar plate. Mutants No. 19 and No. 20 were shown to be deficient in the production of the yellowish, fluorescent pigments phytotoxic to rice seedlings and Mioga plants, whereas the other mutant No. 22 produced the pigments.

Characterization of Tn4431 insertion mutants of *B. glumae*

Southern blot hybridization was performed to verify the number of Tn4431 insertions in the genomes of the three selected mutants of *B. glumae*. When Pest-digested genomic DNA from the mutants was probed with Tn4431 DNA, a single insertion was revealed in mutants No. 19 and No. 20 by the presence of one positive band, and the size of positive DNA fragments was about 12 kb and 13 kb, respectively (Fig. 1). No transposon insertion was detected in mutant No. 22.

Cloning of the genes involved in pathogenicity

DNA probes for cloning the Tn4431-inactivated gene were obtained from the genomic libraries of mutants Nos. 19 and 20, which were constructed in the cosmids pUCD 615 and introduced into *E. coli* DH5α. After selection for Tc resistance with Tn4431, one Tc-resistant clone (pNP19-1) was obtained from 3800 clones of mutant No. 19 library, and five resistant clones were selected from 5200 clones of No. 20. Of these clones, pNP19-1 was used for further experiments. Enzymatic digestion of the clone pNP19-1 showed that 2kb BamHI/Pst I fragments and 3kb Xba I/BamHI fragments were hybridized to Tn4431 DNA probe, so that these fragments contain Tn4431 and its flanking sequences from the mutant No. 19 genomic DNA. Two kinds of these DNA fragments were used as probes for gene cloning.

When the genomic cosmid library of *B. glumae* wild strain was selected by colony hybridization using these two DNA probes, the 2 kb DNA probe showed homology to two clones (pNP142 and 143), and the 3 kb DNA probe reacted positively with five clones (pNP143–147). Clone pNP143 was positive to both of the probes. Introducing
Fig. 2. Mass spectrum of a yellowish toxin produced by B. glumae No. 19-61. The toxin was analyzed by direct inlet method, and gave M+ ion at m/z 193 and its fragment ions at m/z 165, 136, 109 and 66 in EIMS spectrum.

these recombinant clones into mutant No. 19 by triparental mating indicated that clone pNP147, with a genomic insert of about 25 kb, complemented the mutant for toxin production as indicated by pigment production on CaPG medium (Plate I-A) and by phytotoxicity of the culture filtrate to rice leaves (Plate I-B). Also, pathogenicity recovery in mutant No. 19 with pNP147 was observed after inoculating to rice seedlings (Plate I-C) and Miogia leaves (Plate I-D). These results suggest that the genes involved in toxin production by B. glumae were cloned in pNP147. Mutant No. 19 carrying pNP147 was named B. glumae No. 19-61.

**Characterization of a virulence factor**

A yellowish, fluorescent toxin was isolated from a culture of B. glumae No. 19-61. The toxin was purified and crystallized as described in the MATERIALS AND METHODS, and analyzed using UV, Mass and NMR spectrometry. Its UV spectrum had absorption maxima at 262 (ε = 21,500) and 400 (ε = 5900) nm in methanol. Mass spectrum of the toxin gave M+ ion at m/z 193 and characteristic fragment ions at m/z 165, 136, 109, and 66 in EIMS spectrum using the direct inlet method as shown in Fig. 2. Its HRMS spectrum gave M+ ion at 193.0588 (Calcd, for C7H7O2N5: 193.0600). 1H-NMR spectrum (500MHz, in CDCl3) showed three proton signals at δ 3.48 (3H, s, N6-Me), 4.17 (3H, s, N1-Me), and 8.83 (1H, s, C3-H). 13C-NMR spectrum (125MHz, in CDCl3) showed seven carbon signals, tentatively assigned using its HMBC and HMQC spectra as follows: δ 32.9 (q, N6-CH3), 43.29 (q, N1-CH3), 145.14 (d, C3), 145.41 (s, C4a), 150.31 (s, C8a), 154.12 (s, C5 or C7), and 158.38 (s, C5 or C7). The MS and 1H-NMR spectra were identical to those of toxoflavin reported by Sato et al.7). Furthermore, the 13C-NMR spectra verified to be toxoflavin. From these results, the toxin produced by the wild strain or the mutant No. 19-61 of B. glumae was identified as toxoflavin.

**DISCUSSION**

We successfully isolated Tn4431 insertion mutants of B. glumae which were unable to cause rice seedling rot. Some of these mutants were also unable to produce toxoflavin, which was released as a yellowish, fluorescent pigment on CaPG medium. The nontoxigenic mutants Nos. 19 and 20 had been mutagenized by a single insertion of Tn4431, as shown by analysis of genomic DNA by Southern blot hybridization with a probe of Tn4431 DNA. Therefore, the lack of toxoflavin production in mutant Nos. 19 and 20 resulted from destruction of the gene responsible for toxin production. Because toxin production might then be recovered when the mutant is complemented with the corresponding gene from the wild strain of B. glumae, we tried to clone the genes for toxoflavin production by complementation tests of mutant No. 19.

Two kinds of DNA fragments containing Tn4431-flanking sequences from the genomic DNA of mutant No. 19 were used as probes for selection of the genomic DNA library of the wild strain. Six genomic clones showed homology to the probes. When these clones were transconjugated into mutant No. 19, the conjugant with
pNP147 (*B. glumae* No. 19-61) was complemented not only for pathogenicity to rice seedling but also for toxin production. Since clone pNP147 has a large insert of around 25 kb from the genomic DNA, subcloning the insert DNA might be required to confirm that complementation is due to one gene. In preliminary work, experiments showed that toxin production in mutant No. 19 was complemented with a 2.5 kb *Eco*RI fragment of the 25 kb genomic insert. Thus, the *Tn4431*-mutagenized gene in mutant No. 19 may be related to one of the enzymes or regulatory systems responsible for toxin production by *B. glumae*.

The toxin produced by *B. glumae* No. 19-61, mutant No. 19 with pNP147, was identified as toxoflavin, which had been isolated from the culture filtrate of *B. glumae* by Sato et al.7). Since mutant No. 19 did not induce seedling rot of rice plants, toxoflavin is probably an essential virulence factor in the pathogenicity of *B. glumae*. This is also supported by the fact that the *Tn4431*-mutagenized gene in mutant No. 19 may be related to one of the enzymes or regulatory systems responsible for toxin production by *B. glumae*.

Further characterization of the genes cloned in pNP147 is in progress to clarify their specific roles in toxoflavin production.

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**Literature cited**

Plate I

A: Production of yellowish toxins from B. glumae strains grown on a CaPG agar plate. Left: Wild strain. Middle: Clone No. 19 isolated by Tn-insertion mutagenesis. Right: Mutant No. 19 complemented with pNP147 (No. 19-61).


C: Development of rice seedling rot after inoculation with B. glumae strains. From the left tube, wild strain, clone No. 19-61, mutant No. 19 and no inoculation.