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Genome-Wide RNA Sequencing Analysis of Quorum Sensing-Controlled Regulons in the Plant-Associated *Burkholderia glumae* PG1 Strain

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***Burkholderia glumae* PG1 is a soil-associated motile plant-pathogenic bacterium possessing a cell density-dependent regulation system called quorum sensing (QS). Its genome contains three genes, here designated *bgaI1* to *bgaI3*, encoding distinct autoinducer-1 (AI-1) synthases, which are capable of synthesizing QS signaling molecules. Here, we report on the construction of *B. glumae* PG1 Δ*bgaI1*, Δ*bgaI2*, and Δ*bgaI3* mutants, their phenotypic characterization, and genome-wide transcriptome analysis using RNA sequencing (RNA-seq) technology. Knockout of each of these *bgaI* genes resulted in strongly decreased motility, reduced extracellular lipase activity, a reduced ability to cause plant tissue maceration, and decreased pathogenicity. RNA-seq analysis of all three *B. glumae* PG1 AI-1 synthase mutants performed in the transition from exponential to stationary growth phase revealed differential expression of a significant number of predicted genes. In comparison with the levels of gene expression by wild-type strain *B. glumae* PG1, 481 genes were differentially expressed in the Δ*bgaI1* mutant, 213 were differentially expressed in the Δ*bgaI2* mutant, and 367 were differentially expressed in the Δ*bgaI3* mutant. Interestingly, only a minor set of 78 genes was coregulated in all three mutants. The majority of the QS-regulated genes were linked to metabolic activities, and the most pronounced regulation was observed for genes involved in rhamnolipid and Flp pilus biosynthesis and the type VI secretion system and genes linked to a clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* gene cluster.**

Quorum sensing (QS) is a cell density-dependent gene regulation system in bacteria (1) in which the population density is sensed through the accumulation of bacterially produced signaling molecules called autoinducers (AIs). This cell-to-cell signaling process allows the microbial population to synchronize group behavior and alter its gene expression accordingly. QS is involved in a wide array of regulatory circuits, among which are pathogenicity, secretion of extracellular proteins, secondary metabolite production, and others (2). Key QS signaling molecules in many Gram-negative bacteria are *N*-acyl-homoserine lactones (AHLs) (3–5), synthesized mainly through LuxI homologs (EC 2.3.1.184) using *S*-adenosylmethionine (SAM), and an acyl-acyl carrier protein (acyl-ACP) from the fatty acid biosynthesis pathway (6). LuxR-type receptor/regulator proteins are involved in AHL signal perception. Together with LuxR, other proteins may be part of this regulatory circuit.

The motile, rod-shaped Gram-negative soil bacterium *Burkholderia glumae* is considered to be a seed-borne pathogen that causes panicle blight of rice (7). *B. glumae* has also been reported to infect other plant species, like tomato, sunflower, and pepper (8, 9). Although it is not classified as a human pathogen, a single case of the isolation of *B. glumae* from a clinical sample was reported (10), indicating that at least some strains of this pathogen may be associated with opportunistic infections in humans. The phytopathogenicity of *B. glumae* is caused by multiple factors, including the biosynthesis of toxoflavin (11, 12); motility (13); the secretion of virulence factors by a type III secretion system (T3SS) (14); and the production of lipase (15), catalase (16), and pectate lyase (17). Since the expression of many of the respective genes is controlled by AHLs, QS plays a major role during plant infection processes as well (11, 18).

The first evidence of the presence of QS in the *Burkholderia* genus was obtained in 1995 for a strain within the *Burkholderia cepacia* complex (collectively called Bcc) from cross-feeding experiments with *Pseudomonas aeruginosa* (19). Since then, all described *Burkholderia* species have been found to employ at least one AHL-mediated QS system. Several species, like *Burkholderia thailandensis*, *Burkholderia mallei*, and *Burkholderia pseudomallei* (the so-called Bptm group), hold multiple QS systems driven by diverse AHL signaling molecules (20–22). Strain *B. glumae* BGR1 was reported to possess a single QS system homologous to LuxI/LuxR, designated TofI/TofR, involved in the biosynthesis and transport of toxoflavin (11). TofI, which is an AHL synthase belonging to the LuxI protein family (15, 23), catalyzes the synthesis of an *N*-hexanoyl-homoserine lactone (C_6-AHL) and an *N*-octa-

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noyl-homoserine lactone (C_8-AHL) (8, 11, 24, 25). The *tofR* gene encodes its cognate LuxR-type receptor protein. In BGR1, C_8-AHL forms a complex with TofR which regulates subordinated cellular processes like motility and toxoflavin biosynthesis, which are pivotal for the pathogenicity of *B. glumae*.

Recently, the complete genome sequence of *B. glumae* strain PG1 (here called BGPG1) was determined (26). Interestingly and in contrast to the findings for BGR1, the 7.9-Mbp genome of BGPG1 codes for three distinct AHL synthase genes designated *bgaI1* to *bgaI3*. BgaI1 is highly similar to the AHL synthase TofI found in BGR1 (95% identity at the amino acid level). BgaI2 is similar to the AI synthase from *B. thailandensis* (GenBank accession no. WP_006029278) with 53% identity at the amino acid level, and BgaI3 has 46% identity at the amino acid level to an AI synthase from *B. mallei* (GenBank accession no. WP_004195479) (see Fig. S1 in the supplemental material). Although the presence of multiple AHL synthase genes is a common feature within the *Burkholderia* genus (20), the presence of three AHL synthase genes within the species *B. glumae* is currently a unique feature of BGPG1 only.

Until now, only a few high-resolution RNA sequencing (RNA-seq) studies that give a detailed first insight into the expression profiles of QS-regulated genes in selected *Burkholderia* species have been published. These studies analyzed the QS-dependent expression profiles in *Burkholderia cenocepacia* (27), *B. thailandensis* (28), *Burkholderia gladioli* (29), and *B. mallei* and *B. pseudomallei* (30). While *B. thailandensis* is a nonpathogenic tropical soil microorganism, *B. cenocepacia*, *B. mallei*, and *B. pseudomallei* are considered human and animal pathogens (31–33). Interestingly, the *B. thailandensis* and *B. pseudomallei* strains that have been analyzed were found to harbor three AHL-based systems, while the genome of the *B. mallei* isolate analyzed codes only for two AHL synthases. Very recently, the results of an RNA-seq analysis for *B. gladioli*, which causes diseases in both plants and humans and possesses two systems homologous to LuxI and LuxR, were published (29). Also, a low-coverage RNA-seq analysis of the plant-associated strain *B. glumae* BGR1 was reported (34, 35).

This report describes the expression profiling by high-resolution RNA-seq of the BGPG1 wild-type strain and compares the profile obtained to the profiles of three newly constructed deletion mutants, namely, BGPG1 Δ*bgaI1* (BGPG2), BGPG1 Δ*bgaI2* (BGPG3), and BGPG1 Δ*bgaI3* (BGPG4). Additionally, the phenotypes of the wild-type and mutant strains were comparatively studied, and a common set of orthologous QS-regulated genes was identified in *B. glumae* PG1 and the recently studied model organisms from the Bptm group (28, 30).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *B. glumae* PG1 strains and *Chromobacterium violaceum* CV026 were grown in lysogeny broth (LB) medium (36) at 30°C; *Escherichia coli* strains were cultivated in LB medium at 37°C. *Agrobacterium tumefaciens* NTL4 (37) was grown in LB medium or AT medium (38) at 30°C. Antibiotics were added to the cultures, when required, at the following final concentrations: for *B. glumae*, chloramphenicol was added at 25 μg/ml and gentamicin (Gm) was added at 20 μg/ml; for *C. violaceum*, chloramphenicol was added at 25 μg/ml; for *E. coli*, ampicillin was added at 100 μg/ml, gentamicin was added at 10 μg/ml, and kanamycin was added at 25 μg/ml; and for *A. tumefaciens*, tetracycline was added at 5 μg/ml and spectinomycin was added at 50 μg/ml. For the growth of *E. coli* WM3064, *meso*-diaminopimelic acid (DAP) was added to a final concentration of 300 μM. Motility assays were done as previously described (39), but the incubation time was 3 days and a glucose concentration of 45 mM was used. Agar concentrations were 0.45% (wt/vol) for swarming tests and 0.25% (wt/vol) for swimming tests. Sedimentation assays were performed in LB medium according to the previously described method (40), and the sedimentation time was extended to 42 h. For the tributyrin (TBT) plate assay, LB medium was homogenized with 1% TBT as an indicator substrate for lipolytic activity prior to autoclaving. Tetrazolium chloride (TZC) medium was prepared as described previously (41).

Molecular methods. DNA cloning and PCR procedures were performed according to standard protocols (36); the primers are listed in Table 2. Genomic DNA of BGPG1 and the corresponding mutants was isolated using an Aqua Pure genomic DNA kit (Bio-Rad Laboratories, Hercules, Canada). Isolation of plasmid DNA was performed with a high-speed plasmid minikit (Geneaid Biotech Ltd., Taiwan, China). Plasmid transformation in *E. coli* was done following standard heat shock protocols (36). Conjugations in *B. glumae* were performed by biparental mating using *E. coli* WM3064 as the donor and incubation at 30°C overnight (36).

Construction of *B. glumae* PG1 AHL synthase mutants. Three insertion cassettes, namely, Δ*bgaI1*-Gm, Δ*bgaI2*-Gm, and Δ*bgaI3*-Gm, were assembled using the flanking regions of the *bgaI1*, *bgaI2*, and *bgaI3* genes (400- to 770-bp up- and downstream fragments of the respective genes) and cloned into a gentamicin resistance gene derived from the broad-host-range cloning vector pBBR1MCS-5 (42). The three cassettes were then separately inserted into the suicide vector pNPTS138-R6KT (43), and the three constructs obtained were introduced into BGPG1 by conjugation. Mutants resulting from the first crossover event were selected on LB plates containing gentamicin and kanamycin and subsequently plated onto LB plates containing 10% (wt/vol) sucrose but lacking kanamycin. Kanamycin-sensitive colonies were selected and verified by direct colony PCR using different primer pairs flanking the respective *bgaI1*, *bgaI2*, and *bgaI3* genes. The mutations obtained were confirmed by DNA sequencing, and the mutants were designated BGPG2 for *B. glumae* PG1 Δ*bgaI1*, BGPG3 for *B. glumae* PG1 Δ*bgaI2*, and BGPG4 for *B. glumae* PG1 Δ*bgaI3* (Table 1). The corresponding deletion positions or the locations of the gentamicin resistance gene insertions in all three mutants are shown in Fig. 1A. Complementation of the mutants was achieved by reintroducing the three AHL synthase genes (*bgaI1* to *bgaI3*) back into the respective mutant strains, BGPG2 to BGPG4, using the broad-host-range vector pBBR1MCS-2 (42). The complemented mutants were designated BGPG2_c for complemented *B. glumae* PG2, BGPG3_c for complemented *B. glumae* PG3, and BGPG4_c for complemented *B. glumae* PG4 (Table 1), and their correctness was verified by PCR. Recombinant *E. coli* strains carrying the *bgaI1* to *bgaI3* genes were constructed using a Qiagen PCR cloning kit (Qiagen, Hilden, Germany) and employed for determination of the acyl side chain length as outlined below.

Detection of AHLs by *C. violaceum* CV026 and *A. tumefaciens* NTL4. BGPG1 and the three mutant strains were grown in 100 ml LB for 28 h at 30°C, and *E. coli* harboring the *bgaI1* to *bgaI3* genes was grown in 100 ml LB for 18 h at 37°C. AHLs were extracted from stationary-phase cultures with a 3-fold volume of ethyl acetate, concentrated *in vacuo*, and resuspended in 500 μl ethyl acetate. The reporter strain, *C. violaceum* CV026 (44), was used for the detection of AHL molecules in cell extracts by quantification of violacein as previously described (45) with minor modifications. For the initial separation and subsequent detection of AHLs, thin-layer chromatography (TLC) was carried out, followed by AHL detection using the *A. tumefaciens* reporter strain NTL4 as previously described (40).

Onion maceration and rice pathogenicity assays. The plant maceration capabilities of BGPG1 and the mutant strains were tested by an onion maceration assay (46) after incubation for 72 h at 30°C. Rice (*Oryza sativa* cv. Baldo) seeds were sterilized with 70% (vol/vol) ethanol for 5 min and washed once with sterile water, followed by 20 min of incubation in 3% H_2O_2 and rinsing three times with sterile water. Pretreated seeds were

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)[a]	Source or reference
Strains		
B. glumae		
PG1	Wild-type strain CBS 322.89 (CBS, Utrecht, The Netherlands)	84
PG2	Δ*bgaI1* mutant of *B. glumae* PG1; Gm^r	This study
PG3	Δ*bgaI2* mutant of *B. glumae* PG1; Gm^r	This study
PG4	Δ*bgaI3* mutant of *B. glumae* PG1; Gm^r	This study
PG2_c	BGPG2 carrying the *bgaI1* gene in pBBR1MCS-2	This study
PG3_c	BGPG3 carrying the *bgaI2* gene in pBBR1MCS-2	This study
PG4_c	BGPG4 carrying the *bgaI3* gene in pBBR1MCS-2	This study
C. violaceum CV026	Reporter strain for AI-1; mini-Tn*5* mutant of *C. violaceum* ATCC 31532	44
E. coli		
WM3064	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 *RP4-1360* Δ(*araBAD*)*567* Δ*dapA1341*::[*erm pir*(wt)]	W. Metcalf, University of Illinois, Urbana-Champaign, USA
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*argF-lacZYA*)*U169 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1*	85
A. tumefaciens NTL4(pCF218)(pCF372)	Reporter strain for AHL detection; *traI*::*lacZ* Tet^r Sp^r	86
Plasmids		
pDrive	Vector for PCR cloning, Qiagen PCR cloning kit; Amp^r Km^r	Qiagen, Hilden, Germany
pGEM-T	Vector for PCR cloning; Promega Easy Vector systems; Amp^r	Promega, Mannheim, Germany
pNPTS138-R6KT	Suicide plasmid for in-frame deletions; *mobRP4*$^+$ *ori-R6K sacB* Km^r	43
pNPTS138-R6KT::Δ*bgaI1*-Gm	Deletion cassette Δ*bgaI1*-Gm in pNPTS138-R6KT	This study
pNPTS138-R6KT::Δ*bgaI2*-Gm	Deletion cassette Δ*bgaI2*-Gm in pNPTS138-R6KT	This study
pNPTS138-R6KT::Δ*bgaI3*-Gm	Deletion cassette Δ*bgaI3*-Gm in pNPTS138-R6KT	This study
pBBR1MCS-2	Broad-host-range vector, low copy no.; Km^r	42
pBBR1MCS-5	Broad-host-range vector, low copy no.; Gm^r	42
pBBR1MCS-2::*bgaI1*	pBBR1MCS-2 carrying the *bgaI1* gene in the MCS	This study
pBBR1MCS-2::*bgaI2*	pBBR1MCS-2 carrying the *bgaI2* gene in the MCS	This study
pBBR1MCS-2::*bgaI3*	pBBR1MCS-2 carrying the *bgaI3* gene in the MCS	This study
pBBR1MCS-2::P*abc1*::*mCherry*	pBBR1MCS-2 carrying the promoter of the Abc1 gene and mCherry in the MCS	This study
pBBR1MCS-2::P*cysB*::*mCherry*	pBBR1MCS-2 carrying the promoter of the *cysB* gene and mCherry in the MCS	This study
pBBR1MCS-2::P*rhlA*::*mCherry*	pBBR1MCS-2 carrying the promoter of the *rhlA* gene and mCherry in the MCS	This study
pDrive::*bgaI1*	pDrive vector carrying the *bgaI1* gene in the MCS	This study
pDrive::*bgaI2*	pDrive vector carrying the *bgaI2* gene in the MCS	This study
pDrive::*bgaI3*	pDrive vector carrying the *bgaI3* gene in the MCS	This study

[a] wt, wild type; MCS, multiple-cloning site.

infected with 10^7 cells/ml of BGPG1 or the mutants and pregerminated for 2 days at 37°C, transferred into sterile petri dishes containing three layers of filter paper (diameter, 90 mm; Whatman no. 1) that had been wetted with 60 ml of sterile water, and subjected to growth cycles of 16 h in the light and 8 h in the dark at 28°C. Germination rates were calculated after 7 days, and seedling lengths were measured after 2 weeks.

Preparation of *B. glumae* transcriptome samples. The culture samples of BGPG1 used for the transcriptome analysis are summarized in Table 3. Prior to cultivation of 100-ml cultures, precultures were established from cryocultures in 5 ml LB medium and cultivated at 30°C and 200 rpm. For the transcriptome analyses of early-stationary-growth-phase cultures, 100 ml LB medium was inoculated with freshly grown precultures of the BGPG1 wild type as well as the BGPG1 mutant strains, and the bacteria were cultivated as batch cultures for approximately 28 h at 30°C and 200 rpm. After 28 h the cultures were separated into fractions of 45 ml, which were then transferred into Falcon tubes, chilled on ice, and centrifuged at 13,000 rpm for 5 min. The supernatants were discarded, and samples were directly frozen in liquid nitrogen and stored at −70°C until further use.

RNA extraction, library construction, sequencing, and analysis of transcriptome samples. For the BGPG1 wild-type and mutant strains, RNA-seq libraries were constructed from independent biological triplicates of RNA samples. Total RNA was extracted using an RNeasy minikit (Qiagen, Hilden, Germany) and the protocol for purification of total RNA from the bacteria, with the following exceptions: to include the small RNA fraction (<200 nucleotides), the proportion of ethanol to supernatant was raised to 1.5:1 (vol/vol) after cell disruption (step 6), and buffer RWT was used instead of buffer RW1 to wash the column (step 8). The residual genomic DNA was removed from the isolated total RNA by DNase I (Fermentas, St. Leon-Rot, Germany) treatment. To reduce the amount of rRNA-derived sequences, the samples were subjected to rRNA depletion using a Ribo-Zero magnetic kit (Epicentre Biotechnologies, Madison, WI, USA). The strand-specific cDNA libraries for sequencing were constructed with a NEBNext Ultra directional RNA library preparation kit for Illumina (New England BioLabs, Frankfurt am Main, Germany). The cDNA libraries obtained were sequenced by using a GAIIx instrument (Illumina Inc., San Diego, CA, USA) in the single-read mode and running 75 cycles. For the 12 samples analyzed, we retrieved between 32.5 million and 45.3 million raw reads (Table 3). To ensure high sequence quality, the remaining sequencing adaptors were removed and the reads with a cutoff phred-33 score of 15 were trimmed by the program Trimmomatic (47). The remaining sequences were mapped with the Bowtie (version 2) program (48) using the implemented end-to-end mode, which requires that the entire read align from one end to the other. Differential expression

TABLE 2 Primers used in this study

Oligonucleotide	Sequence[a]
M13-20 for	GTAAAACGACGGCCAGT
M13 rev	CAGGAAACAGCTATGACC
T7 promoter	TAATACGACTCACTATAGGG
SP6 promoter	CATTTAGGTGACACTATAG
BgaI1_f	ACGACATCGAGTTCGGCGTGTTC
BgaI1_r	AGCAGACCGTGTCTTCGGCATTG
BgaI2_f	GAGGCGGCGCGATACTATCAAC
BgaI2_r	CGCGAGATCGACGTGCTCAAGTG
BgaI3_f	AAAGATTGGGCACGCGATCGAATCC
BgaI3_r	ATCTTCAGCTTCCGCAGCTACCG
BgaI1_uf	CGGATCCGCGGACTATCCGGTTGCGATCCAC
BgaI1_ur	CAAGCTTGATCGACATCGACGCGCAGAC
BgaI1_df	CAAGCTTGCGGGAACACTTCCTGCAACAGGTAG
BgaI1_dr	GACGCGTCGTCGGCTGGGACTGGTATCTCGAAC
BgaI2_uf	GGGATCCGAGCTGCTCGAGGAATAC
BgaI2_ur	AGCAAGCTTCCAGTTTCTCGACGAACAC
BgaI2_df	ACTAAGCTTGCTTCAGCGCAGCAAAC
BgaI2_dr	GGAATTCGGGATCGTCGAGGGATG
BgaI3_uf	TGGATCCGTCATCGCTTGATGCTTGG
BgaI3_ur	CGAAAGCTTCAGGTGCTTGACGAAC
BgaI3_df	ACAAAGCTTACCGGAAGAAGGGATTCAG
BgaI3_dr	AGAATTCAGACCGCCGAGAACATCGTG
BgaI1_in_1f	GAACAGCCGCTCGATGCTGCAGAAC
BgaI1_in_1r	GTTCTGCAGCATCGAGCGGCTGTTC
BgaI1_out_2f	CGTGACGAACATGAGCGAACCCATC
BgaI1_out_2r	ACAGCTCCCACGCTGTCATTCTTGC
BgaI2_in_1f	CCTATCTGCTCTCCGACGTGTTC
BgaI2_in_1r	TGAGCTCGATCCAGCAGGCGAAG
BgaI2_out_2f	AGGCGGACTTCTTCGGCTACCAG
BgaI2_out_2r	CAGACCGTGATGATCTCGAACTACC
BgaI3_in_1f	AAGTGGGACCTGCCGATGGTCTC
BgaI3_in_1r	CTTCCGGTAGAGCCGCATCATGG
BgaI3_out_2f	GCTTGTTCGCAGTGTAGTCCGAAGC
BgaI3_out_2r	GTCGCGCTGATCTCGACGATCAACG
BGPG1_abc1_f	CGGGATCCCGTTGGGCTTGAAGTCGTTGAG
BGPG1_abc1_r	GGAATTCCATCGCCACCAGCACGAACAC
BGPG1_cysB_f	CGGGATCCCGACGTGCAGAACAAGAAGGTC
BGPG1_cysB_r	GGAATTCCAATTCGCACTTGCCGTGCAG
BGPG1_rhlA_f	CGGGATCCCGACATAGCACGGAATGCATGG
BGPG1_rhlA_r	GGAATTCCTCTCGAACGACGGATCGTAG

[a] Underlined nucleotides indicate restriction sites.

analyses were performed with the baySeq program (49). Genes with a fold change in expression of ≥2.0, a likelihood value of ≥0.9, and an adjusted *P* value of ≤0.05 (the *P* value was corrected by the false discovery rate [FDR] on the basis of the Benjamini-Hochberg procedure) were considered differentially expressed.

Quantitative RT-PCR (qRT-PCR). RNA from a 2-ml culture was routinely extracted using the RNeasy minikit (Qiagen, Hilden, Germany). DNase I digestion was performed both on column with an RNase-free DNase set (Qiagen, Hilden, Germany) and after RNA elution with DNase I (RNase-free) from Ambion (Life Technologies, Darmstadt, Germany) according to the manufacturers' instructions. The transcription of isolated mRNA into cDNA was carried out with a Maxima first-strand cDNA synthesis kit for reverse transcription (RT)-quantitative PCR (qPCR) (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. In a separate reaction, each sample was treated without reverse transcription to exclude the possibility of DNA contamination. The analysis of the transcriptional levels of selected genes was performed by real-time qPCR (35 cycles) using the $\Delta\Delta C_T$ threshold cycle (C_T) method (50). Here, 50 ng of the reverse-transcribed cDNA was used as the template in a real-time 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) with Maxima SYBR green–carboxy-X-rhodamine qPCR master mix (2×; Thermo Scientific, Vilnius, Lithuania), primers specific for the genes of interest (Table 4), and the constitutively expressed gene *rpoD* as a reference (see Fig. S2 in the supplemental material). Primers were designed using the Primer3 tool (51). The amount of PCR product was calculated as the C_T value by a sequence detection system (version 2.3; Applied Biosystems, Foster City, CA, USA). PCR efficiencies were determined with the tool LinRegPCR (52). Calculations of the changes in transcript levels were performed and statistically analyzed with REST software (53). A change in the transcript level was assumed to be significantly different from that in the control sample if the *P* value was ≤0.05.

Analysis of gene expression using mCherry-based promoter fusions. Promoter fusions were constructed employing the red fluorescent protein mCherry (designated *mCherry* in the fusions). All constructs were inserted in the broad-host-range cloning vector pBBR1MCS-2 (42) and mobilized via conjugation into strains BGPG1 to BGPG4. Strains BGPG1 to BGPG4 harboring the constructs were grown in LB medium at 30°C for 48 h, and then culture aliquots of 200 μl were transferred into microtiter plates and analyzed. The fluorescence of mCherry was measured with an excitation filter (590/20-nm filter set) and an emission filter (645/40-nm filter set) and by determination of the absorbance at 600 nm with a Synergy HT multimode microplate reader, and the results were analyzed with Gen5 software (BioTek Instruments Inc., Winooski, VT, USA). The number of relative fluorescence units (RFUs) were corrected by the optical density at 600 nm (OD_{600}) of the analyzed cultures. Data were recorded for a minimum of three independently grown cultures, and each measurement was repeated three times.

SRA accession number. The trimmed reads have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession no. SRP047507.

RESULTS AND DISCUSSION

AHL profiles of *B. glumae* PG1 and *bgaI1* to *bgaI3* deletion mutants. The genome of wild-type strain *B. glumae* PG1 encodes three AHL synthases, and these are flanked by their cognate LuxR-type regulators (Fig. 1A). This is an unusual feature within the species *B. glumae*, since the genomes of all other currently sequenced strains contain only a single AHL synthase gene. To investigate the QS-mediated gene regulation of these three QS regulons in BGPG1, we initially constructed mutants with a deletion of each of the three identified AHL synthase genes. The obtained mutant strains were designated BGPG2 for *B. glumae* PG1 Δ*bgaI1*, BGPG3 for *B. glumae* PG1 Δ*bgaI2*, and BGPG4 for *B. glumae* PG1 Δ*bgaI3* (Fig. 1A). Each mutant strain was verified by DNA sequencing and phenotypic analyses.

Production of AI by the *B. glumae* PG1 wild-type strain and the Δ*bgaI* mutant strains was tested by TLC analysis with subsequent AHL detection using reporter strain NTL4. Recombinant *E. coli* strains carrying the *bgaI1* to *bgaI3* genes produced single spots on the TLC plates (Fig. 1B). For *E. coli* carrying the *bgaI1* gene, a signal that most likely corresponded to the oxo-C_8 standard was detected, the clone carrying *bgaI2* produced a spot that most likely corresponded to the oxo-C_{10} standard, and the clone carrying *bgaI3* produced a spot that corresponded to either the C_{10} or the oxo-C_{12} standard. The parent strain BGPG1 reproducibly produced three spots on such TLC plates which corresponded to the above-described spots of the individual recombinant *E. coli* clones. Further, each of the Δ*bgaI* mutants lacked one of these spots, and the individual AHL profiles of strains BGPG2, BGPG3, and BGPG4 were different (Fig. 1C).

Finally, production of AI by the *B. glumae* PG1 wild-type strain and the Δ*bgaI* mutants was tested with the reporter strain CV026 (Table 1), which is unable to synthesize AHLs and is therefore

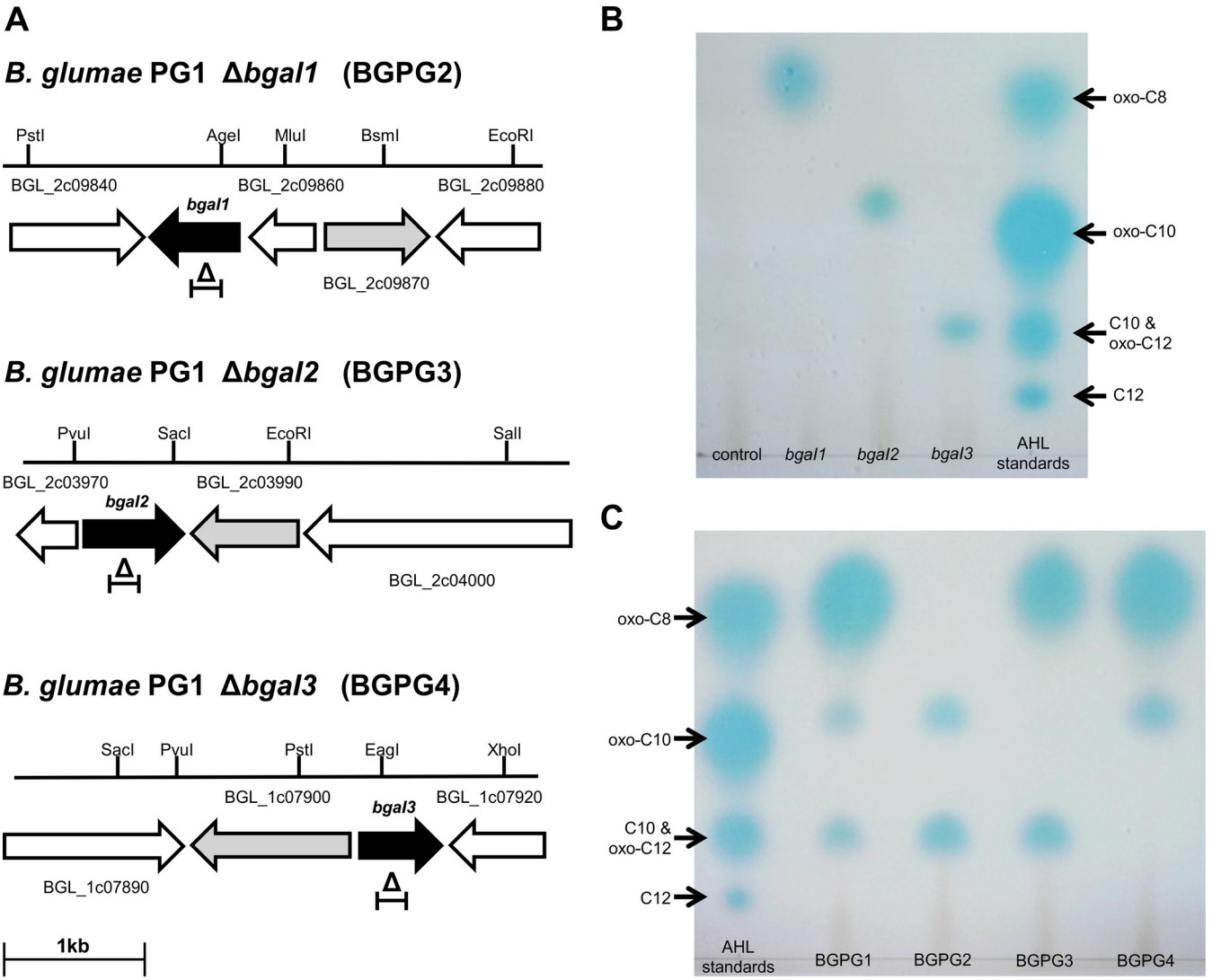


FIG 1 Partial physical maps of the three *B. glumae* PG1 AHL synthase mutants and AHL detection on TLC plates using reporter strain *A. tumefaciens* NTL4. (A) Physical maps showing the AHL synthase genes *bgaI1*, *bgaI2*, and *bgaI3*, the positions of their deletions, and their flanking regions. Black arrows, the *bgaI1*, *bgaI2*, and *bgaI3* genes; gray arrows, their putative LuxR-type regulatory genes; white arrows, flanking open reading frames. Open reading frames are designated using the numbers from the sequence with NCBI GenBank database accession no. CP002580 and CP002581. Triangles, the positions of the deletions, which were located from 1,237,902 bp to 1,238,222 bp (chromosome 2) for the Δ*bgaI1* mutant, 486,784 bp to 487,479 bp (chromosome 2) for the Δ*bgaI2* mutant, and 899,130 bp to 99,528 bp (chromosome 1) for the Δ*bgaI3* mutant. The bars at the top show the identified restriction sites and their positions. (B) TLC chromatogram of AHL profiles of *B. glumae* PG1. The chromatogram illustrates the AHLs obtained from recombinant *E. coli* clones carrying *bgaI1* to *bgaI3* separated by TLC and detected by using the *A. tumefaciens* NTL4 biosensor as an overlay. (C) TLC analysis of the AHLs produced by parent strain BGPG1 and mutants BGPG2 to BGPG4 using the *A. tumefaciens* NTL4 biosensor as an overlay. AHL standards consist of oxo-C_8 (3-oxo-C_8-AHL), oxo-C_{10} (3-oxo-C_{10}-AHL), C_{10} (C_{10}-AHL), oxo-C_{12} (3-oxo-C_{12}-AHL), and C_{12} (C_{12}-AHL). All AHL standards used were obtained from Sigma-Aldrich and Biomol GmbH, Hamburg, Germany.

impaired in QS-regulated violacein production. While a culture extract of BGPG1 could reproducibly restore violacein production in the reporter strain, extracts of BGPG2 could not complement CV026 at all. However, culture extracts of BGPG3 and BGPG4 complemented CV026 at significantly reduced levels (data not shown). Since CV026 can detect only AHLs with an acyl side chain length ranging from C_6 to C_8, the data obtained fit nicely with the chain lengths of AHLs estimated using TLC analyses.

Phenotypic analyses of *B. glumae* PG1 and its AHL synthase mutants. (i) Bacterial motility. Swimming and swarming assays were performed with BGPG1 and the mutant strains BGPG2 to BGPG4, and it was found that both types of motility were clearly affected in the AHL synthase mutant strains. Figure 2A and B indicate typical results of the motility assays for strains BGPG1 to BGPG4. Complementation analyses revealed that swarming motility could be restored (see Fig. S3 in the supplemental material).

Interestingly, BGPG1 and BGPG2 settled to the bottom of the test tubes after incubation for 42 h. BGPG3 and BGPG4 did not sediment within 42 h. These phenotypes were in part restored by complementation (Fig. 2C), and they indicated a different regulation of the flagellar genes in these QS mutants. Similar sedimentation phenotypes were observed for *Sinorhizobium fredii* NGR234 (40) and *Legionella pneumophila* (54, 55) AI synthase mutants.

TABLE 3 Overall transcriptome statistics for the 12 samples from strains BGPG1 to BGPG4 analyzed[a]

Sample no.	BGPG1 genotype	OD_{600}	No. of reads generated (10^6)	No. of uniquely mapped reads (10^6)
1	wt	3.13	39.3	32.7
2	wt	3.15	45.3	37.9
3	wt	3.09	39.8	34.4
4	Δ*bgaI1*	2.84	43.4	33.0
5	Δ*bgaI1*	2.84	32.5	25.0
6	Δ*bgaI1*	2.81	32.8	28.6
7	Δ*bgaI2*	3.08	44.8	32.1
8	Δ*bgaI2*	3.05	33.5	26.1
9	Δ*bgaI2*	3.07	34.7	26.4
10	Δ*bgaI3*	2.98	42.1	33.4
11	Δ*bgaI3*	2.92	34.6	26.8
12	Δ*bgaI3*	2.91	33.5	24.6

[a] Cultures were harvested after 28 h of growth in the transition from exponential to stationary growth phase. wt, wild type.

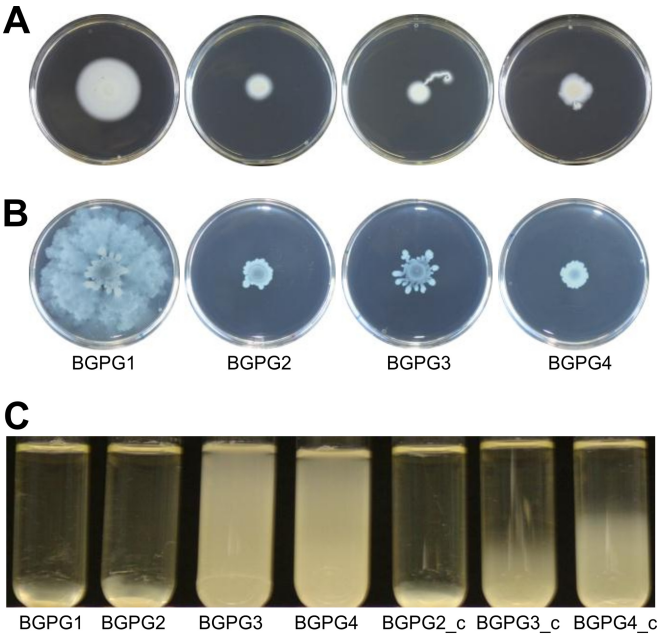


FIG 2 Assays of *B. glumae* PG1 and mutant strain motility. (A) Altered swimming motility of BGPG2, BGPG3, and BGPG4 compared to that of wild-type strain BGPG1 on agar plates containing 0.25% Eiken agar (Eiken, Tokyo, Japan). The plates were inoculated with 1×10^7 cells and incubated for 3 days at 28°C. (B) Phenotypes of reduced swarming of BGPG2, BGPG3, and BGPG4 compared to that of wild-type BGPG1 on agar plates containing 0.45% Eiken agar. The plates were inoculated with 1×10^7 cells and incubated for 3 days at 28°C. (C) Sedimentation phenotypes of BGPG1, BGPG2, BGPG3, and BGPG4 in liquid tryptone-yeast extract medium. BGPG1 and BGPG2 show clear sedimentation phenotypes, whereby cultures of BGPG3 and BGPG4 did not settle after 42 h of incubation at room temperature. The sedimentation phenotypes of the BGPG3_c and BGPG4_c strains were partially restored by reintroducing the wild-type *bgaI2* and *bgaI3* genes into BGPG3 and BGPG4, respectively.

(ii) Lipolytic activity. All *bgaI* deletion mutants were significantly affected in the production and/or secretion of lipolytic enzymes. Clearing zones on indicator plates were, in general, reduced 2.5-fold in the mutant strains compared to the sizes of the clearing zones for the parental strain (Fig. 3A). This observation was confirmed by activity assays using *p*-nitrophenyl octanoate as the substrate (see Fig. S4 in the supplemental material). Thus, our experimental findings are in line with data obtained for the *B. glumae* strains AU6208 and ATCC 33617 (15).

(iii) Colony variation and plant-pathogenic phenotypes. The colony variation of the *B. glumae* parent strain and the *bgaI* deletion mutants was assayed by cultivation of the strains on TZC agar plates. In this assay, the colony morphology and color were influenced by the formation of extracellular or capsular polysaccharides, which were, furthermore, directly correlated with virulence (41). Colonies of the AHL synthase mutants could be readily distinguished by a deep red color (Fig. 3B). Since this test is well-known to differentiate between avirulent and virulent strains (41), we speculated that all the mutations would affect the ability to macerate plant tissue. Therefore, we set up onion maceration assays, where the wild-type strain as well as the mutant strains was tested for pathogenicity on detached onion bulb scales. As expected, each of the constructed mutant strains appeared to be attenuated in its ability to macerate onion tissue (Fig. 3C). Here, clearly macerated tissue around the wound was shown for BGPG1, whereas no maceration could be observed for BGPG2 and BGPG4 and clearly reduced maceration could be observed for BGPG3. These tests were done at least three to five times.

Furthermore, to study the virulence of *B. glumae* PG1 mutants for rice, we monitored the germination rate and recorded the length of developing rice seedlings. All mutant strains showed a reduced pathogenicity on rice seedlings compared to that of BGPG1 (Fig. 3D and E).

Altogether, these findings confirm earlier reports on QS-dependent gene regulation in *B. glumae* with respect to the role of TofI-dependent genes (11, 13, 15, 18, 56). Since mutations in

TABLE 4 Oligonucleotides used for qRT-PCR

Gene locus	Gene name	Primer sequence (5′-3′)	
		Upstream	Downstream
BGL_2c18660	*lipA*	CTATCCGGTGATCCTCGTC	GAGAGATTCGCGACGTACAC
BGL_2c18650	*lipB*	GTGGCAGACGCGCTATCAAG	CGTGAAAGTCTGCTGCCTGAG
BGL_2c21380	*rpoD*	GATGACGACGCAACCCAGAG	GAACGCTTCCTTCAGCAGCA
BGL_2c07470	*rhlA*	TGAAGCCGGAGGCCTATCTC	TTGCCGATCGTCTCGAACTC
BGL_2c07480	*rhlB*	TACGTGTCGGTGCAGGTGTC	GTGATGAGCCCCGTCTTCAG
BGL_1c18830	*csy1*	TCGCCGTGCAGAAACTTGGC	GCAGATGGTTGAGGCGGCTG
BGL_1c18840	*csy2*	TATCGAGGCGCTGCTGGTCC	TTGCAGCGCCCACATCAACC
BGL_1c01710	*flhA1*	TCAAGCGGATCAAGAGCATCC	GAGGTTGTCGCGGATATGGA
BGL_1c35020	*flgB2*	CGTTCGCTCGTACCGGCAG	CGACGTCGCGGGCCTGGTAG

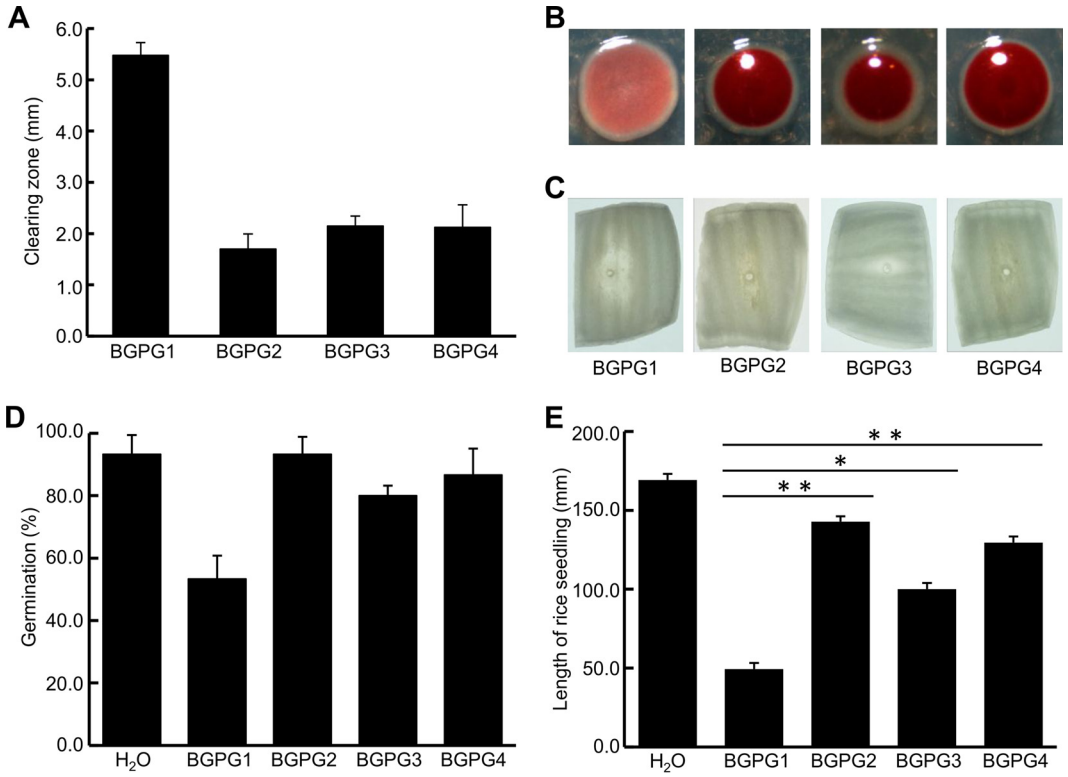


FIG 3 (A) Decreased lipase production. Clearing zones were assayed on agar plates containing TBT. Data are mean values for five colonies analyzed. (B) Colony color and morphology analyzed after growing BGPG1 to BGPG4 on TZC-containing medium for 3 days at 30°C (41). (C) Maceration of onion tissue caused by BGPG1 to BGPG4. Slices of onion bulbs were infected with the wild-type and mutant strains and incubated for 72 h at 30°C. (D and E) AHL synthase mutations reduce inhibition of seed germination (D) and favor fruit development (E). The data in panels D and E are mean values for 10 individual seedlings analyzed per treatment. *, $P < 0.05$; **, $P < 0.01$.

bgaI2 and *bgaI3* also had strong effects on motility, lipase production, and pathogenicity, our findings suggest that the QS-dependent regulatory network is more complex in BGPG1 than in BGR1 and other currently studied *B. glumae* isolates (15, 56, 57).

Global pattern of QS-dependent gene expression in BGPG1. The global patterns of gene expression in wild-type strain BGPG1 and the three AHL synthase mutants (BGPG2 to BGPG4) were analyzed at the transition from exponential to stationary growth phase (see Fig. S5 in the supplemental material). We choose this time point, since we speculated that many QS-dependent processes are turned on during the onset of the stationary growth phase. For these experiments, cells were grown for 28 h at 30°C to an OD_{600} ranging from 2.81 to 3.15 prior to total RNA extraction. Twelve individual samples representing three independent biological samples for each of the four strains were analyzed by RNA-seq (Table 3). Alignments were established, and for each sample a minimum of 24.6 million cDNA reads could be uniquely mapped to the *B. glumae* genome, resulting in 24.6 million to 37.9 million uniquely mapped reads per treatment.

In the comparative analysis of the RNA-seq data, we considered genes with a fold change of ≥2.0, a likelihood value of ≥0.9, and an FDR value of ≤0.05 to be statistically significantly differentially expressed between the BGPG1 parent strain and the mutant strains. Only values that complied with these three requirements were used for subsequent analyses (see Table S1 in the supplemental material). Expression analysis by use of the qRT-PCR technology was used in part to confirm the RNA-seq data. For this, we analyzed the expression profiles of the genes *lipA*, *lipB*, *rhlA*, *rhlB*, *csy1*, *csy2*, *flhA1*, *flgB2*, and *rpoD* as an internal control in the early-stationary-growth phase. The *lipA* gene (BGL_2c18660) codes for a lipase, and the *lipB* gene (BGL_2c18650) codes for a corresponding foldase. The *rhlA* (BGL_2c07470) and *rhlB* (BGL_2c07480) genes are involved in rhamnolipd biosynthesis and motility, the *csy1* (BGL_1c18830) and *csy2* (BGL_1c18840) genes are part of a clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* system, and the *flhA1* (BGL_1c01710) and *flgB2* (BGL_1c35020) genes are involved in the buildup of the bacterial flagella. The expression data obtained for these eight genes and the data obtained by qRT-PCR largely confirmed the data obtained by RNA-seq (see Fig. S2 and Table S1 in the supplemental material).

Furthermore, we constructed fusions of the promoters of selected and differentially regulated genes (e.g., the Abc1 gene, *cysB*, and *rhlA*) and mCherry and monitored their fluorescence in the BGPG1 wild-type strain and the three QS mutants. The Abc1 gene (BGL_2c03920) codes for a predicted ABC transporter family protein, the *cysB* gene (BGL_2c03850) codes for a predicted cysteine synthase, and the *rhlA* gene (BGL_2c07470) codes for the predicted rhamnosyltransferase I subunit A (see Table S1 in the supplemental material). All these genes were chosen because of their assumed QS-dependent regulation and importance for *B. glumae* metabolism. Comparison of the number of RFUs of strain BGPG1 to the number of RFUs of strains BGPG2 to BGPG4 confirmed in part the observed RNA-seq data and the QS-dependent

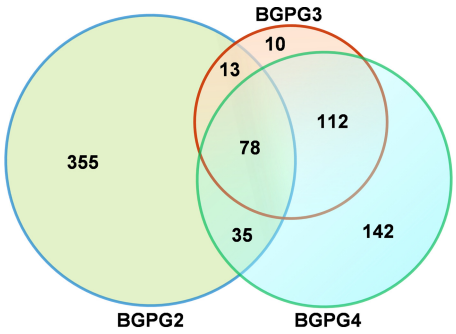


FIG 4 Venn diagram showing the relationship of differentially expressed genes (fold change, ≥2.0; likelihood, ≥0.9; adjusted FDR, ≤0.05) among the BGPG1 AHL synthase mutants. The circles display the number of genes uniquely regulated in each BGPG1 mutant compared with their regulation in the wild-type strain. The circles also show the number of commonly regulated genes within particular relationships. Overall, AHL synthase mutants BGPG2 to BGPG4 share a core set of 78 differentially regulated genes.

regulation of these genes (see Fig. S6 in the supplemental material). Thereby, the downregulation of the Abc1 gene could be confirmed for BGPG2 and BGPG3 but not for BGPG4. The use of the P*cysB*::*mCherry* promoter fusion confirmed the downregulation of the *cysB* gene only in BGPG2 and not in BGPG3 and BGPG4. Lastly, the P*rhlA*::*mCherry* promoter fusion demonstrated the downregulation of the *rhlA* gene in the QS mutant BGPG2. This finding is in line with the RNA-seq data obtained for the *rhlA* gene, where no downregulation was also observed in the background of BGPG3 and BGPG4.

The partly different results in the fluorescence measurements obtained for BGPG3 and BGPG4 may be explained in part by the multicopy effects caused by the use of the promoter fusions in a self-replicable plasmid with multiple copies, which thereby outcompeted the corresponding single-copy regulator. Furthermore, the deviation of the results might also be linked in part to the phenotypic heterogeneous expression of these genes.

***bgaI1*-, *bgaI2*-, and *bgaI3*-specific gene regulation.** The genome of BGPG1 consists of a total of 6,502 predicted genes located on two large chromosomes. Chromosome 1, with a size of 4.164 Mbp, codes for 3,562 genes, and chromosome 2 has a size of 3.733 Mbp and codes for 2,940 genes.

A total of 481 genes (6.5% of all predicted genes) were found to be differentially regulated in the BGPG2 deletion mutant compared to their regulation in the wild-type strain, and the expression of a total of 213 genes (2.9% of all predicted genes) was significantly altered in the BGPG3 mutant. Moreover, the expression of a set of 367 genes (4.9% of all predicted genes) was specifically altered in the BGPG4 mutant strain (Fig. 4). Altogether, a common subset of 78 genes was differentially regulated in the three mutant strains in comparison to their expression in the wild type. In total, 355 genes appeared to be specifically regulated in the BGPG2 strain, 10 genes appeared to be specifically regulated in the BGPG3 strain, and 142 genes appeared to be uniquely regulated in the BGPG4 deletion mutant. Thus, a total of 745 genes (11.5% of all predicted genes) were regulated in a QS-dependent way (Fig. 4). However, with 340 QS-regulated genes being on chromosome 1 and 405 being on chromosome 2, the majority of all QS-regulated genes were located on the second replicon.

Interestingly, two of the QS systems of BGPG1 are found on chromosome 2 (i.e., *bgaI1* and *bgaI2*), while the third one (*bgaI3*) is encoded on the larger chromosome 1, which harbors the housekeeping genes. This differs from the situation observed in *B. thailandensis* (28). Further distribution analyses of QS-regulated genes implied that no obvious correlation exists between the de-

TABLE 5 Shared functional and QS-regulated homologous genes in BGPG1 versus selected members of the Bptm group with multiple AHL QS systems

Predicted function	Presence of gene for the indicated protein in[a]:			
	BGPG1	*B. thailandensis*	*B. pseudomallei*	*B. mallei*
Flagellum biosynthesis	+ (21)	+ (6)	−	−
AHL synthases	+ (3)	+ (3)	+ (1)	−
LuxR proteins	+ (5)	+ (3)	−	+ (1)
Polyketide biosynthesis (PKS)	+ (5)	+ (5)	+ (4)	−
Nonribosomal peptide synthases (NRPS)	+ (5)	+ (1)	+ (2)	+ (2)
Rhamnosyltransferase I	+ (3)	+ (2)	−	−
T1SS	+ (2)	+ (1)	−	−
T2SS	−	+ (1)	−	−
T3SS	+ (4)	+ (1)	+ (3)	−
T4SS	−	+ (2)	−	−
T6SS	+ (13)	−	−	−
Flp pilus assembly	+ (9)	−	−	−
Lipase A	+ (1)	−	−	−
Histidine utilization system	+ (3)	+ (3)	+ (1)	−
Phosphate metabolism	+ (6)	−	−	−
Major facilitator family transporter	+ (10)	+ (5)	+ (4)	−
Malleilactone biosynthesis	−	+ (2)	+ (2)	+ (2)
ABC transporter	+ (17)	+ (14)	+ (7)	+ (2)
Polysaccharide biosynthesis	+ (6)	+ (6)	+ (1)	−
Lipoproteins	+ (11)	+ (8)	+ (1)	−
Ribosomal proteins	+ (7)	+ (1)	+ (26)	−

[a] Transcriptome data sets for members of the Bptm group (*B. thailandensis*, *B. pseudomallei*, *B. mallei*) were obtained from references 28 and 30, and the number of homologous genes identified is given by numbers in parentheses.

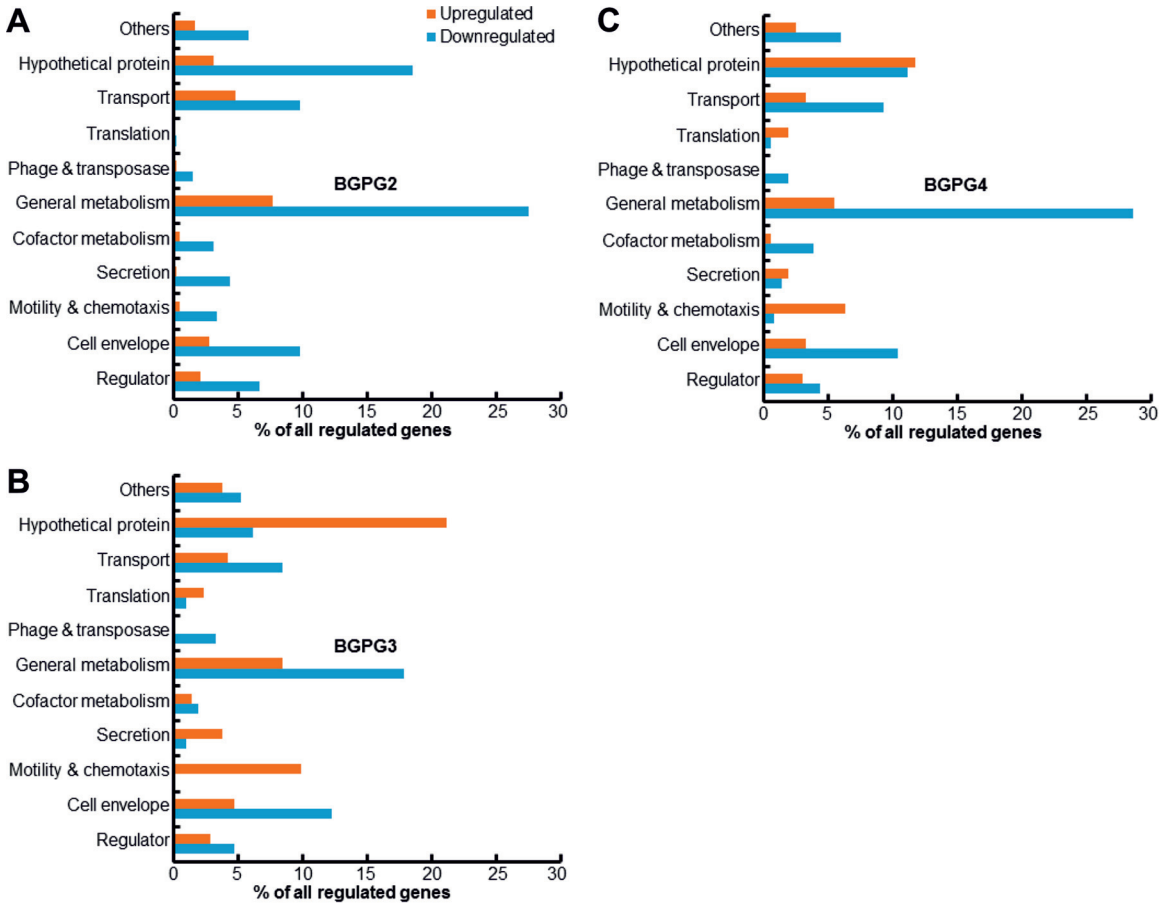
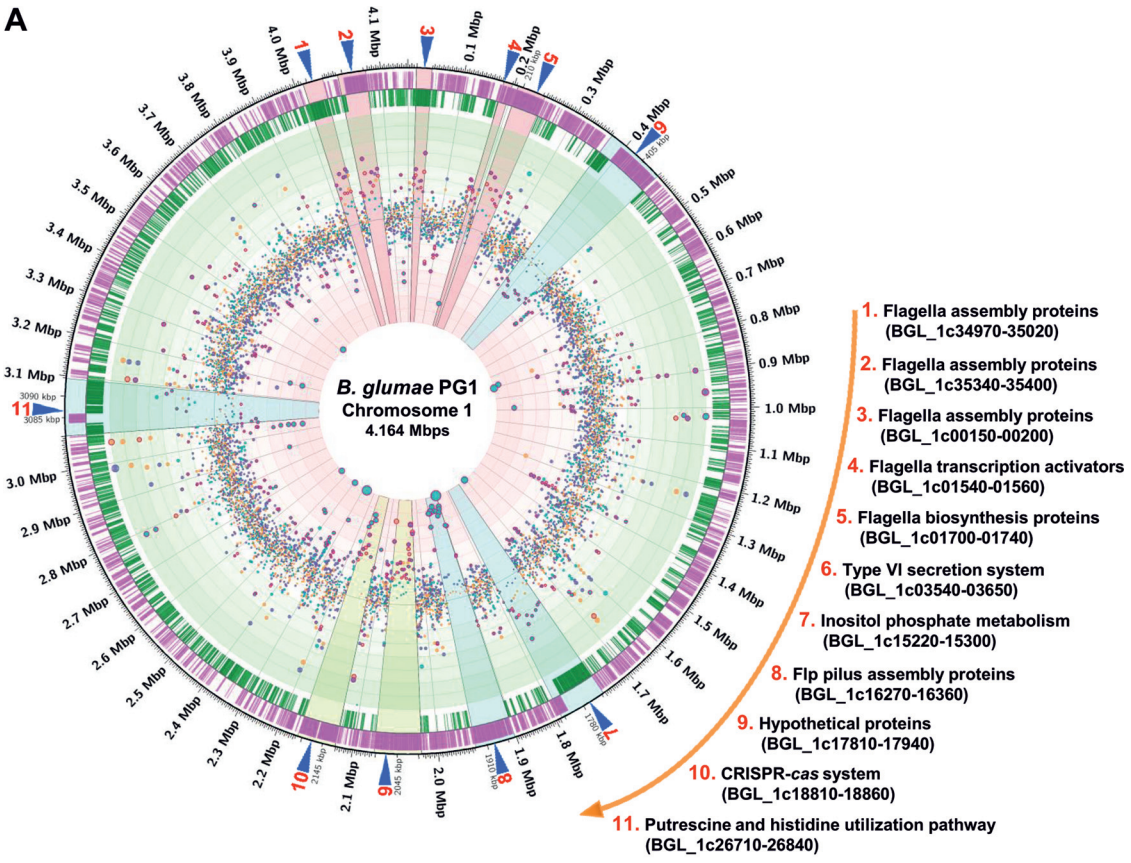


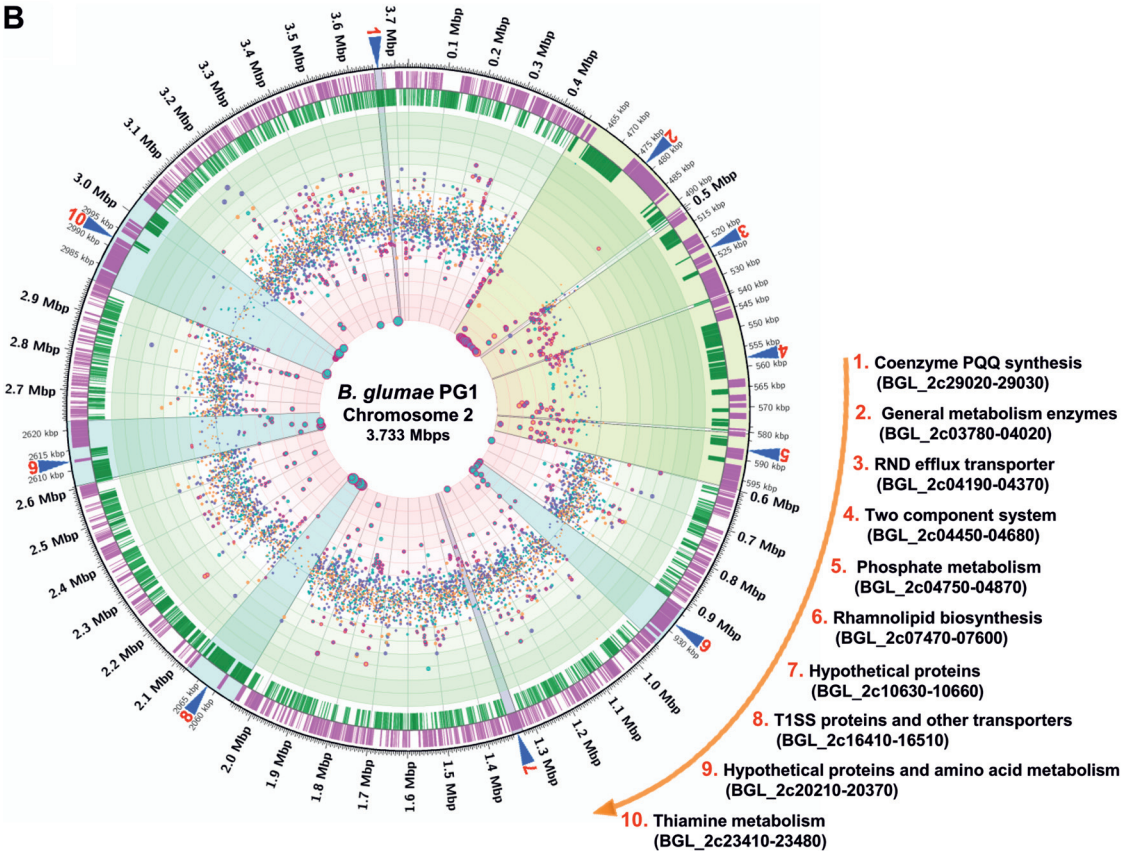
FIG 5 Genes differentially expressed (in percent) in BGPG1 mutant strains in the background of BGPG2 versus the wild-type strain (A), BGPG3 versus the wild-type strain (B), and BGPG4 versus the wild-type strain (C). The classification was based on the KEGG database (http://www.genome.jp/kegg/pathway.html).

leted AI synthase gene and the corresponding number of differentially regulated genes on the respective chromosome. Overall, the distribution over both replicons is balanced in the background of all three mutant strains.

To date, only a few studies have been performed using genome-wide transcriptome analyses to identify QS-regulated gene expression patterns. Each study focused on a different organism, and the growth conditions also differed; hence, the number of genes that were differentially regulated in response to QS processes varied. In recent studies, the QS regulons represented up to 6.2% of the coding sequences in the *P. aeruginosa* genome (for *lasI* and *lasR* and for *rhlI* and *rhlR*) (58), up to 8.1% of the coding sequences in the *Yersinia pestis* genome (59), up to 8.0% coding sequences in the *B. thailandensis* genome (28), 0.8% of the coding sequences in the *B. mallei* genome, 3.6% coding sequences in the *B. pseudomallei* genome (30), and between 4.9 and 7.3% of the coding sequences in the in *S. fredii* NGR234 genome (40). We observed that 11.5% of all genes were differentially regulated by the three BGPG1 QS systems, and it was recently reported that up to 19.6% of the BGR1 genes were regulated in a QS-dependent manner in a *tofI* mutant (34). This value, however, differs from our data, since a different cutoff was used and the overall read coverage of the BGR1 genome was 8- to 35-fold lower. Also, cells were harvested after 8 and 10 h of growth at 37°C in the study by Kim and colleagues (34), while RNA was extracted from our strains after 28 h of growth at 30°C. Finally, it is noteworthy that the data represent those from a comparison of the *B. glumae* PG1 wild-type strain to its AHL synthase deletion mutants and not those from a comparison of the *B. glumae* PG1 wild-type strain to its LuxR regulator deletion mutants. Thus, we cannot exclude the possibility of cross talk between the different regulons due to the promiscuity of the LuxR regulator proteins with respect to the recognition of the AHL signaling molecules.

Interplay between the three *bgaI* QS systems in BGPG1. Results from our transcriptome analyses implied that deletion of the *bgaI1* gene caused a 10.5-fold downregulation of the expression profile of the *bgaI2* gene and a 2.9-fold downregulation of the *bgaI3* gene (see Table S1 in the supplemental material). The deletion of *bgaI2* had no obvious effect on expression of both *bgaI1* and *bgaI3*. The deletion of *bgaI3* caused a 5.7-fold downregulation of the expression of *bgaI2* but had no obvious effect on the expression of *bgaI1* (see Table S1 in the supplemental material). These data suggest that in BGPG1 the three QS systems form a network, with *bgaI1* most likely being at the top of the hierarchy, followed by *bgaI3*. Similarly, other Gram-negative bacteria employ multiple AHL-dependent QS systems to control group behaviors during their life cycle, such as *P. aeruginosa* and members of the Bptm group but not rhizobial or other isolates (30, 40, 60, 61).


A
B. glumae PG1
Chromosome 1
4.164 Mbps
1. Flagella assembly proteins (BGL_1c34970-35020)
2. Flagella assembly proteins (BGL_1c35340-35400)
3. Flagella assembly proteins (BGL_1c00150-00200)
4. Flagella transcription activators (BGL_1c01540-01560)
5. Flagella biosynthesis proteins (BGL_1c01700-01740)
6. Type VI secretion system (BGL_1c03540-03650)
7. Inositol phosphate metabolism (BGL_1c15220-15300)
8. Flp pilus assembly proteins (BGL_1c16270-16360)
9. Hypothetical proteins (BGL_1c17810-17940)
10. CRISPR-cas system (BGL_1c18810-18860)
11. Putrescine and histidine utilization pathway (BGL_1c26710-26840)


B
B. glumae PG1
Chromosome 2
3.733 Mbps
1. Coenzyme PQQ synthesis (BGL_2c29020-29030)
2. General metabolism enzymes (BGL_2c03780-04020)
3. RND efflux transporter (BGL_2c04190-04370)
4. Two component system (BGL_2c04450-04680)
5. Phosphate metabolism (BGL_2c04750-04870)
6. Rhamnolipid biosynthesis (BGL_2c07470-07600)
7. Hypothetical proteins (BGL_2c10630-10660)
8. T1SS proteins and other transporters (BGL_2c16410-16510)
9. Hypothetical proteins and amino acid metabolism (BGL_2c20210-20370)
10. Thiamine metabolism (BGL_2c23410-23480)

QS-regulated orthologous genes in other *Burkholderia* species. Since *B. glumae* is a plant pathogen, we asked which of the QS-regulated genes are involved in plant infection and which ones are involved in life in the soil. Fortunately, Majerczyk and colleagues (28, 30) have recently described the QS regulons in the tropical soil bacterium *B. thailandensis* and the human- and animal-pathogenic species *B. mallei* and *B. pseudomallei*. The *B. thailandensis* genome codes for three AHL synthase genes, and *B. thailandensis* has a nonpathogenic saprophytic lifestyle. A comparison of the QS-dependent genes reported for *B. thailandensis* and the genes identified in this study uncovered a common set of 61 orthologous genes coregulated in both microbes. Of these, 41 genes were coregulated by *bgaI1*, 17 genes were coregulated by *bgaI2*, and 26 genes were coregulated by *bgaI3*. Further, a direct comparison of the data published by Majerczyk et al. (30) and our data allowed us to identify eight shared and QS-regulated genes in *B. pseudomallei* but no QS-regulated genes shared between *B. mallei* and BGPG1. Our data, together with the data set from Majerczyk et al. (28, 30), now allow us to draw the conclusion that no common core set of genes is shared and QS regulated in the four strains analyzed (Table 5). However, a small number of QS-regulated and shared genes can be identified in *B. thailandensis*, *B. pseudomallei*, and BGPG1. The absence of shared genes between BGPG1 and *B. mallei* may indicate a wider phylogenetic distance between these bacteria. It should be noted that a direct functional comparison may add shared functional homologs. Applying this strategy and comparing the functional homologs of QS-regulated genes in the *Burkholderia* isolates from our study and the two studies by Majerczyk et al. (28, 30), we identified two shared functional homologs in all strains, namely, nonribosomal peptide synthases genes and ABC transporters (Table 5).

Function-based evaluation of the BGPG1 transcriptome data. The genes differentially expressed in the three AHL synthase mutants and the parent strain were classified into 11 functional categories on the basis of the KEGG database (http://www.genome.jp/kegg/pathway.html). As indicated in Fig. 5A to C, the genome-wide transcriptome data analysis revealed that the regulated genes were mainly linked to general metabolism (Fig. 6A, cluster 11, and B, clusters 2, 5, and 10) and hypothetical proteins (Fig. 6A, cluster 9, and B, clusters 7 and 9; see also Table S1 in the supplemental material) in all three mutants. Striking changes in the expression of genes in selected important functional categories are discussed below. A complete list of all QS-regulated genes is given in Table S1 in the supplemental material. Some of the identified genes were mentioned in an earlier low-resolution RNA-seq study of *B. glumae* strain BGR1 and a corresponding AI synthase and receptor mutant (34). Since the BGR1 genome codes for only a single AI synthase gene, overall large differences in the regulatory networks associated with the QS-dependent gene regulation occur in BGR1 and our strain BGPG1.

QS-dependent regulation of flagellar genes and their regulators. In many bacteria, motility on surfaces and in liquids is controlled by QS (13, 62–64). While in BGR1 a single AHL synthase is involved in the QS-dependent regulation of flagellar movement, our experimental data suggested that in BGPG1 all three different AHL synthase genes are involved in the regulation of swimming and swarming motility (Fig. 2A and B). The QS-dependent phenotype observed in this work is in line with the QS-dependent BGR1 motility (13, 18). In *B. glumae* BGR1, flagellum-driven motility is essential for the infection of rice plants and subject to QS- and temperature-dependent regulation. Nonmotile mutants are attenuated in their virulence (13). In BGR1, flagellar motility is subject to a complex system of regulation, where FlhDC together with the QsmR regulatory protein is a key regulator of flagellum-dependent motility (13, 18). Thereby, TofR, the AHL receptor/regulator, activates QsmR transcription, and QsmR activates the flagellar master activator FlhDC, which subsequently activates flagellar biosynthesis (13, 18). In BGPG1, BGL_1c10570 encodes the QsmR homologue, and our data indicate that *qsmR* in BGPG1 is not affected by QS.

Interestingly, our RNA-seq data indicate that the majority of the structural genes were upregulated 2.0- to 5.3-fold in the *bgaI2* and *bgaI3* mutant strains compared to their level of regulation in the parent strain (Fig. 6A, clusters 1 to 5; see also Table S1 in the supplemental material). The higher level of transcription of the flagellar genes is in line with reports from *S. fredii* NGR234 in the background of two AHL mutants (40) and is consistent with the increased transcription of flagellar genes in BGR1 (34) and *B. gladioli tofI* mutants (29). The overall stronger transcription of the flagellar genes is most likely responsible for the delayed sedimentation of the mutants BGPG3 and BGPG4 compared with the time to sedimentation of the parent strain that was observed (Fig. 2C).

Within this framework, it is noteworthy that in other Gram-negative bacteria, the *qseBC* genes are involved in the regulation of the flagellum regulator FlhDC (65–68). While the direct involvement of the *qseBC* genes in motility has not been shown for *B. glumae*, it is perhaps reasonable to suggest that they are involved in the regulation of motility in BGPG1 as well. Within this framework, we observed that *qseBC* genes were downregulated in the three mutants analyzed in this work (Fig. 6B, cluster 4; see also Table S1 in the supplemental material). This finding implies that the expression levels of the different regulators rather than their structural features were responsible for the flagellum-dependent motility phenotypes observed on agar plates (Fig. 2A and B).

The BGPG1 genome encodes a QS-regulated type IVb/Flp pilus on chromosome 1. We further asked to what extent genes involved in the synthesis or formation of type IVb/Flp pili are regulated in a QS-dependent manner. Genes for these pili have been identified at up to four copies per genome in a wide variety of

FIG 6 Circular transcriptome map representing the genome-wide RNA-seq data for mutants BGPG2 to BGPG4 versus parent strain BGPG1 obtained with Circos software (version 0.64) (83). Cyan dots, orange dots, and deep purple dots, genes from strains BGPG2, BGPG3, and BGPG4, respectively; dots with red-violet circles, differentially expressed genes. The cutoff was set to a fold change of 2.0 with an FDR of ≤0.05 (the sizes of the dots correspond to the values obtained). The circles from the outside to the innermost circle are as follows: the first circle indicates the genome coordinates of BGPG1 in mega-base pairs; the second and third circles indicate open reading frames on the leading (purple) and the lagging (deep green) strands. The light green circles represent genes with $\log_2$ fold changes of 4, 3, 2, and 1 (from the outside to inside); the light red circles represent genes with log2 fold changes of −1, −2, −3, and −4 (from outside to inside). Highlighted areas (labeled with numbers) are magnified 5-fold and show gene clusters which are differentially expressed in the mutant strains. Clusters in light cyan and light purple, genes differentially expressed only in BGPG2 and BGPG4, respectively; clusters in light pink, genes differentially expressed in both BGPG3 and BGPG4; clusters in pale green, genes differentially expressed in BGPG2, BGPG3, and BGPG4. (A) Circular map representing RNA-seq data from chromosome 1 of BGPG1; (B) circular map representing RNA-seq data from chromosome 2 of BGPG1.

different bacteria and archaea (69–71). Flp pili are involved in surface attachment and in pathogenic interactions with eukaryotic hosts. The *tad* (tight adherence) macromolecular transport system represents an ancient subtype of the type II secretion system (T2SS) and is essential for pilus biogenesis (72, 73). The BGPG1 genome encodes a single cluster of Flp pilus genes on chromosome 1. In the three mutants, the *flp* gene cluster was significantly downregulated (Fig. 6A, cluster 8). The strongest effects were observed in BGPG2, with this gene cluster being downregulated more than 10-fold compared to the levels of expression in the wild-type strain (see Table S1 in the supplemental material). We can only speculate that the *B. glumae* Flp pilus plays a crucial role during the infection process, as recently shown for the plant pathogen *Ralstonia solanacearum* (73). It is likely that the downregulation of Flp pilus genes is also responsible in part for the motility phenotypes observed in Fig. 2A and B.

T1SS and T6SS are subject to QS-dependent regulation. Although the BGPG1 genome encodes many genes linked to the formation of secretion systems, only genes for the type I secretion system (T1SS) and T6SS were regulated in a QS-dependent manner in BGPG1. We identified four QS-regulated T1SS-associated genes, one on chromosome 1 and three on chromosome 2 (Fig. 6B, cluster 8; see also Table S1 in the supplemental material). T1SS is widely distributed in Gram-negative bacteria and is able to transport a variety of substrates, including proteins, antibiotics, toxins, and metal ions, by a one-step mechanism (74). Further, we observed T6SS-affiliated and QS-regulated genes on both chromosomes, but they were spread out in several clusters. One larger cluster (T6SS-1) was identified on chromosome 1 (Fig. 6A, cluster 6; see also Table S1 in the supplemental material). This cluster is similar to a cluster identified in *B. thailandensis* strain E264 (75). This secretion pathway has been shown to be involved in bacterial virulence and interaction with other organisms using a contact-dependent protein translocation mechanism (76).

Genes linked to a CRISPR-*cas* gene cluster are QS dependent. Clustered regularly interspaced short palindromic repeats (CRISPRs) are observed in the genomes of many prokaryotes, and they protect them from invasion by bacteriophages or foreign plasmid DNA (77, 78). Each cluster is linked to a subset of specific CRISPR-associated *cas* genes (79, 80). CRISPR systems have been associated with virulence and biofilm formation in different human-pathogenic microorganisms, and their expression can be modulated through stress, temperature shifts, and other environmental stimuli (81). Three CRISPR arrays were identified in BGPG1 (26). Two of the clusters were accompanied by *cas* genes. Interestingly, our data searches indicated that only two other *B. glumae*/*B. gladioli* isolates (i.e., *B. glumae* 3242-8 and *B. glumae* A.1) have a CRISPR-*cas* system. This feature appears to be unique to a small group of *B. glumae*/*B. gladioli* isolates and may be of importance for the plant interaction.

In the course of the transcriptome data analysis, we identified a CRISPR cluster together with the associated *cas* genes spanning from BGL_1c18810 to BGL_1c18860. The cluster was differentially transcribed in all three AHL mutants compared to their transcription in the wild-type strain (Fig. 6A, cluster 10; see also Table S1 in the supplemental material). Thereby, we observed 2- to 10-fold decreased transcription in BGPG2, 1.2- to 3.8-fold decreased transcription in BGPG3, and 1.1- to 5.6-fold decreased transcription in BGPG4. Data from qRT-PCR analyses verified these findings (see Fig. S2 in the supplemental material).

Altogether this finding suggests the QS-dependent regulation of cell immunity in bacteria. In fact, this is the first report on the QS-dependent regulation of CRISPR-*cas* genes.

Genes linked to secondary metabolite synthesis and general metabolism. Altogether, 284 QS-regulated genes were linked to the main metabolic activities of BGPG1. Similar to the findings for *B. thailandensis* (28), several polyketide biosynthesis clusters were subject to QS regulation. Further, rhamnolipid biosynthesis genes were differentially regulated (Fig. 6B, cluster 6; see also Table S1 in the supplemental material). The rhamnolipid synthesis genes *rhlABC* are known to be controlled by QS in, for example, *P. aeruginosa* (82). This is in line with our findings for BGPG2 as well (see Fig. S2 in the supplemental material). Further, selected genes from phosphate metabolism (Fig. 6B, cluster 5; see also Table S1 in the supplemental material) as well as a large cluster involved in inositol phosphate biosynthesis (Fig. 6A, cluster 7; see also Table S1 in the supplemental material) were subject to QS-dependent regulation. The observation that genes linked to central phosphate metabolism are QS regulated shows the importance of AHL-dependent regulatory circuits, and especially, the observation that genes involved in inositol phosphate biosynthesis are QS regulated raises the question of whether inositol phosphate is involved in plant-microbe signaling.

In addition, several clusters involved in amino acid and cofactor biosynthesis were QS regulated (BGL_1c26710 to BGL_1c26840 and BGL_2c20210 to BGL_2c20370; Fig. 6A, cluster 11, and B, cluster 9; see also Table S1 in the supplemental material). With respect to the QS-dependent biosynthesis of cofactors, coenzyme pyrroloquinoline quinone (PQQ) and thiamine biosynthesis appeared to be regulated in a QS-dependent manner as well (Fig. 6B, clusters 1 and 10; see also Table S1 in the supplemental material).

Because of the relatively high number of QS-regulated genes which are linked to metabolite biosynthesis and general metabolism, one can speculate that QS is of strong importance for growth and survival in soil or saprophytic growth on the plant surface.

Conclusions. A set of 745 QS-regulated genes was identified in BGPG1 and its AHL synthase mutants using RNA-seq. Among them were the genes linked to flagellum, Flp pilus, T1SS, T6SS, and metabolite biosynthesis as well as a CRISPR-*cas* gene cluster. With respect to the QS-dependent expression of motility, pathogenicity, and lipase-related genes, phenotypic analyses confirmed these findings. Finally, it is noteworthy that BGPG1 shared most QS-dependent functional orthologs with *B. thailandensis*, suggesting a common evolutionary origin of QS-dependent regulatory circuits in these microorganisms.

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