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The Type 2 Secretion Pseudopilin, *gspJ*, Is Required for Multihost Pathogenicity of *Burkholderia cenocepacia* AU1054[▽]

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***Burkholderia cenocepacia* AU1054 is an opportunistic pathogen isolated from the blood of a person with cystic fibrosis. AU1054 is a multihost pathogen causing rapid pathogenicity to *Caenorhabditis elegans* nematodes. Within 24 h, AU1054 causes greater than 50% mortality, reduced growth, emaciated body, distended intestinal lumen, rectal swelling, and prolific infection of the nematode intestine. To determine virulence mechanisms, 3,000 transposon mutants were screened for attenuated virulence in nematodes. Fourteen virulence-attenuated mutants were isolated, and the mutant genes were identified. These genes included *paaA*, previously identified as being required for full virulence of *B. cenocepacia* K56-2. Six mutants were restored in virulence by complementation with their respective wild-type gene. One of these contained an insertion in *gspJ*, predicted to encode a pseudopilin component of the type 2 secretion system (T2SS). Nematodes infected with AU1054 *gspJ* had fewer bacteria present in the intestine than those infected with the wild type but still showed rectal swelling. The *gspJ* mutant was also defective in pathogenicity to onion and in degradation of polygalacturonic acid and casein. This result differs from previous studies where no or little role was found for T2SS in *Burkholderia* virulence, although virulence factors such as zinc metalloproteases and polygalacturonase are known to be secreted by the T2SS. This study highlights strain specific differences in *B. cenocepacia* virulence mechanisms important for understanding what enables environmental microbes to function as opportunistic pathogens.**

The betaproteobacterium *Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (BCC), now consisting of 17 classified species (72). Members of the BCC are ubiquitous in the environment, metabolically diverse, and beneficial or pathogenic to a variety of organisms and have large and dynamic multireplicon genomes (11, 48). Originally described as a pathogen to onion plants (9), members of the BCC have emerged as opportunistic pathogens of serious concern to persons with cystic fibrosis (CF) or chronic granulomatous diseases (26, 29, 36). Although *Pseudomonas aeruginosa* is more commonly isolated from CF infections, infections with BCC also occur and are a serious concern because of their inherent multidrug resistance and correlation with the severe loss of lung function, sepsis, and fatality referred to as cepacia syndrome (36). Infections of CF patients arise from patient-to-patient transmission of epidemic clones or sporadically from a presumed environmental source (29). Epidemic strains of *B. cenocepacia* and *B. multivorans* are correlated with the incidence of *cepacia* syndrome (49, 75). The PHDC epidemic clone strain, *B. cenocepacia* AU1054 (called AU1054 hereafter), is a multihost pathogen with high virulence to the nematode *Caenorhabditis elegans* and to onions (13, 44, 64).

The *C. elegans* model for the study of bacterial pathogenicity was originally developed for *P. aeruginosa* (20, 69), which causes nematode mortality by two mechanisms termed fast killing (within hours, toxin mediated) (46, 69) and slow killing (in days, infection mediated) (69). Virulence factors required for *C. elegans* killing are sometimes also involved in multihost virulence (e.g., plant and animal) (45). The *C. elegans* model has now been established for the study of virulence of a large variety of pathogens including BCC (10, 19, 41). AU1054 is highly pathogenic to *C. elegans* killing nematodes fast on minimal medium where *P. aeruginosa* kills slowly by an unknown mechanism (64).

Type 2 secretion systems (T2SSs) are required for the secretion of many toxins and enzymes that contribute to virulence, notably exotoxin A and cholera toxin produced by *P. aeruginosa* and *Vibrio cholerae*, respectively (23, 62). Previous studies did not detect a significant defect in virulence of *B. pseudomallei*, *B. cenocepacia*, and *B. vietnamiensis* strains mutant in T2SS, although the T2SS is required for *B. gladioli* pathogenicity to mushrooms (14, 21, 24, 41). However, expression of the T2SS genes was found to be induced in synthetic CF sputum medium relative to soil extract by strains AU1054 and J2315; although the latter was unexpectedly found to have a 110-bp deletion in *gspL*, known to be required for T2SS function (34, 73, 74). These contradictory results suggest that it is possible that the T2SS is important for BCC to function as opportunistic pathogens.

We describe here the application of the *C. elegans* model for the study of multihost pathogenicity of the CF epidemic *B. cenocepacia* strain AU1054. Our goal was to identify genes required for AU1054 pathogenicity using the *C. elegans* model and to

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TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Relevance or genotype[a]	Source or reference
Strains		
Nematode		
C. elegans SS104	*C. elegans* strain SS104 [glp-4(bn2)I], *ts* sterile at 25°C	*Caenorhabditis* Genetics Center, University of Minnesota
Bacteria		
B. cenocepacia AU1054	CF clinical isolate, U.S. PHDC epidemic clone	J. J. LiPuma, U.S. *Burkholderia cepacia* Research Laboratory and Repository, University of Michigan
P. aeruginosa PA14	Control CF pathogen	69
E. coli OP50	Nematode food source	8
E. coli BW29427	*dap* auxotroph, *tra pir*	K. A. Datsenko and B. L. Wanner, Purdue University
E. coli EC100 *pir-116*	π protein for replication of $ori_{R6K\gamma}$	Epicentre Biotechnologies
Plasmids		
pBBR1MCS	Broad-host-range cloning vector; Cm^r	42
pURR25	Mini Tn*7*KsGFP, GFP driven by P_{lac} ($P_{A1/04/03}$) promoter, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6k\gamma}$; Ap^r (*bla*)	D. Lies and D. Newman, Caltech
pURE10	Mini-*HimarGm* transposon, C9 transposase, suicide $ori_{R6K}\gamma$; Gm^r Ap^r	D. Lies and D. Newman, Caltech
pUX-BF13	Tn*7* transposase genes *tnsABCDE*, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6k}\gamma$; Ap^r (*bla*)	4
pCS238	Source of *flmAB-ccdAB* toxin-antitoxin cassette	12
pTCV2	pBBR1MCS *flmAB-ccdAB* (toxin-antitoxin cassette); Cm^r	This study
pTCV3	pTCV2 Ptac::GFP; Cm^r	This study
p*bnvR*	pTCV2 *bnvR*	This study
p*nhaX*	pTCV2 *nhaX*	This study
p*argG*	pTCV2 *argG*	This study
p*aroA*	pTCV2 *aroA*	This study
p*gspJ*	pTCV2 *gspJ*	This study
p*purF*	pTCV2 *purF*	This study

[a] Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Ap^r, ampicillin resistance.

determine the role of these genes in pathogenicity to multiple hosts.

MATERIALS AND METHODS

Strains and media. The bacterial and nematode strains and plasmids used in the present study are listed in Table 1. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Strain AU1054 was chosen for this study because it was isolated from the blood of a person with cystic fibrosis, is a member of the PHDC epidemic clonal lineage, is highly virulent to *C. elegans* nematodes and onions (64), produces high antifungal activity against *Rhizoctonia solani* AG4, and hence was an ideal candidate for analysis of multihost pathogenicity. AU1054 was cultured on LB medium modified to contain 5 g of NaCl/liter and amended with 1.5% agar (Becton Dickinson, Franklin Lakes, NJ), 150 μg of chloramphenicol/ml, and 300 μg of gentamicin/ml when required. For *Escherichia coli*, LB medium was amended with 100 μg of ampicillin/ml, 120 μg of chloramphenicol/ml, 10 μg of gentamicin/ml, 30 μg of kanamycin/ml, 20 μg of streptomycin/ml, and 300 μg of diaminopimelic acid (DAP)/ml when required. The cultures were grown aerobically at 37°C.

GFP labeling. Green fluorescent protein (GFP)-labeled strains of *E. coli* OP50, *P. aeruginosa* PA14, and *B. cenocepacia* AU1054 were made by inserting a mini-Tn*7* transposon containing P_{lac}::GFP into the bacterial strains by triparental mating as described previously (17). Briefly, donor strain BW29427 (K. A. Datsenko and B. L. Wanner, unpublished data) containing either pURR25 (plasmid containing the miniTn*7*KSGFP) or pUX-BF13 (encoding Tn*7* transposase) were grown and mated with the AU1054 cells by triparental mating on LB agar containing DAP. GFP-labeled recipient cells were selected by resistance to kanamycin and streptomycin (BW29427 requires DAP to grow) and screened for GFP fluorescence by using a MZ16F stereo-fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Molecular biological techniques. Standard molecular techniques were used as previously described (60). Enzymes used were obtained from New England Biolabs (Ipswich, MA) or Invitrogen (Carlsbad, CA) unless otherwise indicated. Genomic DNA was purified by using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) and plasmid DNA was purified by using a Mini or Midi plasmid kit (Qiagen).

Transposon mutagenesis. GFP-labeled AU1054 was mutagenized by the hyperactive mariner HimarGm transposon present on the suicide plasmid pURE10. pURE10 was purified from EC100 *pir-116* (Epicentre, Madison, WI) prior to electroporation into AU1054. Electrocompetent AU1054 cells were prepared from 1 ml of a mid-exponential-phase culture that was washed and concentrated using sterile 10% (wt/vol) glycerol solution. AU1054 HimarGm mutants were selected on LB agar containing gentamicin. Three hundred mutants were screened in each of 10 independent mutagenesis experiments.

Screening for virulence attenuated mutants by nematode pathogenesis assays. *C. elegans* strain SS104 [glp-4(bn2)I], which is sterile at 25°C but fertile at 15°C, was used for nematode pathogenicity assays on nematode growth medium (NGM), *P. aeruginosa* PA14 was used as a positive control, and *E. coli* OP50 served as the negative control. The nematodes were maintained on *E. coli* OP50 lawns on NGM plates at 15°C and synchronized by collecting eggs from the gravid nematodes using bleach-alkali solution. The eggs were transferred to OP50 lawns and allowed to develop to early L4 stage (25°C for 36 to 38 h) used for the assays.

Bacterial strains were grown in LB medium in 96-well microtiter plates (Corning, Inc., Corning, NY) for 14 to 16 h at 37°C, with shaking at 250 rpm. Then, 75 μl of these liquid cultures was spread on 60-by-15-mm petri plates containing NGM, followed by incubation at 37°C for 16 to 18 h to get confluent bacterial lawns of all strains on the NGM assay plates. A total of 40 to 60 early L4-stage *C. elegans* nematodes were added to the plates containing bacterial lawns, and the number of surviving nematodes was determined after 3 days as those moving or responding to touch. Putative mutants attenuated in *C. elegans* pathogenicity were verified in three independent experiments consisting of three replicates each.

The mean of 3-day nematode survival data from mutant screens and complementation experiments was compared by using Student *t* test to determine the statistical significance ($P = 0.05$). Further survival analysis of nematodes on lawns of AU1054 mutant and complemented strains was performed by counting the number of surviving nematodes daily for 7 days and then analyzing the data

TABLE 2. Oligonucleotides used in this study

Oligonucleotide or complementation primer	Sequence (5′–3′)[a]	Restriction enzyme
Oligonucleotides		
TetR-Forward	GATC**CTGCAG**GCGGCCGCCCACCGCGGTGGGATATCATGAAATCT AACAATGCGCTC	PstI
TetR-Reverse	GATC**CTGCAG**TGCACCGCGACGCAACGC	PstI
MarOUT	CCGGGGACTTATCAGCCAACC	
GMRFor	CGGTAAATTGTCACAACGCC	
GFPFor	GA**ACTAGT**CTCGAGAAAATTTATCAAAAAGAGTGTTGACTT	SpeI
GFPRev	GA**ACTAGT**TTATTTGTATAGTTCATCCATGCCATGTGTAATC	SpeI
Lower flm	TTCG**TCTAGA**CCTGGCAGTCTGGTTGTTCAT	XbaI
Lower ccd	CCGA**TCTAGA**CTGCAGACTGGCTGTGTATAAC	XbaI
Complementation primers		
B-cen_0002 C-F	GCAT**GGTACC**ACAATCCAGATCGGCAACCT	KpnI
B-cen_0002 C-R	CTAG**ACTAGT**TCATGCTTTCGGTGATTTCC	SpeI
B-cen_0015 C-F	GCAT**GGTACC**GGCGAACAGAGTTTCGACTG	KpnI
B-cen_0015 C-R	CTAG**ACTAGT**GATCACGCGCTGGTTCTC	SpeI
B-cen_0566 C-F	GCAT**GGTACC**GACGAACTCGACGGCTACAC	KpnI
B-cen_0566 C-R	GCTA**ACTAGT**CTGTCGAGCAGGTGAAAGC	SpeI
B-cen_3021 C-F	GCAT**GGTACC**GGGACTGACGATTCGCATAC	KpnI
B-cen_3021 C-R	CTGA**ACTAGT**CTTCGAGTACCTCGCGTTCT	SpeI
B-cen_4402 C-F	GCAT**GGTACC**AACTGCCCAAACAGGATTTC	KpnI
B-cen_4402 C-R	CTAG**ACTAGT**AAAACAAAAAGCCCGCTATG	SpeI
B-cen_4651 C-F	GCAT**GGTACC**GTTCTCCAGCCGGCTCAC	KpnI
B-cen_4651 C-R	CTAG**ACTAGT**ATTCGCAGCTTGCCATGA	SpeI

[a] Restriction enzyme sites are indicated in boldface.

by using Kaplan-Meier statistical method. The log-rank test was used to compare the survival differences for statistical significance ($P < 0.05$) using software GraphPad Prism (version 5; GraphPad Software, Inc., La Jolla, CA). Survival experiments were performed in triplicate and repeated at least twice.

Determination of the DNA sequence flanking the HimarGm transposons in the virulence-attenuated mutants. The HimarGm transposon contains an R6Kγ ori, which is functional in EC100 *pir-116* (Epicentre), allowing the transposon and flanking DNA to be cloned by marker rescue (17). Briefly, 5 to 10 μg of mutant genomic DNA was digested with SphI that cuts in DNA flanking the transposon, ligated, and concentrated before transformation into EC100 *pir-116*. Transformants containing the HimarGm were selected on LB+Gm plates. Plasmids purified from three clones of each mutant were digested with SacI and SphI to determine the presence of the HimarGm (835-bp fragment) and size of the retrieved plasmid, respectively. The DNA sequence of unique plasmids was determined by sequencing using the primers MarOUT and GMRFor (Table 2), performed at the Research Technology Support Facility at Michigan State University. The exact physical location of the HimarGm was determined by BLASTn analysis of flanking DNA to the AU1054 genome present on one of several genome databases (e.g., the Burkholderia Genome Database [www.burkholderia.com]) (2).

Complementation. We improved a previously described broad host range vector pBBR1MCS (42) for the complementation assays for better stability in *Burkholderia* by adding two toxin-antitoxin cassettes. The *flmAB-ccdAB* toxin-antitoxin cassette was amplified from pCS238 by using primers with XbaI sites (Table 2). The plasmid pBBR1MCS and the *flmAB-ccdAB* toxin-antitoxin cassette were digested with XbaI and ligated to create the pBBR1MCS *flmAB-ccdAB*, which was named pTCV2. The reporter cassette P_{lac}::GFP was PCR amplified from the plasmid pURR25 by using the primers GFPFor and GFPRev with SpeI restriction sites added to the termini (Table 2), digested with SpeI, and cloned into the SpeI site in the multiple cloning site (MCS) of pTCV2 to create pTCV3, which was used to check the stability of this plasmid vector in *Burkholderia* prior to complementation assays.

Vector pTCV2 was used for the complementation assays. The genes to be complemented were amplified from wild-type AU1054 by using Platinum *Taq* DNA polymerase high fidelity (Invitrogen) and cloned directionally downstream of the T3 promoter of pTCV2 after digestion with the appropriate restriction enzyme(s) (Table 2). All of the constructs made as described above were first transformed into *E. coli* strain DH5α and then transformed into the respective AU1054 mutant strains as described above.

***C. elegans* pathology.** To observe bacterial infection- and pathogenesis-related symptoms, the nematodes were inoculated onto GFP-labeled mutants, wild-type AU1054, *P. aeruginosa* PA14, and *E. coli* OP50 lawns as described above. After 24 to 48 h, the nematodes were washed off the lawns in 1.5 ml of Ringer's (100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES [pH 6.9]) and washed again prior to imaging. Nematodes were observed by fluorescent and differential interference contrast microscopy using a Leica DM5000 compound microscope (Leica) equipped with an X-cite 120 fluorescence illuminator (EXFO, Quebec, Canada), a Spot Pursuit charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI), and a GFP filter set (Leica).

Onion pathogenesis assays. Onion pathogenicity assays were performed as described previously (64). Briefly, quartered onion scales were wounded with a sterile pipette tip and then inoculated with 10^7 bacterial cells in 5 μl, followed by incubation at 37°C for 48 h. The degree of maceration was then determined by probing with a sterile toothpick, and the area of water-soaking symptoms was measured with an absolute digimatic caliper (Mitutoyo Corp., Aurora, IL). Area measurement treatment means were separated by using a two-tailed *t* test ($P < 0.05$). Uninoculated King's B (KB) media or *B. cepacia* ATCC 25416 served as negative and positive controls, respectively. Experiments were performed at least four times in triplicate.

Polygalacturonic acid degradation assays. The polygalacturonase enzyme activity of AU1054 wild type and transposon mutants was examined on Hildebrand's polygalacturonic acid gel medium at pH 4.5 to 4.7 (31). One microliter of an overnight-grown bacterial culture in KB medium was spotted in quadruplicate onto the gel surface of a Hildebrand medium plate, followed by incubation at 30°C for 48 h. The degradation of polygalacturonic acid was evaluated by measuring the diameter of the zone of degraded pectin substrate. Uninoculated KB and *Burkholderia ambifaria* AMMD served as negative controls. Three independent experiments with four replications were performed.

Fungal antibiosis assays. Antifungal activity was determined against *Rhizoctonia solani* as described previously (64). Briefly, 2 μl of culture grown overnight in KB was spotted in quadruplicate and in a grid pattern onto potato dextrose agar (Sigma-Aldrich), followed by incubation at 37°C for 48 h. A 7-mm plug of *R. solani* AG4 was added central to the bacterial spots, followed by incubation at 25°C for 96 h. Inhibition of fungal growth was then assessed visually using a qualitative rating scale. Experiments were performed at least twice in triplicate.

***In vitro* growth curves.** Bacterial growth rates were determined in LB medium, defined medium (DM) (0.25× M63 salts supplemented with 0.2% glucose, 0.4% glycerol, 1 mM $MgSO_4$, thiamine [1 μg/ml], and leucine, isoleucine, valine,

TABLE 3. *B. cenocepacia* virulence-attenuated mutants

Strain[a]	Disrupted gene	Mean nematode survival ± SE at day 3[b] (%)	LT_{50} (days)	Predicted function[c]
B. cenocepacia				
AU1054 (wt)	NA	0 ± 0	1	NA[d]
BPV134	Bcen_1509	10.5 ± 4.2	2	Unknown, predicted to be a secreted protein
BPV189	*trpB* (Bcen_4409)	26.8 ± 1.0	3	Tryptophan synthase subunit beta
BPV419*	*bnvR* (Bcen_3021)	30.7 ± 5.2	3	Unknown, predicted to contain a helix-turn-helix DNA-binding motif
BPV522	Bcen_2123	14.8 ± 4.6	2	Unknown, FAD-oxidase-like
BPV1017*	*nhaX* (Bcen_0002)	16.2 ± 1.6	2	Sodium/hydrogen exchanger, COG0475-Kef-type K^+ transport systems
BPV1367*	*argG* (Bcen_4651)	17.7 ± 2.9	3	Argininosuccinate synthase, COG0137
BPV1406	Bcen_3147	8.5 ± 5.4	2	Unknown, contains AAA ATPase domain
BPV1573	*paaA* (Bcen_2776)	10.8 ± 5.5	2	Phenylacetate-coenzyme A oxygenase
BPV1599	*leuS* (Bcen_0171)	14.7 ± 4.2	2	Leucyl tRNA synthetase
BPV1852*	*aroA* (Bcen_0566)	59.1 ± 13.5	4	5-Enolpyruvylshikimate-3-phosphate synthase COG0128
BPV2031	Bcen_1489	13.8 ± 4.7	2	LysR-family transcriptional regulator
BPV2225*	*gspJ* (Bcen_0015)	49.5 ± 11.3	3	Type II secretion system, protein J, COG4795 (*pulJ*)
BPV2246*	*purF* (Bcen_4402)	70.1 ± 5.6	5	Amidophosphoribosyltransferase, COG0034
BPV2391	Bcen_2245	27.2 ± 13.7	2	Unknown, contains UBA/THIF-type NAD/FAD binding fold
P. aeruginosa PA14	NA	42.8 ± 3.4	3	Positive control for nematode assays
E. coli OP50	NA	92.5 ± 1.7	NA	Negative control for nematode assays

[a] *, Mutant successfully complemented by expressing the respective wild-type gene in *trans* that caused a significant restoration of pathogenicity to nematodes.
[b] That is, the mean of three independent experiments. Each experiment was performed with three replicates; approximately 50 nematodes were used for each replicate.
[c] COG, cluster of orthologous groups.
[d] NA, not applicable.

tryptophan, glutamic acid, and glutamine [40 μg/ml each]) (70), NGM, and peptone glucose sorbitol (PGS; 1% Bacto peptone, 1% NaCl, 1% glucose, 0.15 M sorbitol) broths. Growth of 200-μl cultures normalized to an optical density at 600 nm (OD_{600}) of 0.01 was analyzed in triplicate at 37°C in a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA). The specific growth rate/h (μ) was calculated by using the formula: $\mu = 0.6931/\text{doubling time (in hours)}$.

Additional phenotypic characteristics. Congo red binding properties of the strains were determined on LB agar plates containing Congo red dye (0.01% [wt/vol]) (6), incubated at 37°C for 24 to 78 h. The antibiotic activity was determined from spot cultures grown for 24 h at 37°C, killed by chloroform exposure, and then overlaid with *Micrococcus luteus* culture in LB top agar (0.7% agar). The zone of inhibition of growth of *M. luteus* was measured after 24 h at 37°C. Biofilm production for AU1054 was determined by optimization of previously described methods (18, 55). Strains grown for 18 h at 37°C on tryptic soy agar were suspended in LB medium containing 0.6% yeast extract and 0.8% glucose (LBYG) to an OD_{520} of ~0.6 and serial 10-fold diluted in LBYG, and then 200 μl of the 10^{-3} and 10^{-4} dilutions were added to polystyrene microtiter plates (catalog no. 3595; Corning). After 24 h at 37°C, the growth was measured as OD_{540} by using a SpectraMax M5 microtiter plate reader (Molecular Devices). Biofilms were stained with crystal violet, 200 μl of 33% acetic acid was added to release the stain, and the biofilm was measured as the absorbance at 590 nm. Three biological and technical repeats were done for each sample. Biofilm production by each mutant was compared to wild-type AU1054 by using the Student *t* test. The production of siderophore activity was performed as described previously (63) except that a chrome azural S solution was added to the LB agar. Protease activity was assayed on skim milk agar (LB agar containing 1% skim milk) from spot cultures grown for 72 h at 37°C. Experiments were performed at least twice in triplicate.

RESULTS

Identification of genes required for AU1054 pathogenicity to nematodes. Fifteen virulence-attenuated mutants were isolated by screening 3,000 mutants in 10 independent experiments. Southern analysis revealed that all but one mutant contained a single HimarGm insertion, and no gross chromosomal rearrangements were detected by BOX- and REP-PCR analyses (data not shown). The mutant containing the double insertion was not analyzed further. Nematode survival was significantly increased from 0% on AU1054 to 8.4 to 70.1% on the mutants after 72 h. Nematode survival was 42.8% on PA14 (positive) and 92.5% on OP50 (negative) (Table 3).

Determination of the DNA sequence flanking the HimarGm insertions revealed genes predicted to encode the GspJ T2SS pseudopilin, an uncharacterized sodium/proton exchanger, a LysR-family transcriptional regulator, a *leuS* tRNA synthetase, five hypothetical proteins of unknown function (locus tags Bcen_1509, Bcen_3021, Bcen_2123, Bcen_3147, and Bcen_2245) and five metabolic enzymes (*trpB*, *argG*, *paaA*, *aroA*, and *purF*) (Table 3). The *paaA* gene (Bcen_2776) encodes the phenylacetate-coenzyme A oxygenase subunit required for the ring opening reaction of the phenylacetic acid catabolic pathway previously identified as necessary for *B. cenocepacia* K56-2 pathogenicity to nematodes (43). This gene was also found to be induced under CF conditions relative to soil (73). The LysR regulator is a paralog to the shiny variant regulator, ShvR (an AU1054 ortholog of ShvR is encoded by Bcen_5634) previously identified as being required for K56-2 virulence to alfalfa and rough colony morphology (6). The protein encoded by Bcen_1509 is predicted to be secreted, and related proteins were only detected in 18 of the 60 draft and completed *Burkholderia* genome sequences present on the Integrated Microbial Genome Database (51). The six mutants that were successfully complemented with their respective wild-type genes expressed in *trans* (see below) are the focus of the remainder of the present study.

BPV419 was found to contain a HimarGm insertion into Bcen_3021, which is predicted to encode a hypothetical protein of unknown function and is 6 bp upstream of Bcen_3022, which is also predicted to encode another protein of unknown func-

tion. A helix-turn-helix motif was detected in amino acids 191 to 211 of the predicted protein encoded by Bcen_3021 using HTH and GYM 2.0 algorithms (22, 53), suggesting that this protein may bind DNA and regulate gene expression. We named Bcen_3021, *bnvR*, for *B*urkholderia nematode virulence regulator. An orthologous predicted protein 100% identical is present in the *B. cenocepacia* HI2424 genome, which is an environmental strain of the same multilocus sequence type (ST-122) as AU1054 (44). Another ortholog (94.2% identical) was detected in the genome of *B. ambifaria* MC40-6 but was not detected in the genomes of *B. cenocepacia* strains MC0-3, J2315, and PC184 and 55 other *Burkholderia* genomes currently present on the Integrated Microbial Genome Database (51).

In contrast, the other five virulence-attenuated mutants that were successfully complemented contained HimarGm mutations in genes that are conserved in all 60 *Burkholderia* genomes currently available in the Integrated Microbial Genome Database. BPV2225 was found to have an insertion in Bcen_0015 predicted to encode general secretion protein J (*gspJ*), a component of the type 2 secretion system (T2SS). The T2SS is evolutionarily related to type IV pili, and GspJ is a minor pseudopilin whose molecular function for general secretion is not well understood; however, GspJ is known to be essential for T2SS function (3, 16, 61). The T2SS is used by pathogenic and environmental bacteria to secrete a variety of toxins and enzymes. The T2SS is necessary for secretion of protease, lipase, and phospholipase C exoenzymes in *B. pseudomallei* but not for virulence in a hamster model of melioidosis (21). Virulence factors such as two predicted polygalacturonases (Bcen_4887 and Bcen_5664) and two zinc metalloproteases (ZmpA [Bcen_1233] and ZmpB [Bcen_3302]), which are known to be important for pathogenicity to onions and in the rat agar bead model of chronic infection, respectively (27, 40), are likely secreted by T2SS (1).

BPV1017 contained an insertion in Bcen_0002 predicted to encode a protein containing a putative sodium-proton exchanger domain (Kef-type K^+ transport system, E-value 4e−04) (Table 3, Fig. 1). Sodium-proton exchangers are known to be involved in maintaining pH and ionic homeostasis (30, 56). This gene was named *nhaX* for Na/H antiporter.

The remaining three virulence-attenuated mutants identified in the present study contained mutations in conserved central metabolic genes. BPV1367 contained a HimarGm inserted into *argG* (Bcen_4651) predicted to encode a protein that functions as an argininosuccinate synthase (COG0137). For *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, mutations in genes required for arginine biosynthesis result in severe attenuation of virulence, suggesting the importance of arginine metabolism for *in vivo* growth and virulence (28, 39, 47).

BPV1852 contained an insertion in *aroA* (Bcen_0566), predicted to function as a 5-enolpyruvylshikimate-3-phosphate synthase, EPSPS (COG0128), required for the biosynthesis of the chorismate precursor (shikimate pathway) for aromatic amino acid synthesis. EPSPS is present in bacteria, plants, and fungi and is the target of the herbicide glyphosate (65). For several pathogens, mutations in the shikimate pathway result in severely attenuated virulence, and these mutants have been of great utility for the generation of live vaccines (7, 33, 58).

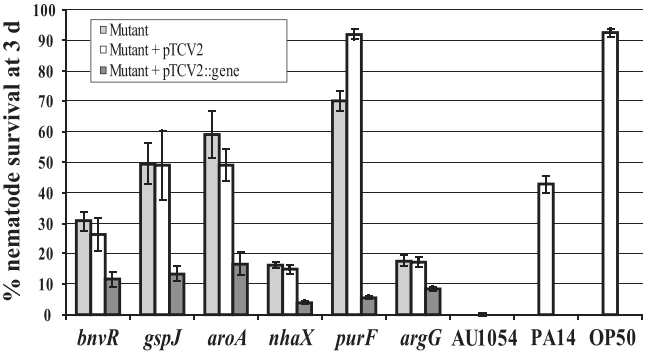

FIG. 1. Attenuated virulence of AU1054 mutants to *C. elegans* on NGM. Six mutants were significantly ($P < 0.05$) attenuated in virulence to *C. elegans* at 3 days. Light gray bars represent mutant strains, white bars are mutant strains containing an empty pTCV2 vector, and dark gray bars are mutant strains containing pTCV2 plus complementing gene. The disrupted genes are denoted in parentheses below each mutant. The *y* axis shows the percent nematode survival on lawns of the strains 3 days after the addition of early L4 nematodes, and the *x* axis indicates the bacterial strains. *B. cenocepacia* AU1054 (wild type), *P. aeruginosa* PA14 (positive control), and *E. coli* OP50 (negative control) are also shown. Error bars indicate standard error of the mean.

The physiological basis for attenuated virulence of *aroA* strains is not completely known and can be due to the inability to synthesize *p*-aminobenzoic acid and folic acid (67), aromatic amino acids (54), or menaquinone required for aerobic respiration (68).

BPV2246, the most severely attenuated mutant in pathogenicity to nematodes, was found to contain an insertion in *purF* (Bcen_4402), predicted to encode a protein that functions as a amidophosphoribosyltransferase (COG0034), the first step in the *de novo* synthesis of purines. Like mutations in the shikimate pathway, mutations in the *de novo* synthesis of purines result in severe attenuation of virulence for some pathogenic bacteria (37, 52, 59).

Complementation of AU1054 virulence-attenuated mutants. As mentioned above, six of the mutants were successfully complemented by expressing their respective wild-type genes in *trans* (Fig. 1A, Table 3). Nematode survival on lawns of the six complemented mutants was significantly less ($P < 0.05$) than for the mutant or the mutant containing the empty vector. Mutants with the greatest virulence defect—BPV2246 (*purF*), BPV2225 (*gspJ*), and BPV1852 (*aroA*)—which showed 70, 49.5, and 59% worm survival, respectively, at 3 days showed the greatest restoration of virulence by complementation with 5.6, 13.4, and 16.7% worm survival, respectively, at 3 days. Complementation of the other three mutants BPV419 (*bnvR*), BPV1017 (*nhaX*), and BPV1367 (*argG*), which showed 30.5, 16.3, and 17.6% worm survival, respectively, at 3 days showed significant ($P < 0.05$) restoration of the virulence and reduced survival of nematodes to 11.5, 4, and 8.4%, respectively, at 3 days.

Characterization of attenuated pathogenicity to *C. elegans*. To better understand the virulence characteristics of the strains, we compared the Kaplan-Meier nematode survival plots for each mutant to the wild-type AU1054. All of the mutants, except BPV1017 (*nhaX*), showed significantly ($P < 0.05$) greater nematode survival over 7 days than did AU1054

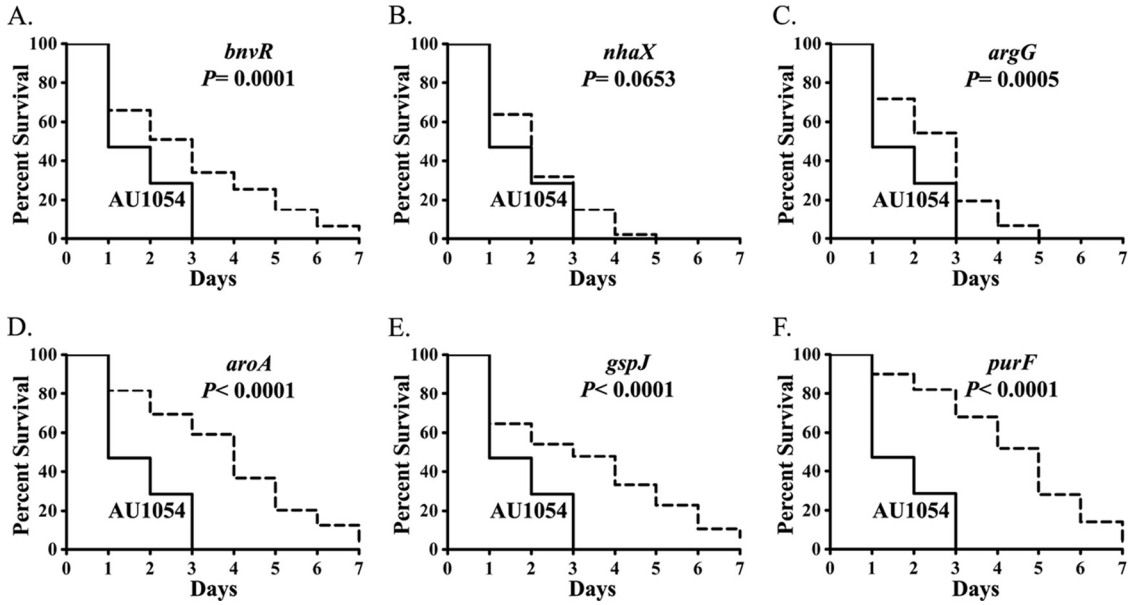

FIG. 2. The virulence of the mutants is attenuated compared to the wild-type *B. cenocepacia* AU1054 in the *C. elegans* infection model. Kaplan-Meier survival plots of *C. elegans* nematodes ($n = 450$) on BPV419 (*bnvR*) (A), BPV1017 (*nhaX*) (B), BPV 1367 (*argG*) (C), BPV1852 (*aroA*) (D), BPV2225 (*gspJ*) (E), and BPV2246 (*purF*) (F) compared to the wild-type AU1054 are shown. AU1054 is indicated by solid lines, and mutants are indicated by dashed lines. All of the experiments were performed three independent times, each time in triplicate with approximately 50 nematodes on each plate. A log-rank test was used to compare survival differences and calculate the probability (*P*).

(Fig. 2). As expected, the mutants displaying the greatest virulence defect at 3 days, e.g., BPV2246 (*purF*), BPV2225 (*gspJ*), and BPV1852 (*aroA*), also showed significantly higher median survival times (LT_{50}) of 5, 3, and 4 days, respectively, than did wild-type AU1054 (1 day). Both the mutants BPV1367 (*argG*) and BPV419 (*bnvR*) showed LT_{50} of 3 days. However, BPV1017 (*nhaX*) ($P = 0.0653$) was not significantly different from AU1054 in the Kaplan-Meier survival analysis but showed a higher LT_{50} of 2 days. All mutants exhibited pathogenicity to nematodes resembling a slow kill reaction by PA14 ($LT_{50} = 3$ days), where 0 to 18% of animals were surviving at 7 days.

In summary, five of six mutants with significant attenuation of nematode pathogenicity after 3 days were also significantly different from the wild type up to 7 days, where they exhibited a slow kill reaction with nematodes compared to the fast kill of the wild type.

Kaplan-Meier survival analysis revealed significant ($P < 0.05$) reduction in worm LT_{50} for five of six complemented mutants compared to the mutants or mutants with empty vector pTCV2. Complementing the mutants with the respective gene reduced the LT_{50} from 5 to 2 days for BPV2246 (*purF*); from 4 to 2 days for BPV1852 (*aroA*); and from 3 to 2 days for BPV2225 (*gspJ*), BPV1367 (*argG*), and BPV419 (*bnvR*) (data not shown). Complemented BPV1017 (*nhaX*) showed a nonsignificant ($P > 0.05$) reduction in LT_{50} from 2 days to 1 day.

Pathology of wild-type and mutant AU1054-infected *C. elegans*. To understand how AU1054 exhibits rapid pathogenicity to *C. elegans* nematode on NGM, we observed nematodes for infection and other pathologies by using GFP-labeled wild-type and mutant AU1054 strains, *E. coli* OP50, and *P. aeruginosa* PA14 (Fig. 3). AU1054-infected nematodes showed reduced growth, severe intestinal infection, distended intestinal lumen, and swollen rectum at 24 to 48 h (Fig. 3A and B) in all nematodes observed (27/27), whereas no or few bacterial cells were observed in the intestines of nematodes associated with OP50 (Fig. 3C and D). *P. aeruginosa* PA14 caused a less prevalent infection of posterior nematode intestine and no rectal swelling (Fig. 3E and F). The swollen rectum, also referred to as the deformed anal region (Dar), phenotype caused by AU1054 infection is unusual because it is known to be caused by *Microbacterium nematophilum* that infects the rectum and Dar is thought to be an innate immune response to rectal tissue colonization (32). Fluorescent AU1054 cells were eliminated from nematodes transferred to OP50, suggesting that AU1054 establishes a transient infection (data not shown). Nematodes observed from lawns of mutant strains BPV419 (*bnvR*) (19/20), BPV1017 (*nhaX*) (22/22), BPV1367 (*argG*) (18/21), BPV1852 (*aroA*) (18/18), and BPV2246 (*purF*) (17/17) showed severe infection and pathology similar to the wild-type AU1054 (Fig. 3G to J and M to R). However, most of the observed nematodes (19/24) from the BPV2225 (*gspJ*) mutant lawns showed weakly infected intestines (Fig. 3K to L) compared to the wild-type AU1054 but still showed a swollen rectum. In summary, AU1054 and all mutants except BPV2225 (*gspJ*) caused severe pathology to *C. elegans*, including rectal swelling.

Onion pathogenicity and antifungal activity. Since AU1054 is a multihost pathogen that exhibits high pathogenicity to onion and produces strong antifungal activity, these characteristics were assessed for the virulence-attenuated mutants isolated in the nematode model (Table 4). Three of the six mutants—BPV1367 (*argG*), BPV1852 (*aroA*), and BPV2246 (*purF*)—showed significantly decreased antifungal activity compared to AU1054. Three of the six mutants—BPV1852 (*aroA*), BPV2225 (*gspJ*), and BPV2246 (*purF*)—showed significantly reduced pathogenicity to onion, measured as a smaller

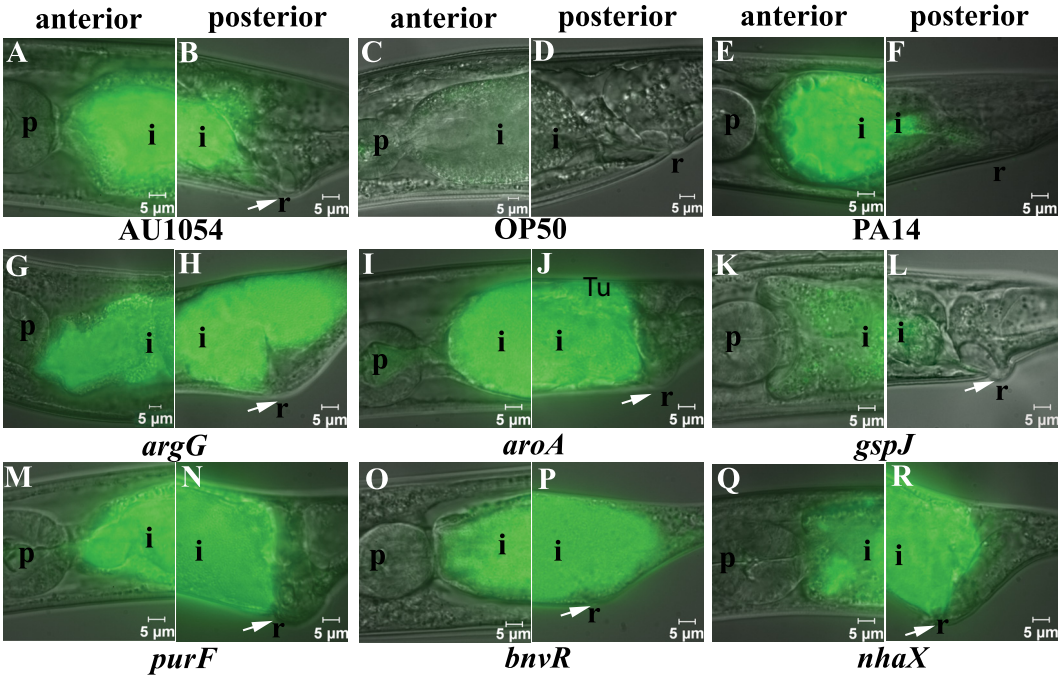

FIG. 3. Pathology of *C. elegans* infected by AU1054 mutants on NGM lawns at 24 to 48 h. Images are overlays of Nomarski and epifluorescence micrographs of the anterior and posterior *C. elegans* intestine at 24 to 48 h after exposure to GFP-labeled strains. The epifluorescent micrographs were taken using the same exposure (200 ms); hence, the absence of green color indicates the lack of GFP-labeled bacterial strains, and the presence of green color indicates infection of the anterior and posterior intestine. The positive control AU1054 (A and B), negative control OP50 (C and D), and *P. aeruginosa* PA14 (E and F) are shown. *E. coli* OP50 did not infect the nematodes. Mutants BPV1367 (*argG*) (G and H), BPV1852 (*aroA*) (I and J), BPV2246 (*purF*) (M and N), BPV419 (*bnvR*) (O and P), and BPV1017 (*nhaX*) (Q and R) infected the nematodes similar to wild-type AU1054, whereas strain BPV2225 (*gspJ*) (K and L) showed reduced infection of nematode intestines. Infected *C. elegans* nematodes showed stunted growth, distended intestinal lumen, and deformed anal region (indicated by arrows) compared to *E. coli*. The pharynx (p), intestine (i), and rectum (r) are indicated.

area of macerated tissue. The reduced pathogenicity to onion by BPV2225 (*gspJ*) is likely due to the inability to secrete endopolygalacturonase(s), which utilize the T2SS (43). The defect in secreted polygalacturonase activity was confirmed by a lack of polygalacturonic acid degradation by mutant BPV2225 (*gspJ*) on Hildebrand's medium (Table 4 and Fig. 4) and also for the two other mutants, BPV1852 (*aroA*) and BPV2246 (*purF*), attenuated in virulence to onion (Table 4). BPV1367 (*argG*) also showed a significant reduction in polygalacturonic acid degradation, although the area of onion maceration by this mutant was not different from AU1054. It is important to note that, as in the nematode assays for virulence, the inocula contained high numbers of cells so that defects in onion virulence or antifungal activity are not simply due to an absence of mutant cells on the assay media. In contrast, a mutation in *bnvR* and *nhaX* was normal in pathogenicity to onion and production of antifungal activity. In summary, four genes (*argG*, *aroA*, *gspJ*, and *purF*) were determined to be required for virulence in multiple hosts, whereas *bnvR* and *nhaX* functions only in virulence to nematode or animal hosts.

***In vitro* growth characteristics of the virulence-attenuated mutants.** Growth characteristics of the mutant and wild-type AU1054 strains was assessed in rich medium (LB medium), defined minimal media (DM), and the nematode virulence assay media NGM and PGS (Fig. 5). In LB medium, strains BPV2225 (*gspJ*) and BPV2246 (*purF*) had significantly lower growth rates and reached a lower final density than AU1054 (Fig. 5A, Table 4). BPV1852 (*aroA*) had a slight decrease in growth rate and reached slightly lower density in LB than wild type, while the other three strains were not significantly different than AU1054. In PGS, only BPV1852 (*aroA*) showed reduced growth (Fig. 5B). In DM, strains BPV1367 (*argG*), BPV1852 (*aroA*), and BPV2246 (*purF*) failed to grow and are likely auxotrophs. BPV2225 (*gspJ*) grew more slowly and reached a lower density in DM than AU1054. BPV419 (*bnvR*) and BPV1017 (*nhaX*) did not differ significantly in the growth rate and maximum density from that reached by AU1054 (Fig. 5C). In NGM, BPV1852 (*aroA*) failed to grow, whereas BPV2225 (*gspJ*) and BPV2246 (*purF*) showed a reduced growth rate and reached a lower final density than the rest of the strains AU1054, BPV1367 (*argG*), BPV1017 (*nhaX*), and BPV419 (*bnvR*) (Fig. 5D).

In summary, BPV1852 (*aroA*) showed a severe growth defect on all tested media except LB medium. BPV2246 (*purF*) showed a severe to moderate growth defect on all media except PGS. BPV2225 (*gspJ*) showed a moderate growth defect on all of the tested media except PGS, whereas BPV1367 (*argG*) was defective only on DM. The other strains, BPV419 (*bnvR*) and BPV1017 (*nhaX*), did not exhibit any significant growth defect and were similar to the wild-type AU1054.

Additional phenotypic characteristics of the virulence-attenuated mutants. Phenotypic variants of *B. cenocepacia* are known to occur with altered virulence characteristics and Congo red binding characteristics (15). Colonies of mutant

FIG. 4. Role for *gspJ* in secretion of exoenzymes. The mutant BPV2225 (*gspJ*) shows reduced onion maceration (A), polygalacturonic acid degradation (B), and protease activity on skimmed milk agar (C) compared to AU1054 (wild type) and restoration of these activities by expressing *gspJ* in *trans* (*gspJ* + p*gspJ*).

TABLE 4. Phenotypic characterization of AU1054 mutants attenuated in pathogenicity to *C. elegans*

Characteristic	Strain[a]							
	AU1054 (wild type)	BPV419 (*bnvR*)	BPV1017 (*nhaX*)	BPV1367 (*argG*)	BPV1852 (*aroA*)	BPV2225 (*gspJ*)	BPV2246 (*purF*)
Antifungal activity[b]	3.6 ± 0.1	3.3 ± 0.2	3.3 ± 0.1	**2.7 ± 0.1**	**2.4 ± 0.2**	3.4 ± 0.1	**3 ± 0.1**
Virulence against onion (area, mm^2)[c]	914.3	860.7	694.7	784.8	**645.7***	**440.7****	**406.0****
Polygalacturonic acid degradation[d]	26.3	27.0	26.6	**20.0***	**14.7****	**11.1****	**21.0***
Growth rate/h (μ) in rich medium (LB)	0.64	0.64	0.64	0.62	0.60	**0.40**	**0.15**
Growth rate/h (μ) in minimal medium (DM)	0.15	0.14	0.14	**ND**[e]	**ND**	**0.08**	**ND**
Growth rate/h (μ) in assay medium (NGM)	0.13	0.13	0.11	0.13	**ND**	**0.09**	**ND**
Growth rate/h (μ) in assay medium (PGS)	0.43	0.44	0.45	0.44	**0.14**	0.44	0.42
Biofilm formation[f] (biofilm/growth)	0.50 ± 0.09 (0.49 ± 0.09)	0.57 ± 0.07 (0.59 ± 0.08)	0.61 ± 0.05 (0.62 ± 0.06)	0.42 ± 0.06 (0.43 ± 0.09)	0.68 ± 0.06 (0.65 ± 0.06)	0.60 ± .07 (0.57 ± 0.06)	**0.23 ± 0.04 (0.31 ± 0.04)**
Siderophore production[g]	+++ (3 mm) ++ (2 mm)	+++ (3 mm) ++ (2 mm)	+++ (3 mm) ++ (2 mm)	+++ (3 mm) ++ (2 mm)	**++ (1.6 mm) + (1 mm)**	+++ (3 mm) ++ (2 mm)	**ND** **ND**
Antibiotic activity[h]							

[a] Phenotypic characteristics significantly different from those of AU1054 are indicated in boldface type.
[b] Based on a mean rating scale (0 to 4) ± the standard error of the mean.
[c] Presented as the area (in mm^2) of maceration determined with an absolute digimatic caliper; treatment means were separated by using a two-tailed *t* test. *, $P < 0.05$; **, $P < 0.01$.
[d] Mean diameter of degradation zone of 12 replicates from three experiments spotted onto Hildebrand's medium. Treatment means were separated by using a two-tailed *t* test. *, $P < 0.05$; **, $P < 0.01$.
[e] No growth detected.
[f] Absorbance at 590 nm of crystal violet stain (A_{590} crystal violet stain/A_{540} planktonic culture). Means and standard errors of mean from 1 representative experiment are indicated in parentheses. Only BPV2246 developed biofilms that were significantly different from AU1054 in all three experiments.
[g] Diameter (in mm) of clearing of chrome azural S stain.
[h] Diameter (in mm) of growth inhibition of *Micrococcus luteus*.

strains absorbed Congo red to the same extent as AU1054, indicating that they are not variants of this phenotype (data not shown). BPV2246 (*purF*) was the only mutant strain found to reproducibly form a reduced biofilm (Table 4). Four of six mutants produced siderophore and antibiotic activities at levels similar to those of AU1054. BPV2246 (*purF*) produced no detectable siderophore or antibiotic activities. BPV1852 (*aroA*) produced lower siderophore and antibiotic activities than AU1054. The defect of these two mutants was not due to lack of growth since both BPV2246 and BPV1852 formed colonies on the assay media (data not shown).

BPV2225 (*gspJ*) was found defective in onion maceration and degradation of polygalacturonic acid; therefore, we determined whether there is also a defect in protease activity and whether these activities are restored by expressing *gspJ* in *trans*. We found that BPV2225 (*gspJ*) is defective in casein hydrolysis in addition to onion maceration and polygalacturonic acid degradation and that these activities are restored by the expression of *gspJ* (Fig. 4). These results suggest that effectors secreted by the T2SS are required for virulence in both nematodes and onion but not for the production of antifungal or antibiotic activities. In addition, this suggests that the four T2SS genes, *gspKLMN*, downstream of *gspJ* are expressed in the BPV225 (*gspJ*) mutant, possibly from a secondary promoter (Fig. 6). Additional experiments are in progress to determine the optimal conditions for T2SS expression and proteomic analysis of the T2SS secretome (our preliminary results suggest that protease activity is enhanced in DM and NGM relative to LB).

In summary, BPV419 (*bnvR*) and BPV1017 (*nhaX*) displayed phenotypes identical to that of AU1054 except for pathogenicity to nematodes. BPV1852 (*aroA*) and BPV2246 (*purF*) were defective in pathogenicity to onion and growth in minimal medium and were reduced in antifungal, siderophore, and antibiotic activities, and BPV2246 also showed reduced

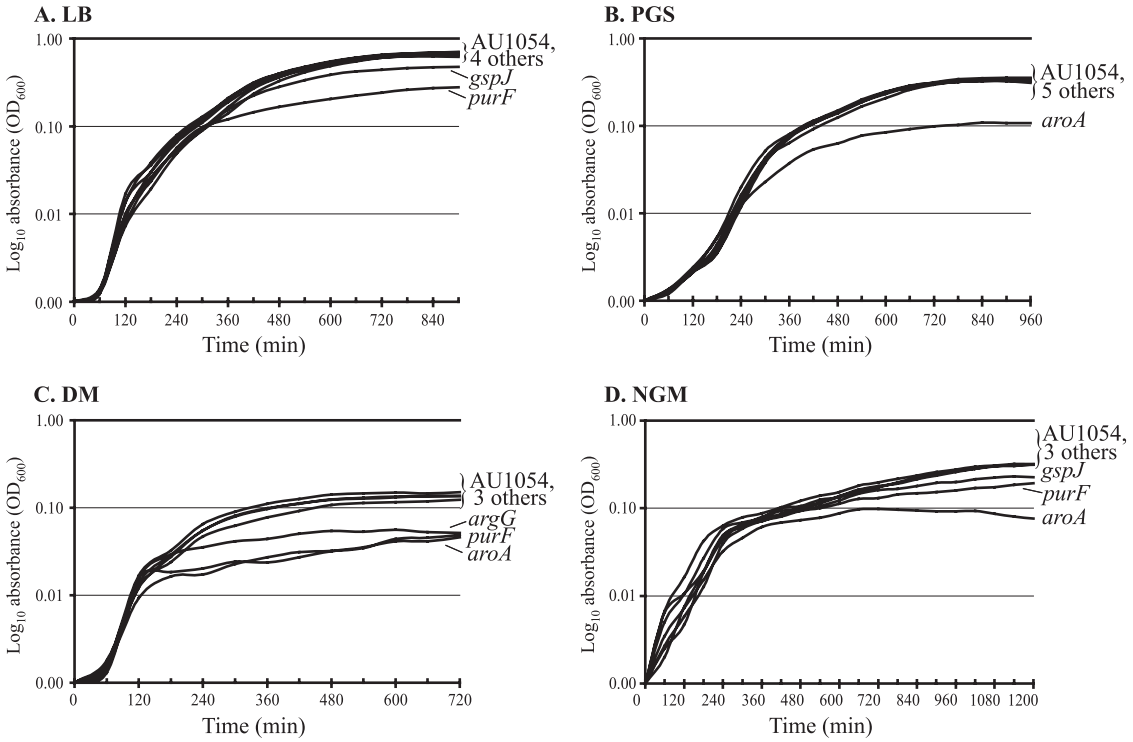

FIG. 5. Growth characteristics of the virulence attenuated AU1054 mutants. (A) In LB medium, *gspJ* and *purF* were defective. (B) In PGS, only *aroA* was defective. (C) In DM, *argG*, *purF*, and *aroA* were defective. (D) In NGM, *aroA*, *purF*, and *gspJ* were defective. The values represented are the mean of two independent experiments, each performed in triplicate until the growth curves reached a plateau.

ability to form biofilms. BPV2225 (*gspJ*) was reduced in pathogenicity to onion and growth in most media except PGS but similar to AU1054 in the production of antifungal, siderophore, and antibiotic activities and biofilm formation. BPV1367 (*argG*) was defective in pathogenicity to onion; the production of antifungal, siderophore, and antibiotic activities; and in growth in minimal medium. Thus, genes were identified that were required for the pathogenicity of AU1054 to multiple and specific hosts.

DISCUSSION

The objective of this study was to determine mechanisms that contribute to virulence and host specificity of the opportunistic pathogen, *B. cenocepacia* AU1054, a strain isolated from the blood of a person with CF that exhibits multihost pathogenicity (13, 64). Our study revealed that the T2SS pseudopilin gene, *gspJ*, and the biosynthetic genes *argG*, *aroA*, and *purF* are required for multihost pathogenicity, while the role for *bnvR* and *nhaX* in virulence is host specific. AU1054 is an ideal model organism because it is a clinical strain that shares a multilocus sequence typing genotype with known environmental strains isolated from onion fields in Michigan and New York (38, 44). Multihost pathogenicity of AU1054 similar to that of the environmental strains suggests limited evolution of pathogenicity of this strain during growth in the cystic fibrosis lung (64). In contrast, strains of the ET12 and Midwest epidemic clonal lineages exhibit variable degrees of pathogenicity to nematodes and contain independent mutations in virulence-related loci, including the T2SS (10, 34, 64; see also below). Clinical isolates lacking T2SS are common, suggesting that these are cheater strains that evolve during chronic lung infection and benefit from coinfection with T2SS-positive strains, while working less to secrete virulence factors.

Since AU1054 exhibits rapid pathogenicity to *C. elegans* under conditions where *P. aeruginosa* kills slowly (46), we utilized the nematode model to identify virulence mechanisms of AU1054. However, the mechanisms of fast killing by AU1054 are not fully understood. Quorum sensing and the quorum-sensing regulated protein AidA are known to be required for *B. cenocepacia* H111, an ET12 epidemic clonal strain, to infect and kill *C. elegans* on slow kill media (35, 41). Mutants defective in quorum sensing or Aid were not obtained in the present study, although we also expect them to be important for AU1054 virulence. The ring-opening enzymatic reactions of the phenylacetic acid catabolic pathway was also known to be required for *B. cenocepacia* K56-2 pathogenicity to nematodes (43), and our results confirm this finding in AU1054. However, we discovered additional genes required for virulence, which may reflect the adaptability of *Burkholderia* in host-bacterium interactions (48).

Evidence for the role of the T2SS for AU1054 pathogenicity was unexpected, because no role was found previously for the T2SS in *B. pseudomallii*, *B. vietnamiensis*, and *B. cenocepacia* H111 (21, 24, 41). T2SS was found to be required for *B. gladioli* pathogenicity to mushrooms (14). In addition, the genome sequence of the ET12 epidemic clone strain, J2315, revealed a 110-bp insertion in *gspL* required for T2SS function (34). Another ET12 strain, K56-2, has a functional T2SS required for

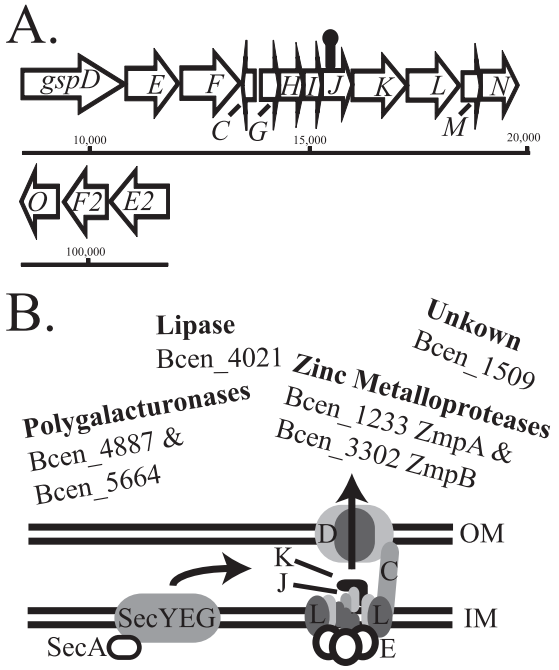

FIG. 6. Physical map and functional model of the AU1054 T2SS. (A) The majority of the genes encoding the T2SS are present in a single region of chromosome one. The HimarGm transposon (lollipop) located *gspJ* is not likely to be polar onto *gspKLMN* since these genes are also known to be essential for T2SS and expression of *gspJ* alone restores virulence and exoenzyme secretion to the BPV2225 (*gspJ*) mutant. The prepilin peptidase gene (*gspO*) and paralogs *gspE2* and *gspF2* are located in a second region of chromosome one. GspO is known to be essential for T2SS, however, the functions of the paralogs is unknown. (B) Model of T2SS in AU1054. T2SS proteins require the Sec pathway for secretion into the periplasm, where they are folded. By a poorly understood mechanism, proteins are recognized by GspK for secretion through the outer membrane pore GspD. GspJ interacts with two other pseudopilins, GspI and GspG, all of which are required for T2SS. Proteins possibly secreted by AU1054 T2SS are two predicted polygalacturonases (Bcen_4887 and Bcen5664), zinc metalloproteases (Bcen_1233 and Bcen_3302), nonhemolytic phospholipase C (Bcen_4021), and unknown proteins such as Bcen_1509 identified in the present study as having a role in AU1054 pathogenicity to nematodes. OM, outer membrane; IM, inner membrane.

the secretion of zinc metalloproteases, although other ET12 strains have different mutations in *gspL*, suggesting that loss of T2SS function is a trait derived in the CF lung (1, 34, 40). Clinical strains with defective T2SS may function as cheaters that benefit from secretion of virulence factors and exoenzymes of other bacteria inhabiting the CF lung. However, the T2SS was found to be induced by AU1054 in synthetic CF sputum relative to soil extract, indicating a possible role for virulence (74). The virulence defect of the *gspJ* mutant to onion can be explained by the inability to secrete two polygalacturonases, Bcen_4887 and Bcen_5664 (Fig. 6). It is not likely that the known nematode virulence factor, AidA, is T2SS dependent because it lacks a secretion signal, as determined by SignalP 3.0, and the T2SS is not required for H111 pathogenicity to nematodes (41). However, for the ET12 strain K56-2, the T2SS-dependent protease ZmpA is required for persistence in the rat lung, and both ZmpA and ZmpB are required for K56-2-induced inflammation in the same rat model (40). Furthermore, strain K56-2 with a functional T2SS was more virulent to nematodes and produces more protease activity than strain J2315 with a nonfunctional T2SS (10, 25). These observations suggest that the ancestral environmental *B. cenocepacia* had a functional T2SS during infection of the cystic fibrosis lung and that cheater strains with nonfunctional T2SS frequently occur during chronic lung infections. In summary, these results suggest that *B. cenocepacia* T2SS is important for virulence and might function by secreting known or novel virulence factors such as the protein encoded by Bcen_1509 identified in the present study (Fig. 6). We are currently conducting experiments to determine the T2SS effectors required for AU1054 pathogenicity.

In addition to *gspJ*, the genetic analysis of rapid killing of *C. elegans* caused by AU1054 revealed roles for two genes, *bnvR* and *nhaX*, in virulence that were previously unknown. *bnvR* is interesting because the predicted protein, identified as a hypothetical with unknown function, contains a helix-turn-helix motif, suggesting that it might bind DNA and regulate gene expression. Furthermore, only a few *Burkholderia* strains contain *bnvR* homologs, suggesting that this gene was acquired by horizontal transfer. The presence of a transcription factor is known to significantly affect host-bacterium interactions of a variety of bacteria (57). For example, a single regulatory protein is sufficient to alter the host range of vibrio symbionts (50), and a *B. cenocepacia* LysR regulatory protein affects virulence and phenotypic variation (6).

The discovery that *nhaX* is required for pathogenicity to nematodes is interesting because it is only distantly related to other studied sodium (cation)/hydrogen (proton) exchangers. NhaA (COG3064) and NhaB (COG0475) domain-containing proteins were not detected in AU1054. However, five predicted proteins contained NhaP (COG0025) and two contained NhaD (COG1055) domains. Sodium/hydrogen exchangers are known to regulate pH and ionic homeostasis and maintenance of the cell shape and to control phagosomal acidification (56, 66). The resting pH of the *C. elegans* intestine is pH 4, although this oscillates with the defecation cycle, where transcellular transport of proton ions by intestinal sodium/proton exchangers triggers muscle contraction (5). Thus, the *nhaX* predicted sodium/hydrogen exchanger may function by maintaining pH or osmotic homeostasis in the nematode intestine.

The AU1054 metabolic *argF*, *aroA*, and *purF* mutants showed growth defects on various media, and it is tempting to conclude that the attenuated virulence of these mutants could be attributed to lack of growth. However, evidence indicates that the attenuation in virulence is not due to lack of growth because (i) nematodes were exposed to visibly confluent bacterial lawns and therefore sufficient infective units; (ii) no visible difference in fluorescent bacteria infecting the nematode intestine was evident except for *gspJ*; (iii) *aroA* and *purF* mutants were defective in producing siderophore, antibiotics, and antifungal activities even though the mutants formed colonies on the assay media; and (iv) *argG* is defective in antifungal activity while still producing siderophore and antibiotic activities, suggesting that this gene is involved in the synthesis of an antifungal metabolite. All three auxotrophs show reduced pathogenicity to onion, suggesting that these metabolic functions are required in multiple hosts. Because *aroA* is only

found in bacteria, plants, and fungi and is the target for the herbicide glyphosate, glyphosate or related compounds may be effective in inhibiting the growth of AU1054 in human, animal, or plant hosts.

In summary, *C. elegans* was used successfully to identify genes involved in multihost pathogenicity of the human opportunistic pathogen *B. cenocepacia* AU1054. A putative novel transcription factor, sodium/hydrogen exchanger, *gspJ* (T2SS), and three metabolic genes were determined to be required for rapid pathogenicity to *C. elegans*, and five of these were also required for multihost pathogenicity. However, some virulence factors, such as those encoded by *bnvR* identified in the present study and those encoding a nematode toxin, *aidA*, the outer membrane protein *opcI*, and a component of T3SS, *hldA*, appear to be host specific (71). We further illustrate here how much remains to be learned about the complex problem of how select environmental microbes function as opportunistic pathogens.

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