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The *Burkholderia cenocepacia* LysR-Type Transcriptional Regulator ShvR Influences Expression of Quorum-Sensing, Protease, Type II Secretion, and *afc* Genes[▽]

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***Burkholderia cenocepacia* is a significant opportunistic pathogen in individuals with cystic fibrosis. ShvR, a LysR-type transcriptional regulator, has previously been shown to influence colony morphology, biofilm formation, virulence in plant and animal infection models, and some quorum-sensing-dependent phenotypes. In the present study, it was shown that ShvR negatively regulates its own expression, as is typical for LysR-type regulators. The production of quorum-sensing signal molecules was detected earlier in growth in the *shvR* mutant than in the wild type, and ShvR repressed expression of the quorum-sensing regulatory genes *cepIR* and *cciIR*. Microarray analysis and transcriptional fusions revealed that ShvR regulated over 1,000 genes, including the zinc metalloproteases *zmpA* and *zmpB*. The *shvR* mutant displayed increased gene expression of the type II secretion system and significantly increased protease and lipase activities. Both ShvR and CepR influence expression of a 24-kb genomic region adjacent to *shvR* that includes the *afcA* and *afcC* operons, required for the production of an antifungal agent; however, the reduction in expression was substantially greater in the *shvR* mutant than in the *cepR* mutant. Only the *shvR* mutation resulted in reduced antifungal activity against *Rhizoctonia solani*. ShvR, but not CepR, was shown to directly regulate expression of the *afcA* and *afcC* promoters. In summary, ShvR was determined to have a significant influence on the expression of quorum-sensing, protease, lipase, type II secretion, and *afc* genes.**

Members of the *Burkholderia cepacia* complex (Bcc) are important in medical, agricultural, and biotechnological research fields (31, 35). Seventeen Bcc species have been identified, including *Burkholderia cenocepacia*, which is an opportunistic pathogen in individuals with the inherited disease cystic fibrosis (CF). *B. cenocepacia* is intrinsically resistant to antibiotics and can persist in the lungs of CF patients (35, 44). In some patients, infection with *B. cenocepacia* can progress from chronic lung infection to what is termed "cepacia syndrome." Cepacia syndrome is associated with a rapid deterioration in lung function associated with necrotizing pneumonia, bacteremia, and sepsis that can result in death (24).

Our laboratory previously identified spontaneous shiny colony variants (shv) that were easily distinguished from the typical rough colony morphotypes of *B. cenocepacia* (4). These shv typically exhibited the absence of an extracellular matrix, reduced biofilm formation, and reduced virulence in alfalfa and rat infection models and displayed differences in *N*-acyl homoserine lactone (AHL) and protease activities, motility, and siderophore production. Expression of BCAS0225, encoding the LysR-type transcriptional regulator (LTTR) ShvR, influenced a number of these phenotypes in shv (4).

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LTTRs are part of a large protein family and display a well-conserved structure with an N-terminal DNA-binding helix-turn-helix motif and a C-terminal coinducer-binding domain (for a review, see reference 33). LTTRs typically engage in negative autoregulation and frequently positively regulate divergently transcribed genes. However, it is now widely accepted that LTTRs can act as global regulators in a positive or negative manner (33).

The locus carrying *shvR* lies adjacent to the predicted *afcA* and *afcC* operons that are divergently transcribed (60). Mutations affecting *afcA*, *afcC*, and *afcD* in *Burkholderia pyrrocinia* BC11 (formerly *B. cepacia* BC11 [56]) were previously shown to impair the production of an antifungal compound and inhibit antifungal activity against the soilborne phytopathogen *Rhizoctonia solani* (25). We previously showed that the 27-kb genomic region encompassing *shvR* and the *afc* operons was influenced by quorum sensing (QS). Expression of *shvR* and many genes in the *afcA* and *afcC* operons was positively regulated by CepR and negatively regulated by CciR (41). QS is a cell-cell communication system involving the production and perception of chemical signals used by diverse bacterial species. In many Gram-negative bacteria, members of the LuxI protein family synthesize AHLs that bind and activate cognate LuxR protein family transcriptional regulators (for reviews, see references 40, 49, and 57). AHLs accumulate during bacterial growth until a threshold is reached at high cell densities, allowing coordinated regulation of gene expression.

B. cenocepacia has two complete AHL-dependent QS sys-

tems as well as an orphan LuxR homolog. CepI is primarily responsible for the synthesis of *N*-octanoyl-L-homoserine lactone (OHL) (29) and minor amounts of *N*-hexanoyl-L-homoserine lactone (HHL) (30). CciI primarily synthesizes HHL, with lesser amounts of OHL produced (36). All members of the Bcc have the CepIR system (19, 32), whereas CciIR is found only in transmissible *B. cenocepacia* strains containing the cenocepacia island and is absent from genomes of other *B. cenocepacia* strains, including representatives of the transmissible PHDC lineage (AU1054, HI2424) (2, 21, 60). CepR2 is an orphan LuxR homolog found in all *B. cenocepacia* strains and can regulate gene expression in the absence of AHLs (37). The *B. cenocepacia* CepIR system positively influences virulence in murine, alfalfa, *Caenorhabditis elegans*, *Galleria mellonella*, and *Danio rerio* infection models (22, 26, 27, 51, 55, 58). A *B. cenocepacia cciI* mutant exhibited reduced virulence in a rat chronic respiratory infection model but did not affect virulence in *Caenorhabditis elegans*, *Galleria mellonella*, or alfalfa infection models (2, 55). CepR, CciR, and CepR2 regulate between 3 and 12% of the genome and thus are considered global regulators (37, 41). CepIR and CciIR regulate protease and lipase activities and type II, III, and VI secretion systems, as well as biofilm formation and maturation (8, 23, 26, 29, 36, 41, 51, 53, 54).

We previously demonstrated an association between shv and the *shvR* mutation with some QS-dependent phenotypes (4). To further investigate this relationship, we studied the effects of ShvR on the AHL-dependent QS regulatory network and examined the coregulation of gene expression by CepR, CciR, and ShvR. We investigated the influence of ShvR on several QS-controlled phenotypes.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were routinely grown at 37°C in Miller's Luria broth (LB) (Invitrogen, Burlington, Ontario, Canada) with shaking or on 1.5% Lennox LB agar plates. For promoter::*lux* assays, strains were grown in LB, Trypticase soy broth (TSB) (Difco, Franklin Lakes, NJ), 0.25% Trypticase soy broth with 5% Bacto peptone (Difco) (PTSB), dialyzed brain heart infusion (D-BHI), and/or 1.5% skim milk (Difco). For some assays, OHL was added in concentrations ranging from 30 to 3,000 pM. For microarray and quantitative reverse transcription-PCR (qRT-PCR) experiments, cultures were grown to stationary phase (16 h) in 10 ml LB in 125-ml Erlenmeyer flasks. For promoter::*lux* assays, samples were cultured in black, clear-bottom, 96-well plates (Corning, Inc., Corning, NY). Cultures entered stationary phase at 18 h (growing at 37°C) or 30 h (growing at 29°C). AHL activity was monitored using *Agrobacterium tumefaciens* A136(pCF218)(pMV26) in a real-time liquid coculture assay with *B. cenocepacia* grown in 96-well plates in TSB at 29°C as previously described (3). Protease activity was determined using D-BHI with 1.5% skim milk agar plates as previously described (50) except that cell pellets were washed twice with PTSB and normalized to an optical density (OD) of 0.3 prior to inoculation. Lipase activity was determined as previously described (23) except that cell pellets were washed twice with LB and normalized to an OD of 0.3 prior to inoculation on 1% polysorbate (Tween) 80 or 1% tributyrin in LB agar plates. Colony morphology was assessed by examining the appearance of rough or shiny morphology of strains on LB agar following incubation at 28°C or 37°C for 72 h and capturing images using a Samsung digital camera. Biofilm formation was assessed as previously described (5) on polystyrene pegs in a 96-well-plate format (Nunc, Roskilde, Denmark) by using cultures grown in TSB for 24 h at 37°C on a rocking platform. Biomass was strained with crystal violet, destained with ethanol, and quantified by measuring OD at 600 nm. When appropriate, the following concentrations of antibiotics were used: 100 μg/ml of trimethoprim (Tp) and 200 μg/ml of tetracycline (Tc) for *B. cenocepacia*, 100 μg/ml of Tp and 50 μg/ml of kanamycin (Km) for *Escherichia coli*, and 25 μg/ml of Km and 4.5 μg/ml of Tc for *Agrobacterium tumefaciens.* Antibiotics were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Antifungal activity. Antagonistic activities against *Rhizoctonia solani* (obtained from the culture collection of the phytopathology group of the Institute of Plant Sciences, Federal Institute of Technology, Zurich, Switzerland) were assayed on malt extract agar (15 g/liter; supplemented with 12 g/liter agar; Becton Dickinson, Sparks, MD) by spotting 10-μl stationary-phase cultures of selected strains at three positions on the plate. Following incubation overnight at 37°C, a 5-mm-diameter fungal inoculum, which was cut from a 1-week-old fungal culture plate, was placed onto the center of the plate. Plates were incubated at 22°C in the dark, and inhibition zones were recorded after 3 days. The experiment was performed in triplicate.

DNA manipulations. DNA manipulations were performed using standard techniques as described previously (45), and genomic DNA was isolated as described previously (1). Oligonucleotide primers (Table 2) were designed with Primer3 (42) and were synthesized by the University of Calgary Core DNA and Protein Services (Calgary, Alberta, Canada). Plasmids were introduced into *B. cenocepacia* by electroporation (12).

Construction of the K56-2*shvR*::Tp and K56-2Δ*shvR* mutants. To construct K56-2*shvR*::Tp, pUCP26-BCAS0225 (4) and pEX18Tc (20) were digested with PstI and EcoRI, respectively. The resulting insert or vector fragments were blunt ended using T4 DNA polymerase (Invitrogen), digested with BamHI, and ligated to create pEX18Tc-BCAS0225. BCAS0225 was inactivated using a Tp cassette from pGSVTp*lux* (4) by using SacI to generate pEX18Tc-BCAS0225::Tp. The K56-2*shvR*::Tp mutant was generated by gene replacement by using the previously described strategy (47). A K56-2Δ*shvR* deletion mutant was constructed by following a described method (16) by using the primers listed in Table 2. PCR confirmed gene replacement or deletion in these mutants.

Transcriptional fusions to *luxCDABE* (*lux*). Promoter regions for *shvR*, *afcC*, *gspC*, and *gspG* were amplified using the described primers (Table 2) and cloned into the XhoI-BamHI site upstream of *lux* in pMS402 (13) or pCS26-*Pac* (6). For the expression reporter system, *cepR* or *shvR* expression constructs in vector pUCP28T were electroporated into pCS26-*Pac* derivatives, and construct presence was confirmed by PCR by using the appropriate primers (Table 2). Luminescence assays were carried out as previously described (8, 41). Cultures for luminescence assays on skim milk agar were prepared as described above, and promoter::*lux* activity was visualized using a Molecular Imager ChemiDoc system (Bio-Rad, Mississauga, Ontario, Canada).

RNA manipulations. RNA was prepared as previously described (41) using a RiboPure bacterial RNA isolation kit (Ambion, Streetsville, Ontario, Canada). DNase treatment was performed, and samples were confirmed by PCR using *Taq* polymerase (Invitrogen) to be free of DNA prior to cDNA synthesis.

Microarray sample preparation. Microarray samples were prepared as previously described (41). Briefly, three independent RNA samples from *B. cenocepacia* strains grown to stationary phase in LB were used in microarray experiments. Gene expression profiles were generated using custom *B. cenocepacia* J2315 microarrays (Agilent, Santa Clara, CA) (21, 28). K56-2 and K56-2*shvR*::Tp cDNA samples were fluorescently labeled with Cy3 and Cy5, respectively. Labeling, hybridization, and scanning were performed by the Mahenthiralingam Laboratory, Cardiff University, Wales.

Microarray data analysis. Microarray data analysis was performed using GeneSpring GX 7.3.1 software (Agilent). Initial data were preprocessed by employing the enhanced Agilent FE import method, and then per-spot and per-chip normalizations were performed using the Affymetrix FE data normalization recommended for Agilent arrays, eliminating the per-gene normalization step. After filtering to include genes present in one of three samples, followed by filtering on confidence ($P < 0.05$, t test with Benjamini-Hochberg and false discovery rate postcorrection), the remaining genes were filtered using a 1.5-fold ratio change to identify genes showing increased or decreased expression in K56-2*shvR*::Tp compared to that in K56-2. Operon prediction was derived from analysis of *B. cenocepacia* genomes at http://www.burkholderia.com (60).

qRT-PCR. Quantitative RT-PCR was performed as previously described on samples from *B. cenocepacia* strains grown to stationary phase in LB (41) except the NADH dehydrogenase gene *ndh* (BCAM0166) was used as a reference standard as described previously (53). Expression of *ndh* was not significantly altered according to microarray analysis (data not shown).

Statistical analyses. Analysis of variance (ANOVA) was performed with GraphPad Prism software (GraphPad Software, San Diego, CA).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
A. tumefaciens		
A136	Ti plasmidless host	C. Fuqua
E. coli		
TOP10	F$^-$ *mcrA*Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (St^r) *endA1 nupG*	Invitrogen
DH10B	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Invitrogen
SY327	*araD* Δ(*lac-pro*) *argE*(Am) *recA56* Rif^r*nalA* λ*pir*	39
B. cenocepacia		
K56-2	CF isolate	34
K56-R2	*cepR*::Tn*5*-OT182 derivative of K56-2, Tc^r	29
K56-2Δ*cepR*	*cepR* derivative of K56-2	41
K56-2*shvR*::Tp	*shvR*::Tp derivative of K56-2, Tp^r	This study
K56-2Δ*shvR*	*shvR* derivative of K56-2	This study
K56-2*cepIcciIb*	Δ*cepI cciI*::Tp derivative of K56-2, Tp^r	36
Plasmids		
pCR2.1Topo	Cloning vector for PCR products, Ap^r Km^r	Invitrogen
pCF218	IncP plasmid expressing TraR, Tc^r	63
pMV26	*traI-luxCDABE* fusion, Km^r	51
pRK2013	ColEl Tra (RK2)$^+$, Km^r	15
pGSVTp*lux*	Mobilizable suicide vector containing *lux* operon, derivative from pGSV3-*lux*, OriT, Tp^r	4
pEX18Tc	Gene replacement vector *oriT*$^+$, *sacB*$^+$, Tc^r	20
pUCP26-BCAS0225	pUCP26 with 1.7-kb PstI-BamHI fragment containing BCAS0225 and upstream region, Tc^r	4
pEX18Tc-BCAS0225::Tp	pEX18Tc containing BCA0225 inactivated with a Tp cassette from pGSVTp*lux*	This study
pGP*I-Sce*I	*ori*$_{R6K,}$ Tp^r, *mob*$^+$, carries *I-Sce*I endonuclease recognition site, Tp^r	16
pDA*I-Sce*I	pDA17 carrying the *I-Sce*I gene, Tc^r	16
pUCP28T	Broad-host-range vector, Tp^r	48
p28T-*shvR*	pUCP28T with 1.7-kb PstI-BamHI fragment containing BCAS0225 and upstream region, Tc^r	This study
pSLR100	pUCP28T with 1.65-kb KpnI-SphI fragment from pSLA3.2 containing *cepR* gene, Tp^r	29
pMS402	*lux*-based promoter reporter plasmid, Km^r Tp^r	13
pCP300	*cepI*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	36
pRM432	*cepR*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	36
pRM445	*cciIR*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	36
pP*afcA*	*afcA*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	41
pP*afcC*	*afcC*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	This study
pBS13	*zmpA*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r Tc^r	37
pBS9	*zmpB*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r Tc^r	26
pP*gspC*	*gspC*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	This study
pP*gspG*	*gspG*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	This study
pP*shvR*	*shvR*::*lux* transcriptional fusion constructed in pMS402, Km^r Tp^r	This study
pCS26-*Pac*	*lux*-based promoter reporter plasmid, Km^r	6
pEPO100	pCS26-Pac with 269-bp XhoI-BamHI fragment from pCP300 containing *cepI* promoter, Km^r	This study
pEPO101	pCS26-Pac with 371-bp XhoI-BamHI fragment from pP*afcA* containing *afcA* promoter, Km^r	This study
pEPO128	pCS26-Pac with 405-bp XhoI-BamHI fragment from pP*afcC* containing *afcC* promoter, Km^r	This study

Microarray data accession number. The entire microarray data set has been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2939.

RESULTS

ShvR expression is temperature regulated and negatively autoregulated. Expression of *shvR* was monitored using promoter::*lux* fusions in wild-type cells (Fig. 1A). In LB or TSB medium incubated at 37°C, *shvR*::*lux* expression levels were highest between 8 and 18 h and dropped after this point in LB but remained relatively steady in TSB. During growth in LB or TSB medium at 29°C, the highest *shvR*::*lux* expression level occurred after prolonged incubation (Fig. 1A). Negative autoregulation is a common feature of LysR-type regulators (33), which prompted us to examine *shvR* expression in an *shvR* mutant versus that in the wild type. Expression of the *shvR*::*lux* fusion was significantly higher in the *shvR* mutant than in K56-2 in both LB and TSB (Fig. 1B and C).

ShvR temporally affects AHL activity and negatively regulates expression of *cepIR* and *cciIR* QS genes. We previously observed that *shvR* mutants had slightly increased AHL activity compared to that of the wild type (3). AHL activity profiles throughout growth were monitored in a real-time liquid coculture assay with K56-2Δ*shvR* and K56-2 by using the biosensor *Agrobacterium tumefaciens* A136(pCF218)(pMV26). AHL activity peaked prior to stationary phase in K56-2Δ*shvR*(pUCP28T) and during stationary phase in K56-2(pUCP28T), although total

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)	Reference
5′-XbaI-UP-BCAS0225	TTTTTCTAGATGCGCATCGAATGCACACCG	This study
3′-KpnI-UP-BCAS0225	TTTTGGTACCCTTCTCAATCGCTTTGCC	This study
5′-KpnI-DWN-BCAS0225	TTTTGGTACCACGTGCTGCCGATGTATCGC	This study
3′-SmaI-DWN-BCAS0225	TTTTCCCGGGATCTTCCAGATTCACGTCCG	This study
XhoI-5′-PBCAS0225	AGCTCTCGAGCGGATTCATCTTGACGGTCG	This study
PBCAS0225-3′-BamHI	AGCTGGATCCGGCAGACTTCTCAATCGCTT	This study
P*gspC*&Gfor1	GGCTCGAGTGTCGAGACGATACAGCTTGAG	This study
P*gspC*&Grev1	GGGGATCCCATATTTGCCATCCATTTGC	This study
P*afcC*for1	GGCTCGAGCGATTCGATCAGACGGTGAATG	This study
P*afcC*rev1	GGGGATCCGACAAGCGATGAGGTGACG	This study
F*ndh*	GCGATCGGGCTGTACAAGTT	53
R*ndh*	AGTGGCTCAGCGACTGGAA	53
BCAS0220qRTfor2	GAACCAGTTCTCGGTGTTCG	This study
BCAS0220qRTrev2	GATCCAGTTGCTCATCGACA	This study
BCAS0215qRTfor1	ATCCTGTCGATGCTGCTGAT	This study
BCAS0215qRTrev1	TACGAGCAGGTCATCCAGTC	This study
BCAS0208qRTfor1	ATCTGCAAGGCGTTCATCTC	This study
BCAS0208qRTrev1	GTAGTTCGTGCCTTCCCAGA	This study
BCAS0204qRTfor1	GCAATTGCAGAAGGTCGAGT	This study
BCAS0204qRTrev1	CGACGATCTCGGATACACG	This study
pUCP28TflankMCSfor1	CGACGTTGTAAAACGACGG	This study
pUCP28TflankMCSrev1	GGAAACAGCTATGACCATGA	This study

AHL activity was similar between the strains (Fig. 2A). There was no significant difference between K56-2(pUCP28T) and K56-2Δ*shvR*(p28T-*shvR*), indicating restoration of parental AHL activity patterns by expression of *shvR* in *trans* (Fig. 2A). These data indicate that ShvR affects AHL activity in terms of timing rather than quantity. This trend was confirmed in experiments performed in the absence of vector pUCP28T, where AHL activity peaked prior to stationary phase in two independently constructed *shvR* mutants compared to that of K56-2 (data not shown).

Promoter::*lux* fusions to *cepI*, *cepR*, and *cciIR* were used to assess the effect of ShvR on the expression of QS genes. In the *shvR* mutant compared to K56-2, *cepR* expression was significantly higher from mid-log phase, and *cepI* and *cciR* expression was significantly higher in stationary phase (Fig. 2B to D). Furthermore, using qRT-PCR, we confirmed that expression of *cepI*, *cepR*, and *cciIR* was increased 3.6-, 5.6-, and 12.5-fold, respectively, in the *shvR* mutant compared to that in K56-2 in stationary phase (Table 2).

ShvR coregulates QS-controlled genes but independently regulates biofilm formation and rough colony morphology. Because ShvR influences timing of AHL activity and expression of *cepIR* and *cciIR*, we compared microarray expression profiles generated from stationary-phase cultures of the *cepR*, *cciR* (41), and *shvR* mutants relative to K56-2. Microarray analysis showed that 1,077 genes were expressed differently in the *shvR* mutant than in K56-2 (Table 3). Among the coregulated genes, patterns of common and reciprocal regulation were observed. CepR and ShvR commonly regulated 263 genes; 159 genes were positively regulated and 100 genes negatively regulated by CepR and ShvR. Only four genes were reciprocally regulated by CepR and ShvR, whereas most of the 156 genes coregulated by CciR and ShvR were reciprocally regulated (Table 3). Of these reciprocally regulated genes, 135 genes were positively regulated by ShvR and negatively regulated by CciR, and 14 genes were negatively regulated by ShvR and positively regulated by CciR. Seven genes were commonly regulated by ShvR and CciR, including *cciI*, which was negatively regulated (Table 3). Negative regulation of *cciI* by ShvR was observed in *cciIR*::*lux* fusions, microarray data, and qRT-PCR (Fig. 2D and Table 4). The expression of 369 and 358 genes was decreased and increased, respectively, in the *shvR* mutant compared to that of K56-2 but unaffected by mutation of *cepR* or *cciR* (Table 3). These represent genes independently regulated by ShvR and include genes encoding 18 LTTRs, exopolysaccharide biosynthesis proteins, ABC transporter ATP-binding proteins, 45 membrane proteins, 20 exported proteins, and 15 lipoproteins.

Both *cepR* and *shvR* mutants have reduced biofilm formation (4, 54) compared to that of the wild type. Since many genes were coregulated by both CepR and ShvR, we sought to determine whether cross-complementation with the other regulator on a high-copy plasmid would allow restoration of biofilm formation or colony morphology changes in *cepR* or *shvR* mutants. Reduced biofilm formation of the *cepR* mutant was restored by expression of *cepR*, but not *shvR*, in *trans*. Similarly, reduced biofilm formation of the *shvR* mutant was restored by expression of *shvR*, but not *cepR*, in *trans* (Fig. 3A). These data suggest that CepR and ShvR independently regulate genes contributing to biofilm formation, as neither regulator can compensate the other for the biofilm defect. Colony morphology was assessed in the *cepR* and *shvR* mutants following growth on agar for 72 h at 28°C (data not shown) and 37°C. The *shvR* mutant exhibited shiny colony morphology, while the *cepR* mutant did not differ from K56-2 and appeared rough (Fig. 3B). Expression of *cepR* or *shvR* in *trans* in the *cepR* mutant had no effect on colony morphology, as cells remained rough. Rough colony morphology was restored in the *shvR* mutant by expression of *shvR*, but not *cepR*, in *trans* (Fig. 3B). This suggests that

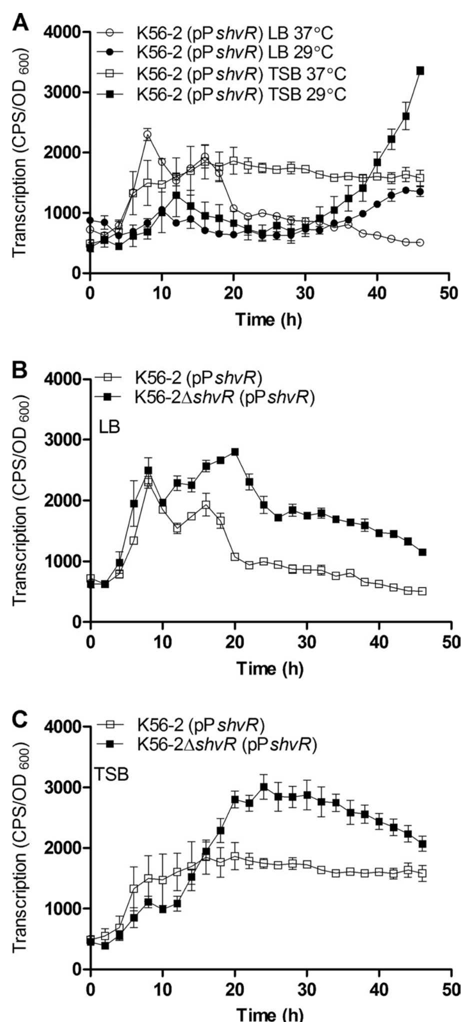

FIG. 1. Influence of medium and temperature on *shvR* promoter activity. Expression of an *shvR* promoter::*lux* fusion (pP*shvR*) in K56-2 in LB and TSB media at 29°C or 37°C (A) and in K56-2 and K56-2Δ*shvR* in LB (B) and TSB (C) at 37°C. Expression was significantly increased in K56-2Δ*shvR* compared to that in K56-2 from 11 to 38 h in LB and from 22 to 35 h in TSB ($P < 0.001$, two-way ANOVA). All values are the means ± standard deviations (SD) of results from triplicate cultures and are representative of results from two individual trials.

ShvR is required for rough colony morphology, and a defect in *shvR* cannot be compensated by overexpression of *cepR*.

ShvR negatively regulates protease and lipase activities and the type II secretion system (T2SS). Analysis of *zmpA* and *zmpB* mutants in a rat chronic respiratory infection model showed that ZmpA and ZmpB contribute significantly to virulence and suggests that ZmpA positively influences persistence (10, 26). Microarray analysis showed that *zmpB* expression was significantly decreased in the *shvR* mutant compared to that in K56-2 (Table 4). Expression of both *zmpA* and *zmpB* promoter::*lux* fusions was significantly decreased in the *shvR* mutant compared to that in K56-2 in LB (Fig. 4A and B) and PTSB (data not shown).

Regulation of *zmpA* and *zmpB* by ShvR was further investigated using phenotypic assays. In contrast to the gene expression data, protease activity was significantly increased in the *shvR* mutant compared to that in K56-2 on D-BHI–1.5% skim milk agar after a 24- and 48-h incubation (Fig. 4C). When *shvR* was expressed on the multicopy pUCP28T plasmid, protease activity was significantly decreased in K56-2Δ*shvR*(p28T-*shvR*) compared to that in either K56-2(pUCP28T) or K56-2Δ*shvR*(pUCP28T) (Fig. 4C). Data from these experiments suggest that ShvR negatively regulates protease activity. ZmpA and ZmpB are secreted via the T2SS, which is comprised of at least 12 open reading frames (ORFs), including *gspC*, and the *gspD* and *gspG* operons. In order to reconcile the apparent contradiction between transcriptional and phenotypic data, we analyzed expression of the T2SS genes. The expression of *gspC* and the *gspG* promoter::*lux* fusions was significantly increased in the *shvR* mutant compared to that in K56-2 (Fig. 4D and E). To determine whether increased expression of the T2SS genes in the *shvR* mutant influenced activity of additional proteins secreted by the T2SS, lipase assays were performed. Lipase activity was significantly increased in the *shvR* mutant compared to that in K56-2 when using 1% Tween 80 (Fig. 4F) or 1% tributyrin (data not shown) as substrates. When *shvR* was expressed in *trans*, lipase activity was significantly decreased in K56-2Δ*shvR*(p28T-*shvR*) compared to that in K56-2Δ*shvR*(pUCP28T) (Fig. 4F). Additionally, the expression of putative polygalacturonase encoded by BCAS0196 was significantly increased in the *shvR* mutant compared to that in the wild type (Table 4). Therefore, expression of at least four enzymes secreted by the T2SS was increased in the *shvR* mutant compared to that in the wild type, suggesting that the increased protease and lipase activity could be primarily due to ShvR regulation of the T2SS.

To further investigate the effects of ShvR on the expression of extracellular zinc metalloprotease and T2SS genes, strains carrying promoter::*lux* fusions were visualized for *lux* activity directly on skim milk agar plates. The intensities of the *zmpA* and *gspC* promoter::*lux* fusion activity appeared equivalent in K56-2 and the *shvR* mutant; however, in support of the protease activity data (Fig. 4C), and in contrast to results obtained in LB liquid cultures (Fig. 4B), *zmpB* promoter::*lux* fusion activity was increased in the *shvR* mutant compared to that in K56-2 (Fig. 5A). Increased expression of *gspG* was also detected in the *shvR* mutant compared to that in K56-2 (Fig. 5A). Expression of *zmpA*, *zmpB*, *gspC*, and *gspG* was more easily detected using promoter::*lux* fusions on D-BHI–1.5% skim milk agar than using L agar plates, suggesting medium-dependent effects (data not shown). To address the regulation by ShvR in different media, promoter::*lux* fusions were compared in the medium constituents used to make D-BHI–1.5% skim milk. Expression of *zmpA* was significantly decreased in the *shvR* mutant compared to that in K56-2 in D-BHI–1.5% skim milk (Fig. 5B) or D-BHI or 1.5% skim milk (data not shown). In contrast, expression of *zmpB*, *gspC*, and *gspG* was significantly increased in the mutant compared to that in the wild type in D-BHI–1.5% skim milk (Fig. 5C to E) or D-BHI or 1.5% skim milk (data not shown). ZmpB has previously been shown to be more potent than ZmpA at degrading casein in the D-BHI–1.5% skim milk agar assay (26). Increased expression of *zmpB* rather than *zmpA*, in addition to

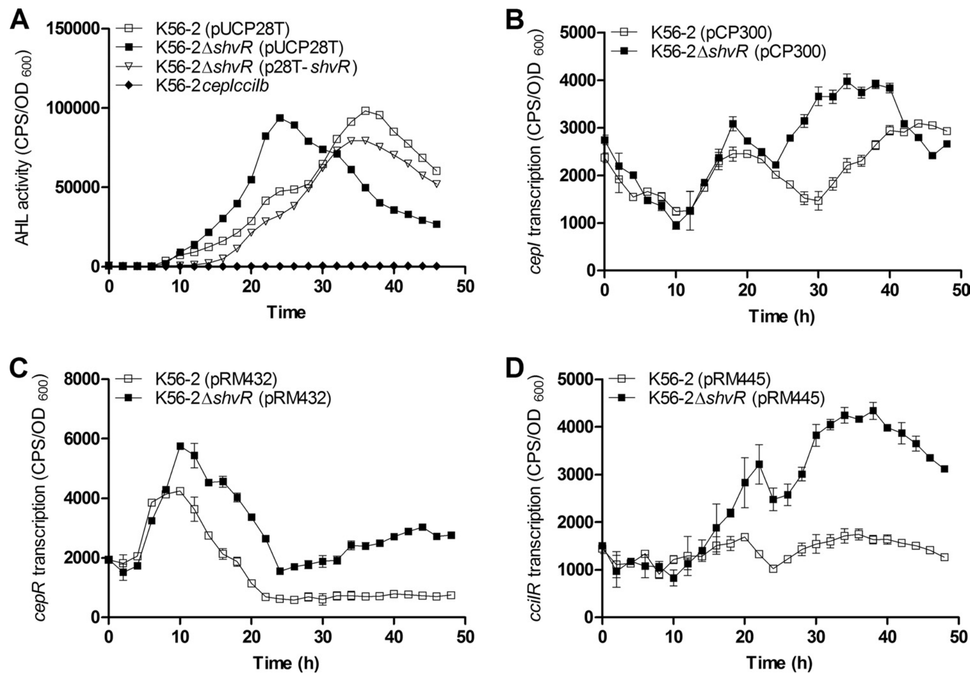

FIG. 2. Effect of the *shvR* mutation on AHL activity and expression of QS genes. (A) AHL activity was measured using the biosensor *A. tumefaciens* A136(pCF218)(pMV26) in coculture with *B. cenocepacia* at 29°C. AHL activity was significantly increased from 22 to 26.5 h (log phase) and significantly decreased from 35 to 45 h (stationary phase) in K56-2Δ*shvR*(pUCP28T) compared to that in K56-2(pUCP28T) ($P <$ 0.05, two-way ANOVA). Expression of *cepI* (B), *cepR* (C), or *cciIR* (D) was monitored using promoter::*lux* fusions (pCP300, pRM432, or pRM445, respectively) in K56-2 and K56-2Δ*shvR* in LB at 37°C. Expression is significantly increased in K56-2Δ*shvR* compared to that in K56-2 from 24.5 to 40.5 h for *cepI*, from 5.5 to 49.5 h for *cepR*, and from 19 to 49.5 h for *cciIR* ($P < 0.001$, two-way ANOVA). All values are the means ± SD of results from triplicate cultures and are representative of results from at least two individual trials.

significantly increased expression of the T2SS, most likely explains the increase in protease activity. Since CepR positively regulates *zmpA* and *zmpB* expression, we also considered the possibility that premature AHL activity in the *shvR* mutant led to deregulated control of *zmpA* and *zmpB* expression by CepR. The exogenous addition of 300 ρM OHL immediately prior to maximal *cepR* transcription in log phase cultures of the *shvR* mutant led to significantly increased *zmpA* and *zmpB* expression (Fig. 4A and B). This trend was not observed when 3,000 pM OHL was added to log-phase cultures or 300 pM OHL was added to stationary-phase (18-h) cultures of the *shvR* mutant (data not shown). These data suggest that appropriate timing and concentrations of exogenous OHL supplementation influenced *zmpA* and *zmpB* expression in the *shvR* mutant.

Expression of the *afcA* and *afcC* operons is ShvR dependent, and ShvR is required for antifungal activity. We recently reported that CepR and CciR reciprocally regulate genes in the 24-kb *afc* genomic region adjacent to *shvR* (41). All 23 genes located in the *afc* genomic region had reduced expression in the *shvR* mutant compared to that in K56-2 according to microarray analysis (Fig. 6A and B). Gene expression levels were reduced up to 100-fold, indicating that these were the most highly regulated genes in the *shvR* mutant. Bioinformatic analysis predicted that the *afcA* operon contains 21 genes, whereas the divergently transcribed *afcC* operon contains two genes (60). Three genes located in different parts of the *afcA* operon were selected for expression studies. Reduced expression of these genes was con-

TABLE 3. Microarray analysis of genes controlled by ShvR, CepR, and CciR

Regulators	Regulation type[e]	No. of genes
ShvR (total no.)[a]	Positive	595
	Negative	482
ShvR (unaffected by *cepR* or *cciR*)[b]	Positive	369
	Negative	358
Coregulated by ShvR and CepR[c]	*cepR*− *shvR*−	100
	cepR− *shvR*+	3
	cepR+ *shvR*−	1
	cepR+ *shvR*+	159
Coregulated by ShvR and CciR[d]	*cciR*− *shvR*−	4
	cciR− *shvR*+	135
	cciR+ *shvR*−	14
	cciR+ *shvR*+	3

[a] Total number of genes positively or negatively regulated in stationary-phase cultures of K56-2*shvR*::Tp that are regulated differently from genes in K56-2.

[b] Number of genes positively or negatively regulated in stationary-phase cultures of K56-2*shvR*::Tp that are regulated differently from genes in K56-2 but unaffected in K56-R2 (*cepR*) and K56-2*cciR* (41).

[c] Number of genes positively or negatively coregulated in stationary-phase cultures of K56-R2 (*cepR*) and K56-2*shvR*::Tp mutants.

[d] Number of genes positively or negatively coregulated in stationary-phase cultures of K56-2*cciR* and K56-2*shvR*::Tp mutants.

[e] Numbers of coregulated genes were grouped as follows: +, positive regulation; −, negative regulation. Previously published data from O'Grady et al., 2009 (41), regarding number of genes positively or negatively regulated in stationary-phase cultures of K56-R2 (*cepR*) or K56-2*cciR* that were regulated differently in K56-2 were used for comparison with K56-2*shvR*::Tp.

TABLE 4. Microarray and qRT-PCR analysis of selected genes showing differential expression in K56-2*shvR*::Tp and K56-2

Gene	Function[a]	Fold change[b]	
		Microarray	qRT-PCR
BCAM1870	AHL synthase CepI	NC	3.6
BCAM1868	AHL regulator CepR	NC	5.6
BCAM0239a	AHL synthase CciI	1.8	12.5[c]
BCAM0240	AHL regulator CciR	NC	12.5[c]
BCAS0204	ABC transporter ATP-binding protein	−100[d]	−34.5
BCAS0215	Putative exported protein	−100	−51.7
BCAS0220	Putative permease	−38.6	−193.4
BCAM2307	Zinc metalloprotease ZmpB	−4.4	ND
BCAS0196	Putative polygalacturonase	2.3	ND

[a] Function derived from *B. cenocepacia* J2315 (21).
[b] Change in stationary-phase cultures of K56-2*shvR*::Tp compared to those of K56-2 as determined by microarray analysis or using qRT-PCR. NC, no change; ND, not determined using qRT-PCR.
[c] *cciI* and *cciR* are cotranscribed (36).
[d] Change in expression of BCAS0204 was included, as it is predicted to be part of the *afcA* operon (60), although it did not meet statistical significance for microarray analysis (detected as "absent" in K56-2*shvR*::Tp).

firmed in the *shvR* mutant by using qRT-PCR (Table 4). Furthermore, analysis of *afcA* and *afcC* promoter::*lux* fusions confirmed significantly lower expression of these operons in the *shvR* mutant than in K56-2 (Fig. 6C and D).

Mean expression levels of genes in the *afcA* and *afcC* operons were reduced 5.7-fold in the *cepR* mutant (41) compared to 77.6-fold in the *shvR* mutant (Fig. 6B). CepR positively regulates *shvR* (41); thus, we considered the possibility that CepR may indirectly regulate the *afcA* operon via positive regulation of ShvR. Cross-complementation experiments were performed to investigate expression of the *afcA* operon in the *cepR* or *shvR* mutant backgrounds with *cepR* or *shvR* provided in *trans*. Three genes located in different parts of the *afcA* operon were selected for expression studies. In the *cepR* mutant background, expression of these genes was reduced between 3.5- and 15.3-fold (Table 5). Expression of these genes increased to levels close to or above parental levels by the presence of either *cepR* or *shvR* in *trans* (Table 5). In the *shvR* mutant background, expression of these genes was reduced between 106.5- and 242.4-fold and was restored to above parental levels by expression of *shvR* in *trans* (Table 5). In contrast, expression of *cepR* in *trans* in the *shvR* mutant background had little effect, in that gene expression levels remained between 69- and 211-fold reduced compared to those of the control (Table 5).

A heterologous host *E. coli* expression reporter system was developed to examine the potential for direct regulation of *afcA* and *afcC* by CepR and ShvR. *E. coli* strains were generated containing two types of plasmids: the first plasmid for expression of CepR or ShvR from pUCP28T in parallel with a second plasmid containing a promoter::*lux* fusion to enable measurement of promoter activity for *cepI*, *afcA*, *afcC*, or *shvR*. CepR binds the *cepI* promoter in the presence of OHL (59) and thus acted as a positive control in this *E. coli* expression reporter system. As expected, CepR activated expression of the *cepI* promoter::*lux* fusion only in the presence of OHL (Fig. 7A) in a concentration-dependent manner (data not shown). CepR in the presence of OHL did not activate expression of promoter::*lux* fusions to *afcA*, *afcC* (Fig. 7B and C), or *shvR* in *E. coli* (data not shown), suggesting that CepR does not directly activate these promoters. In contrast, ShvR activated expression of *afcA* and *afcC* promoter::*lux* fusions (Fig. 7B and C). These data provide evidence that ShvR directly regulates the *afcA* and *afcC* promoter regions and suggest that CepR indirectly positively regulates these genes via positive regulation of ShvR.

Mutations affecting *afcA*, *afcC*, and *afcD* in *B. pyrrocinia* BC11 were previously shown to impair production of an antifungal compound and inhibit antifungal activity against the soilborne phytopathogen *Rhizoctonia solani* (25). *B. pyrrocinia* BC11 AfcA, AfcC, and AfcD show greater than 95%

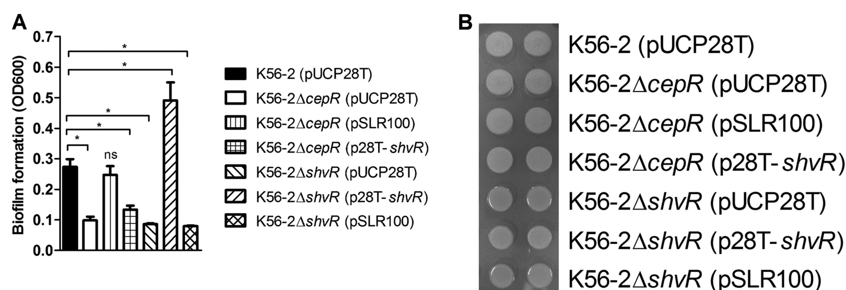

FIG. 3. Regulation of biofilm formation and colony morphology. (A) Biofilm formation was assessed on polystyrene pegs. Statistical significance was determined (*, $P < 0.001$ [one-way ANOVA]). All values are the means ± SD of results from 8 replicate cultures and are representative of results from at least two individual trials. (B) Colony morphology of cultures spot inoculated in 8 replicates onto LB agar. Images are representative of results from two individual trials.

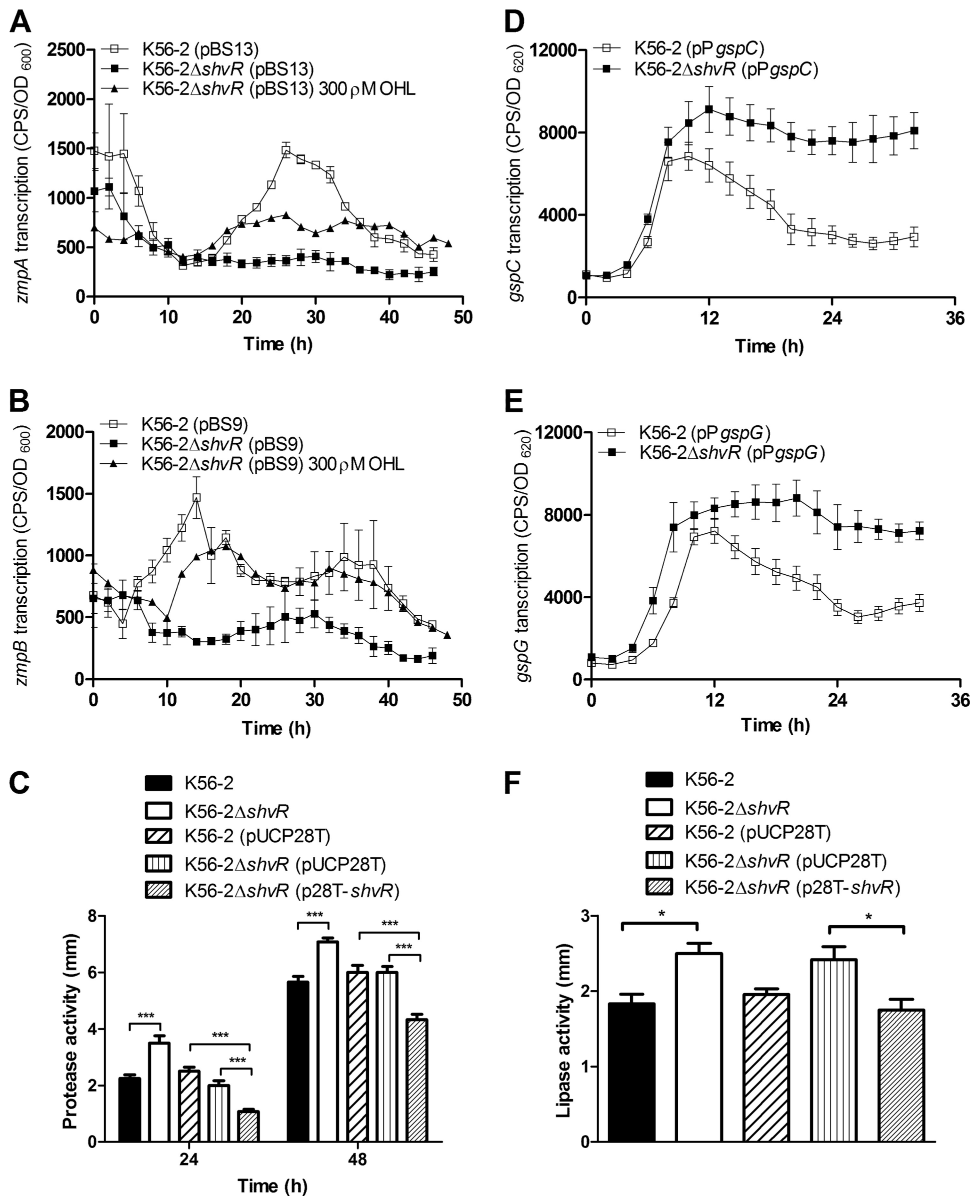

FIG. 4. Expression of *zmpA*, *zmpB*, *gspC*, and the *gspG* operon and protease and lipase activities. Expression of *zmpA* (A), *zmpB* (B), *gspC* (D), or *gspG* (E) was monitored using promoter::*lux* fusions in *B. cenocepacia* in LB at 37°C. Expression is significantly decreased in K56-2Δ*shvR* compared to that in K56-2 from 20 to 37.5 h for *zmpA* and from 8 to 21.5 h for *zmpB*. Expression is significantly increased in K56-2Δ*shvR* compared to that in K56-2 from 16 to 32 h for *gspC* and *gspG*. Expression is significantly increased in K56-2Δ*shvR* including 300 pM OHL compared to that in K56-2Δ*shvR* from 17.5 to 26.5 h and 30.5 to 48 h for *zmpA* and 12 to 23.5 h and 32 to 40.5 h for *zmpB* ($P < 0.001$, two-way ANOVA). All values are the means ± SD of results from triplicate cultures and are representative of results from at least two individual trials. Cultures were spot inoculated in triplicate on D-BHI–1.5% skim agar for protease activity (C) or 1% Tween 80 agar for lipase activity (F), and zones of clearing or precipitation around colony growth were measured, respectively. Statistical significance was determined (*, $P < 0.05$; ***, $P < 0.001$ [one-way ANOVA]).

identity with corresponding proteins in *B. cenocepacia* J2315, AU1054, HI2424, and MCO-3 (60). Considering the regulation by CepR and ShvR of genes in the *afc* genomic region, we compared antifungal activity of *cepR* and *shvR* mutants to that in the wild type. Antifungal activity against *R. solani* was detectable to a similar extent in K56-2 and the *cepR* mutant (Fig. 8). K56-2Δ*shvR*(pUCP28T) did not appear to have any antifungal activity; however, activity was restored to parental levels in K56-2Δ*shvR*(p28T-*shvR*) (Fig. 7). In summary, these data suggest that ShvR-dependent expression of the *afcA* and *afcC* operons was essential for the antifungal activity of K56-2.

DISCUSSION

We previously reported an alteration of virulence-related phenotypes in *B. cenocepacia* that was associated with a mutation of the ORF encoding the LTTR ShvR (4). In this

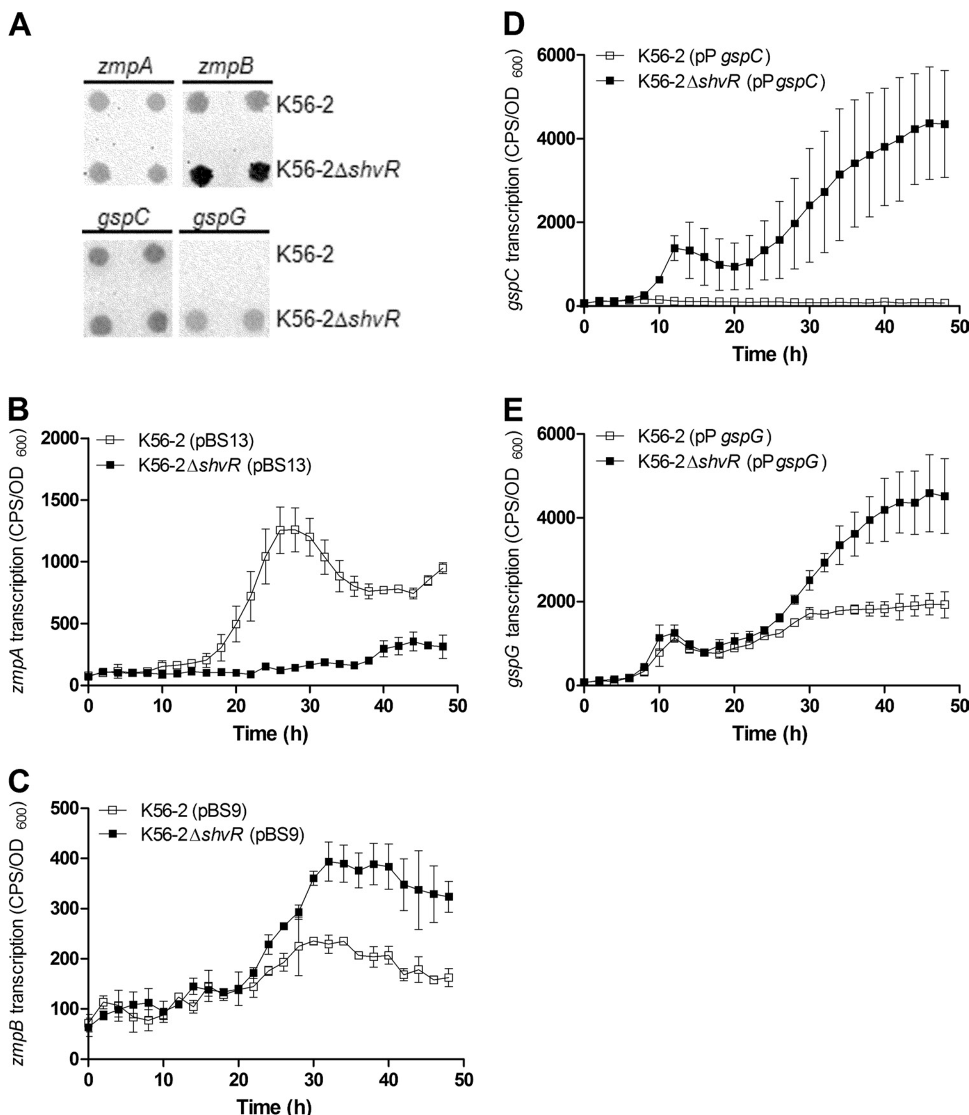

FIG. 5. Expression of *zmpA*, *zmpB*, *gspC*, and the *gspG* operon on D-BHI–1.5% skim milk agar and in D-BHI–1.5% skim milk. (A) Expression of *zmpA*, *zmpB*, *gspC*, and *gspG* was monitored using promoter::*lux* fusions in *B. cenocepacia* from cultures spot inoculated onto D-BHI–1.5% skim milk agar. Images are representative of results from two individual trials. Expression of *zmpA* (B), *zmpB* (C), *gspC* (D), or *gspG* (E) was monitored using promoter::*lux* fusions in *B. cenocepacia* in D-BHI–1.5% skim milk at 37°C. Expression is significantly decreased in K56-2Δ*shvR* compared to that in K56-2 from 18 to 48 h for *zmpA*. Expression is significantly increased in K56-2Δ*shvR* compared to that in K56-2 from 29.5 to 48 h for *zmpB*, 38.5 to 48 h for *gspC*, and 35.5 to 48 h for *gspG* ($P < 0.001$, two-way ANOVA). All values are the means ± SD of results from triplicate cultures and are representative of results from at least two individual trials.

study, we characterized ShvR regulation and determined that it exerts negative autoregulation, a feature typical of LTTRs. ShvR also exerts the transcriptional control of genes that are located in the genomic region adjacent to *shvR* and genes located on all three chromosomes and the plasmid, as well as QS-regulated genes. The *cepIR* and *cciIR* genes are negatively regulated by ShvR, and consequently ShvR has a significant influence on the timing of expression of AHL activity in that AHLs accumulate earlier in an *shvR* mutant than in the wild type, although total AHL activity is similar between the strains. The reduced AHL activity in the *shvR* mutant during stationary phase likely contributed to the identification of numerous genes coregulated by CepR and ShvR, including *zmpA*, *zmpB*, and the *afcA* and *afcC* operons. Despite the identification of coregulated genes, biofilm formation and colony morphology are independently regulated by CepR and ShvR. More than 67% of the total number of genes that were differentially expressed in the *shvR* mutant were not altered in the *cepR* or *cciR* mutants. Independent ShvR-mediated regulation of these genes may play a role in the altered biofilm formation and colony morphology observed in the *shvR* mutant. Reduced alfalfa virulence by *shvR* but not *cepI* mutants of *B. cenocepacia* K56-2 provides additional evidence for independent regulation by ShvR (4, 55).

ZmpA and ZmpB extracellular proteases each contribute

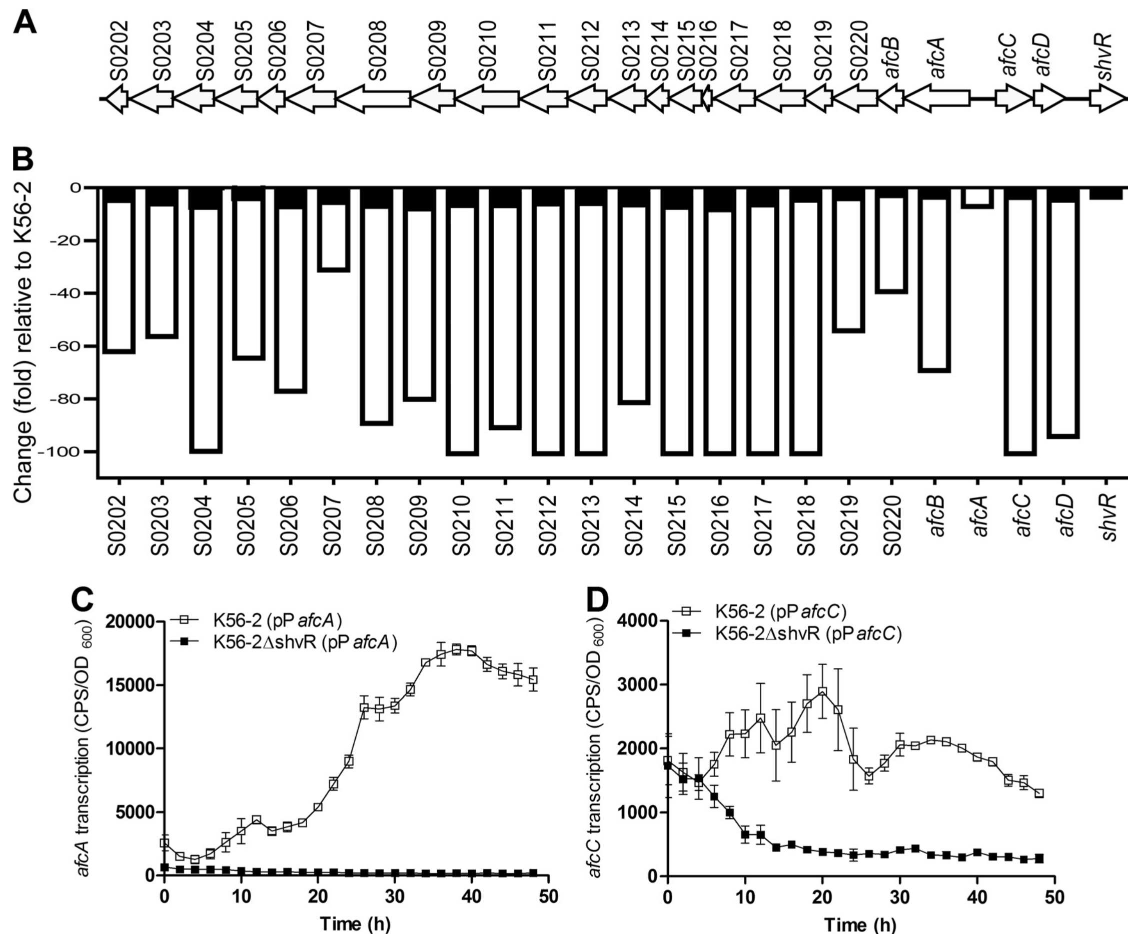

FIG. 6. Genomic organization and expression of genes in the *shvR*/*afc* genomic region. (A) ShvR lies in the genomic region adjacent to the *afcA* and *afcC* operons, which are transcribed divergently. (B) Expression was measured in K56-2*shvR*::Tp (open bars) and K56-R2 (closed bars) compared to that of K56-2 as determined by microarray analysis. Changes in expression of BCAS0204 and BCAS0205 were included, as they are predicted to be part of the *afcA* operon (60), although they did not meet statistical significance for microarray analysis. Expression of *afcA* (C) and *afcC* (D) was monitored using promoter::*lux* fusions in *B. cenocepacia* in LB at 37°C. Expression is significantly decreased in K56-2Δ*shvR* versus K56-2 ($P < 0.001$, two-way ANOVA). All values are the means ± SD of results from triplicate cultures and are representative of results from at least two individual trials.

to virulence in a rat chronic respiratory infection model (26). Several studies highlight the importance of the T2SS, encoded by the *gsp* genes, for secretion of proteases and other extracellular enzymes (14, 17, 27, 52). Protease, phospholipase C, and hemolysin activities were reduced due to a *gspN* mutation in *Burkholderia vietnamiensis* CEP40 (14). *B. cenocepacia* H111 with mutations in the *gspD* or *gspG* operons failed to produce extracellular protease and had significantly reduced lipase activity (27). In *B. cenocepacia* AU1054, a *gspJ* mutation resulted in reduced protease and polygalacturonase activities (52). Analysis of sequenced genomes at http://www.burkholderia.com (60) indicates that *B. cenocepacia* J2315 is the only *Burkholderia* strain which lacks *gspL*, which is part of the *gspG* operon (21). *B. cenocepacia* J2315 produces little or no protease activity on skim milk agar despite detection of high *zmpA* expression levels using qRT-PCR (17).

QS regulation of the T2SS is observed in *Pseudomonas*

TABLE 5. Expression of selected genes in the *afcA* operon

Gene	Fold change[a]					
	K56-2Δ*cepR*(pUCP28T)	K56-2Δ*cepR*(pSLR100)	K56-2Δ*cepR*(p28T-*shvR*)	K56-2Δ*shvR*(pUCP28T)	K56-2Δ*shvR*(p28T-*shvR*)	K56-2Δ*shvR*(pSLR100)
S0220	−4.4	9.3	−1.2	−242.4	3.0	−210.6
S0208	−15.3	4.9	−5.2	−214.1	2.9	−100.9
S0204	−3.5	16.4	−1.3	−106.5	1.4	−69.3

[a] Change (fold) in stationary-phase cultures of *cepR* and *shvR* mutants carrying pUCP28T derivatives as indicated compared to cultures of K56-2(pUCP28T) as determined by qRT-PCR.

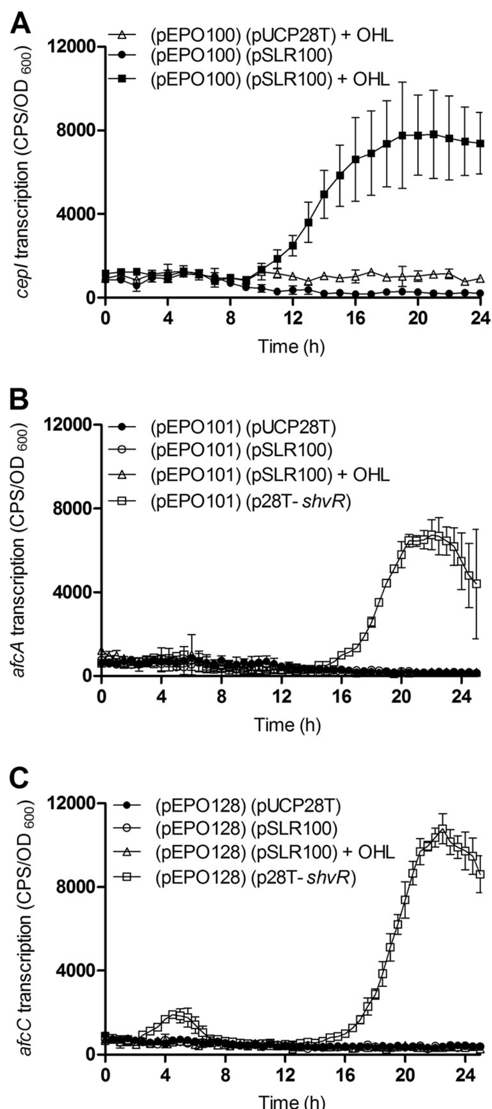

FIG. 7. Expression of *cepI*, *afcA*, and *afcC* in the heterologous host *E. coli* by using an expression-reporter system. Strains harbor two plasmids: a derivative of pUCP28T and a promoter::*lux* fusion for *cepI* (pEPO100), *afcA* (pEPO101), or *afcC* (pEPO128) as indicated. Assays were performed in the absence or presence of 3,000 pM OHL at 37°C. Expression of *cepI* (A), *afcA* (B), or *afcC* (C) was monitored using the appropriate promoter::*lux* fusion in *E. coli* also carrying pUCP28T, or *cepR* in *trans* (pSLR100) or *shvR* in *trans* (p28T-*shvR*). All values are the means ± SD of results from triplicate cultures and are representative of results from at least two individual trials.

aeruginosa and *Burkholderia glumae*. Expression of the XcpR protein in *P. aeruginosa* is most easily detected at the onset of stationary phase, after which protein levels decrease slightly (9). The *B. glumae* TofIR QS system positively regulates the IclR-type transcriptional regulator gene, QsmR, which directly binds the promoters for *gspC*, *gspD*, and *gspG* (18). We previously noted that expression of *gspC* and *gspG* was decreased in the wild type compared to that in a *cepI* mutant or upon addition of OHL to a *cepI* mutant, suggesting negative regulation by CepIR (53). The negative effect of CepIR on *gspC* and *gspG* is consistent with the decrease in *gsp* expression that occurs upon entry into stationary phase in wild-type cells. After a 24-h incubation, *gspC* and *gspG* operon transcription in the wild type is reduced to a steady-state level that remains higher than basal transcription levels observed in starting cultures. Expression of the *P. aeruginosa xcp* gene is activated by QS followed by a decrease in expression to a level that is higher than that of starting cultures (9). Together, these data suggest that expression of the T2SS is tightly regulated by QS in both *B. cenocepacia* and *P. aeruginosa*, and once the apparatus is fully formed, gene expression is maintained to facilitate continued secretion by the T2SS. Expression of T2SS genes was significantly increased in the *shvR* mutant compared to that in the wild type during stationary phase of growth, when AHL activity was reduced. It is possible that unidentified ShvR-regulated factors, in addition to altered AHL activity in the *shvR* mutant, contribute to the sustained and increased expression of the T2SS that resulted in increased protease and lipase activities.

Reduced *zmpA* and *zmpB* promoter activity in the *shvR* mutant in LB cultures was recovered by supplementation of exogenous OHL immediately prior to maximal *cepR* transcription. These data are in concordance with a positive regulatory effect of CepR and provide evidence that reduced expression of these genes in the *shvR* mutant in LB occurred via ShvR influence on AHL activity and QS gene expression. The absence of *shvR* had a different effect on *zmpB* expression in different media. The combination of D-BHI–1.5% skim milk and the *shvR* mutation resulted in increased *zmpB* expression. These conditions may provide a stimulus for an intermediate regulator of *zmpB* that functions in the absence of ShvR, resulting in increased *zmpB* expression. Previous studies demonstrated that CciR regulation of *zmpA* and *zmpB* is influenced by medium and growth phase, indicating that the regulation of these extracellular enzymes is

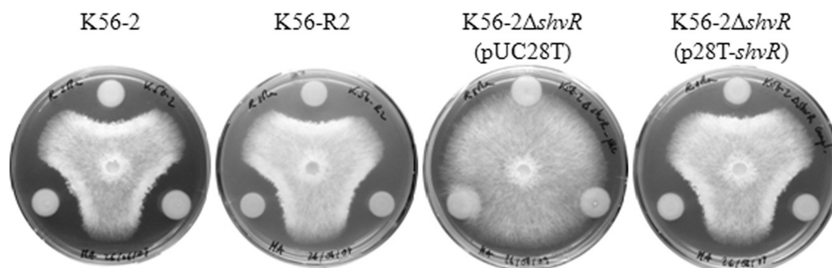

FIG. 8. Antifungal activity against *R. solani*. *R. solani* was grown on malt agar in the presence of K56-2, K56-R2 (*cepR*), K56-2Δ*shvR*(pUCP28T), or K56-2Δ*shvR*(p28T-*shvR*). Fungal growth inhibition was recorded after 3 days. The assay was performed in triplicate; a representative plate for each assay is shown.

complex (26, 36, 41). *B. cenocepacia* produces *cis*-2-dodecenoic acid (*Burkholderia* diffusible signal factor [BDSF]), which is a QS signal (7, 11, 38, 43). While *zmpB* expression was not examined, BDSF positively regulated *zmpA* and *lipAB* (lipase) expression in wild-type and *cepR* mutant backgrounds (11). These data indicate that the BDSF signal is another factor that regulates expression of these extracellular enzymes.

We previously showed that the *shvR*/*afc* genomic region is positively regulated by CepR and negatively regulated by CciR (41). During review of the manuscript, expression of *shvR* and the *afcA* and *afcC* operons was reported to be decreased 2.6-fold in a *B. cenocepacia* J2315 BDSF synthase mutant (BCAM0581 [*rpfF*]) (38). We previously reported a 5.7-fold decrease in expression of these genes in a K56-2 *cepR* mutant (41). In contrast to the effects of QS, expression of the *afcA* and *afcC* operons was substantially lower in the *shvR* mutant (77.6-fold) than in the wild type. Data from cross-complementation experiments and the heterologous host expression reporter system suggested that the expression of the *afcA* and *afcC* operons is ShvR dependent and that the effects of the CepIR system on these operons are likely indirectly controlled via ShvR.

The majority of genes in the *afcA* operon are uncharacterized, although in *B. pyrrocinia* BC11, *afcA* and *afcCD* were shown to be involved in the production of an antifungal with inhibitory activity against the soilborne phytopathogens *Rhizoctonia solani* and *Pythium ultimum* (25). Antifungal activity was substantially reduced in the *shvR* mutant, likely due to the reduced expression of genes in the *afc* genomic region. Antifungal production is QS dependent in certain strains of *Burkholderia ambifaria*, *Burkholderia pyrrocinia*, and *Burkholderia lata* (46) but was not altered by the mutation of *cepR* in *B. cenocepacia* K56-2, providing evidence of species variation in antifungal activity. It is noteworthy that the *B. pyrrocinia* BC11 antifungal activity was not found against pathogenic fungi more commonly associated with human infections such as *Candida albicans* or *Aspergillus* species (25). We determined that *shvR* expression was highest after extended incubation at 29°C, which is consistent with the observation that production of antifungal activity in *B. pyrrocinia* was detected at 30°C (25). Genes in the *shvR*/*afc* genomic region are more highly expressed in the agricultural field isolate *B. cenocepacia* HI2424 in soil-like culture conditions at 22°C than in synthetic CF sputum medium at 37°C (61) and in the CF isolate *B. cenocepacia* J2315 in soil-like culture conditions than in synthetic CF sputum medium at 37°C (62). Together, these data suggest that in addition to ShvR contribution to virulence in plant and animal infection models, ShvR control of the antifungal encoded by *afc* genes may play an important role in the competitive environment of the plant rhizosphere, protecting plants against phytopathogenic fungi.

ShvR exhibited features typical of LTTRs, namely, negative autoregulation and regulation of genes located in the adjacent *afc* genomic region. Negative autoregulation may explain the biphasic expression pattern of *shvR* observed in cultures grown at 29°C. The CepIR and CciIR QS systems display a complex regulatory interrelationship that involves positive or negative autoregulation, CepR-dependent expression of *cciIR*, and negative regulation of *cepI* by CciR (29, 36). While *shvR* expression is inversely regulated by CepIR and CciIR (41), a feedback loop exists where ShvR negatively regulates *cepIR* and *cciIR* expression. A situation may exist where basal *shvR* expression at low cell densities is sufficient to repress *cepIR* and *cciIR* expression and delay AHL accumulation until positive regulators of *cepIR* and *cciIR* become active as cell density increases. Apart from the AHL-dependent effects of ShvR, global transcriptional changes observed in the *shvR* mutant may stem from ShvR regulation of intermediate regulators, including 18 LTTRs identified by using microarray analysis. Characterization of ShvR cofactor(s) may enable the identification of subsets of genes regulated under specific environmental conditions. In the current study, two promoters were determined to be directly regulated by ShvR. The identification of an ShvR-binding motif may reveal other promoters that are directly regulated versus those that are indirectly regulated. Further studies are under way to determine the mechanisms by which ShvR regulates virulence traits in *B. cenocepacia*.

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