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MINIREVIEW

Regulation of Virulence by Two-Component Systems in Pathogenic *Burkholderia*

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ABSTRACT The regulation and timely expression of bacterial genes during infection is critical for a pathogen to cause an infection. Bacteria have multiple mechanisms to regulate gene expression in response to their environment, one of which is two-component systems (TCS). TCS have two components. One component is a sensory histidine kinase (HK) that autophosphorylates when activated by a signal. The activated sensory histidine kinase then transfers the phosphoryl group to the second component, the response regulator, which activates transcription of target genes. The genus *Burkholderia* contains members that cause human disease and are often extensively resistant to many antibiotics. The *Burkholderia cepacia* complex (BCC) can cause severe lung infections in patients with cystic fibrosis (CF) or chronic granulomatous disease (CGD). BCC members have also recently been associated with several outbreaks of bacteremia from contaminated pharmaceutical products. Separate from the BCC is *Burkholderia pseudomallei*, which is the causative agent of melioidosis, a serious disease that occurs in the tropics, and a potential bioterrorism weapon. Bioinformatic analysis of sequenced *Burkholderia* isolates predicts that most strains have at least 40 TCS. The vast majority of these TCS are uncharacterized both in terms of the signals that activate them and the genes that are regulated by them. This review will highlight TCS that have been described to play a role in virulence in either the BCC or *B. pseudomallei*. Since many of these TCS are involved in virulence, TCS are potential novel therapeutic targets, and elucidating their function is critical for understanding *Burkholderia* pathogenesis.

KEYWORDS *Burkholderia*, two-component regulatory systems

The genus *Burkholderia* is composed of Gram-negative betaproteobacteria that were originally described in the 1950s as the pathogenic cause of onion rot (1). *Burkholderia* species are found in the environment, often in water, soil, or in the rhizosphere of plants (1). Some members of the genus can cause disease in humans, animals, and plants, while other members have beneficial effects of agricultural or industrial importance (2). Some strains are capable of producing compounds with antifungal, antibacterial, herbicidal, or insecticidal properties (2). Certain *Burkholderia* strains are capable of bioremediation of hydrocarbons and heavy metals (3). One common theme among members of the *Burkholderia* genus is that most species are highly resistant to many antibiotics. Much of the antibiotic resistance in *Burkholderia* is attributed to efflux pumps, inducible beta-lactamases, and decreased permeability of the outer membrane due to the structure of the lipopolysaccharides (LPS) (4, 5). *Burkholderia* species can produce biofilms, which limit the ability of antibiotics to access the bacterial cell, making antibiotic treatment more difficult. The primary *Burkholderia* species that cause human infections are the members of the *Burkholderia cepacia* complex (BCC) and *Burkholderia pseudomallei*.

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BURKHOLDERIA CEPACIA COMPLEX

The BCC is a group of 20 related species that can cause severe respiratory infections in patients with cystic fibrosis (CF) or chronic granulomatous disease (CGD). CF is an autosomal recessive genetic disorder in which the *cftr* gene contains a mutation that renders the CFTR protein with low or no activity. Lack of full CFTR function leads to dysfunction in multiple organs, but in the lungs it leads to decreased mucociliary clearance, mucus buildup, and defective host defense, which leads to chronic infection (primarily bacterial) and chronic inflammation, leading to bronchiectasis and severe lung damage (6, 7). CGD is also a genetic disorder, often X chromosome linked, in which the gene encoding NADPH oxidase is mutated. These mutations result in phagocytes that are less able to produce reactive oxygen species, making the patient more susceptible to bacterial and fungal infections (8, 9). The prevalence of BCC infection among CF patients is lower than that by more commonly seen CF pathogens such as *Pseudomonas aeruginosa* or *Staphylococcus aureus*, but the infections caused by certain strains of the BCC are sometimes very severe and can result in "cepacia syndrome," which is characterized by rapid respiratory decline, bacteremia, and necrotizing pneumonia. Cepacia syndrome is a nearly uniformly fatal complication of BCC infection even with aggressive antibiotic therapy. *Burkholderia cenocepacia* and *Burkholderia multivorans* are the species most commonly seen in CF patients in the United States, and *B. multivorans* has emerged as the most predominant species in some regions (10–14). Outbreaks of highly pathogenic strains have occurred at CF clinics across the globe, including *B. cenocepacia* outbreaks in Europe (5) and Toronto (Canada) (15), and a *Burkholderia dolosa* outbreak in Boston (USA) (16). The overall prevalence of the BCC is 2% to 3% in North American CF patients (17). In non-CF and non-GCD patients, BCC species have also been associated with serious infections associated with contaminated medications (18–23). Some members of the BCC, such *Burkholderia vietnamiensis*, which is capable of fixing nitrogen, have the potential to be beneficial to plants; however, the use of BCC species in agriculture has been limited due to concerns about the risk it poses to susceptible populations, including those with CF (24, 25).

BURKHOLDERIA PSEUDOMALLEI AND *BURKHOLDERIA MALLEI*

B. pseudomallei is the causative agent of melioidosis, which is a serious systemic infection that can include sepsis, pneumonia, fever, and abscesses. These infections are life-threatening, and mortality can be as high as 50% (26). *B. pseudomallei* is found in soil and water, primarily in the tropics of Southeast Asia and Northern Australia and to a lesser extent in tropical climates across the world (26). Infection with *B. pseudomallei* occurs primarily via wounds or skin abrasions, although infection when contaminated water or soil is inhaled or ingested can also occur. Chronic infections (longer than 2 months) occur in ~10% of cases, and up to 28% of acute cases have recurrence of infection after resolution of initial symptoms (26). Infections most commonly occur in individuals with an underlying condition such as immunosuppression or diabetes. In countries where *B. pseudomallei* is endemic, people with CF can be infected by *B. pseudomallei* (27). *B. pseudomallei* is a potential bioterrorism agent and is classified as a tier 1 agent by the Centers for Disease Control and Prevention. Due to the nonspecific symptoms, infections caused by *B. pseudomallei* are likely underreported, and models predict there could be as many as 165,000 cases and 89,000 deaths per year worldwide (28). Related to *B. pseudomallei* is *Burkholderia mallei*, the causative agent of glanders, which is a disease similar to melioidosis that primarily affects horses, donkeys, and mules. Infections in humans caused by *B. mallei* are extremely rare and are typically associated with exposure to infected animals. Both *B. mallei* and *B. pseudomallei* are able to survive intracellularly by modulating the host response (29). One key difference between *B. mallei* and *B. pseudomallei* is that *B. mallei* is an obligate mammalian pathogen and is not found in the environment (30). *B. mallei* has been eradicated in many parts of the world, including in the United States. *B. mallei*, like *B. pseudomallei*, has the potential to be used as a bioterrorism agent, and was actually used as such during World War I (31).

OTHER PATHOGENIC *BURKHOLDERIA* SPECIES

Other members of the *Burkholderia* genus include species that are beneficial to plants, while other species can be pathogenic to plants. With the exception of some BCC species such as *B. vietnamiensis* (outlined above), strains that are beneficial to plants typically do not pose a risk to humans since most of these strains likely lack the virulence factors needed to cause an infection in humans (24). A subset of species outside the BCC and *B. pseudomallei* that are pathogenic to plants are also pathogenic to humans with CF or CGD. *Burkholderia gladioli* is one such example of an organism that is pathogenic to both plants and humans, and it can cause rare infections in immunocompetent individuals (32, 33). *B. gladioli* is also capable of producing many compounds with antibacterial or antifungal activity; one strain that was isolated from CF sputum produces a macrolide antibiotic named gladiolin that has activity against *Mycobacterium tuberculosis* (34). *Burkholderia glumae* is another pathogen that causes infection in rice and at least a single documented infection in an infant with CGD (35). Interestingly, BCC strains that are virulent in an alfalfa plant model are also virulent in animal models, suggesting that increased virulence can be a trait independent of host (36).

TWO-COMPONENT SYSTEMS

Two-component systems (TCS) are one of many mechanisms that bacteria use to sense and respond to their environment. In the typical TCS, one component, the sensory histidine kinase (HK), senses a signal, autophosphorylates, and then transfers the phosphoryl group to the second component, the response regulator (RR) (37, 38). Sensory histidine kinases contain a domain or domains that can sense the activating signal(s), and the sensory kinase also contains the catalytic domains that allow for the kinase activity to occur. Many RRs contain both a receiver domain that is phosphorylated and a DNA-binding domain that regulates the expression of target genes. RRs function as both transcriptional activators and repressors (38). Other RRs have an RNA-binding domain or an enzymatically active domain instead of a DNA-binding domain that is activated when the receiver domain is phosphorylated (38). In addition to transferring the phosphoryl group to the response regulator, many, but not all, sensory kinases are able to dephosphorylate the response regulator, which is critical for the regulation of signal transduction (38). Typically, both the sensory kinase and the RR are able to form dimers, and in most cases the dimer form is believed to be the active form of the protein. In the classic TCS, the sensor kinase only phosphorylates the associated RR, but cross talk between TCS is possible by the phosphotransfer and/or dephosphorylation of other RRs (39, 40). In addition to the classic TCS, there are hybrid systems in which one protein contains the sensory domain and the kinase domain typically found in HK and a receiver domain that is typically found in the RR (41). Upon stimulation, the hybrid kinase autophosphorylates and then transfers the phosphoryl group on the receiver domain on the same protein. The phosphoryl group is then transferred to a histidine phosphotransfer domain (htp), also on the same protein, and from there the phosphoryl group is then transferred to a receiver domain on a different RR, activating downstream effects of the pathway (37).

Bioinformatic analysis of sequenced *Burkholderia* genomes has shown that all sequenced strains contain at least 40 predicted TCS (42, 43). The majority of these systems have not been analyzed for the function they play in virulence. TCS are attractive targets for new antibiotics, as TCS are not found in animal cells and inhibition of TCS may make the pathogen less virulent (44). The rest of the review will focus on the TCS that have been studied in either the BCC or *B. pseudomallei*. TCS in the BCC are summarized in Table 1, and TCS in *B. pseudomallei* are summarized in Table 2. The majority of the genes encoding the studied TCS are conserved across the reference strains of the multiple species that make up the *Burkholderia* genus (43). Several of the TCS described in *Burkholderia* have homologs involved in virulence in other bacterial pathogens (45).

BURKHOLDERIA CEPACIA COMPLEX TCS

Some TCS in the BCC work in combination with other regulatory mechanisms, such as quorum sensing. One system, the RqpSR system, induces the expression of the enzymes that produce both of the quorum-sensing signaling molecules that BCC members use, *cis*-2-dodecenoic acid (BDSF) and *N*-acyl homoserine lactone (AHL) (46). Disruption of either the sensory histidine kinase *rpqS* or the RR *rqpR* resulted in decreased biofilm formation, motility, toxicity to human lung epithelial cells, and virulence in a murine model (46). Interestingly, the phenotypes seen in the *rpq* deletion mutants appear to be due to both a quorum sensing-dependent and a quorum sensing-independent mechanism (46). This TCS was described in *B. cenocepacia*, but bioinformatic analysis shows that it is distributed across many *Burkholderia* strains, including other BCC species and *B. pseudomallei*, although the role of the *rqpSR* system in these species has not been evaluated.

The BceSR TCS is conserved across many BCC strains that are isolated from CF patients and is 38% identical to that of the GacSA system in *Pseudomonas aeruginosa* PAO1 (47, 48). *B. cenocepacia* lacking *bceR*, which encodes the RR, had reduced swimming motility, made less protease, and had increased *cepI* expression. CepI produces AHL, one of the quorum-sensing molecules (49), and there was an increase in quorum sensing but no difference in the ability of the *bceR* deletion mutant to produce biofilm. Based on these findings, the authors concluded that despite sharing identity with *P. aeruginosa* GacSA, BceSR plays a different role in BCC species than GacSA does in *P. aeruginosa*. *B. cenocepacia* lacking *bceR* had reduced pathogenicity in the alfalfa plant model, but there was no difference seen in a nematode model (48) (mammalian models of infection have not been reported). Another study found that both of the genes encoding BceSR, BCAM1494 and BCAM1493, were upregulated in response to growth under low-oxygen conditions (50).

A study investigating the role of essential genes in *B. cenocepacia* by knocking down expression of these genes identified the EsaSR system as a TCS involved in antibiotic resistance (51). The RR EsaR was essential, but, when depleted, the strain became sensitive to multiple antibiotics, including chloramphenicol, ciprofloxacin, kanamycin, meropenem, novobiocin, and tetracycline. This increased susceptibility is potentially due to a defect observed in membrane integrity and a decrease in efflux pump activity when EsaR was depleted. Interestingly the *B. cenocepacia* EsaSR has a homolog in *B. pseudomallei* (88 to 89% protein identity) encoded by BPSL0127 and BPSL0128 that are required for virulence in murine models (52), described further below. The EsaSR system also is similar to the *Bordetella* PlrSR system (49 and 64% protein identity for the HK and RR, respectively). The *Bordetella* PlrSR system is required for virulence in the lower respiratory tract and controls the activity of the "master virulence regulator" system, BvgAS (53, 54).

Sequencing of sequential BCC isolates from chronically infected CF patients can provide insight into genes that are under selective pressure during infection. One such gene, the sensory histidine kinase encoded by *fixL*, was identified by sequencing over 100 *B. dolosa* isolates collected over a 16-year period from an outbreak among CF patients in Boston (55, 56). Interestingly, *fixL* was also found to be mutated in sequential *B. multivorans* isolates (57). These findings strongly suggest that modulation of this pathway during chronic infection is important and occurs in independent infections in different BCC species. In *B. dolosa*, FixL, along with its RR FixJ, is an oxygen-sensing mechanism that is activated under low-oxygen conditions, regulating multiple phenotypes that include motility, biofilm, and intracellular survival in epithelial cells and macrophages (58). Additionally, the FixLJ system is required for the full virulence of *B. dolosa* in a murine pneumonia model (58). The FixLJ system mediates these phenotypes through the differential regulation of a large number of genes, as mutants lacking *fixLJ* had differential expression of ~11% of the genome (58).

The conversion of BCC species from a mucoid to a nonmucoid phenotype is often observed during chronic infection and is correlated with worse clinical

outcomes (notably different from those with *P. aeruginosa*, where mucoid strains are associated with clinical decline) (59). Mutations occurring in the response regulator *ompR* during chronic infection with *B. multivorans* are a major contributor to this phenotypic switch (60). OmpR is able to regulate the transcription of 701 genes, including many of the *bce* genes that are involved in the production of extracellular matrix. Interestingly, the introduction of the ancestral *ompR* into an evolved, nonmucoid strain carrying an *ompR* mutation was able to restore the mucoid phenotype (60). OmpR has a homolog in *Bordetella pertussis* RisA (77% protein identity) that induces the expression of virulence-repressed genes and works in opposition to the BvgAS system (61).

Another TCS was identified by an *in vitro* evolution experiment of *B. cenocepacia* that selected for biofilm-adapted small colony variants reverting to a planktonic lifestyle (62). Mutations within the response regulator encoded by Bcen2424_1436 allowed for the conversion to a smooth colony phenotype from a wrinkly phenotype. This phenotypic switch was associated with decreased biofilm formation. This TCS made up of Bcen2424_1436 and the associated histidine kinase encoded by Bcen2424_1438 is conserved across *Burkholderia* species (62).

Another TCS was discovered to be part of a six-gene operon involved in thermal and osmotic stress response (63). This operon includes BCAL2831 and BCAL2830, which encode a RR and HK, respectively, along with a protease encoded by $htrA_{BCAL2829}$. Disruption of the gene encoding the response regulator led to a disruption of transcription of the entire operon and decreased ability to survive thermal and osmotic stresses, as well as a reduction in virulence, in a chronic respiratory infection model using rats. Interestingly, the authors found that the TCS likely regulated factors, in addition to the protease $htrA_{BCAL2829}$, that are involved in stress responses. These additional factors were not identified, nor were the activating signal(s). The RR encoded by BCAL2831 is also involved in resistance against polymyxin B in a *B. cenocepacia* strain with truncated LPS devoid of heptose (64). This strain with truncated LPS was used to find additional antibiotic resistance mechanisms that are masked by the robust outer membrane permeability barrier, and this strain is more sensitive to polymyxin B than an isogenic strain with intact LPS (64). Deleting the BCAL2831 gene in the heptoseless LPS strain made the mutant more sensitive to polymyxin B than the heptoseless LPS strain (64). The protein encoded by BCAL2831 has 53% protein identity with the *Pseudomonas aeruginosa* PmrA protein, which plays a role in resistance against polymyxin B and cationic antimicrobial peptides (65).

BCC species also use hybrid TCS to regulate virulence. The gene *atsR* is predicted to encode both a histidine kinase and receiver domain, making it a hybrid TCS. Deletion of *atsR* led to increased biofilm formation and type 6 secretion system (T6SS) activity (66). Subsequent work found that *atsR* also negatively regulated quorum-sensing molecules and quorum sensing-regulated virulence factors (67). Interestingly, *atsR* is predicted to contain transmembrane domains but no DNA binding domains, making the mechanism of *atsR*-regulated gene regulation unclear initially. Subsequent studies identified AtsT as a RR that is phosphorylated by AtsR, which in turn mediates the increased biofilm and T6SS activity (68).

Another hybrid TCS has been found to regulate the transcription of the cable pilus that is expressed in the *B. cenocepacia* epidemic ET12 lineage and is a major virulence factor in this lineage (5). This system includes 2 hybrid sensor kinases, CblT and CblS, and a response regulator, CblR, all of which are required for expression of the cable pilus (69). Another hybrid sensor, encoded by BCAM0227, was identified as a novel BDSF sensor mediating expression of the cable pilus. BCAM0227 is required for virulence in several models, including a mouse infection model (70). The hybrid sensor encoded by BCAM0227 is part of a family of diffusible signal factor (DSF) sensing molecules that are important in the virulence of other pathogens such as *P. aeruginosa* and are also involved in intraspecies signaling (71).

TABLE 1 TCS in the *Burkholderia cepacia* complex

System	Description	Reference(s)
rqpSR	Regulates production of quorum sensing and biofilm formation; required for full virulence in a murine model	46
bceSR	Regulates production of *cepI*; required for virulence in an alfalfa plant model	48
esaSR	Essential genes; required for resistance to several antibiotics	51
fixLJ	Under selective pressure during chronic infection; regulates biofilm formation and motility; required for survival in macrophages and virulence in murine pneumonia model; ~11% of genome regulated by *fixLJ*	58
ompR	Mutations confer mucoid phenotype switch; regulates *bce* cluster, which regulates extracellular matrix	60
Bcen2424_1436 and Bcen2424_1438	Mutations within *Bcen2424_1436* confer wrinkly colony to smooth colony switch; regulates biofilm formation	62
BCAL2831 and BCAL2830	Involved in heat and osmotic stress response; required for virulence in a rat infection model	63
atsRT	Hybrid TCS; regulates biofilm formation and T6SS[a]	66–68
cblTSR	Hybrid TCS; regulates cable pilus in ET12 *B. cenocepacia*	69
BCAM0227	Hybrid TCS that responds to BDSF; regulates cable pilus; required for full virulence	70

[a]T6SS, type 6 secretion system.

B. PSEUDOMALLEI AND *B. MALLEI* TCS

One of the best characterized TCS in *B. pseudomallei* and *B. mallei* is the VirAG system. The VirAG system regulates the expression of one cluster of T6SS genes that is required for virulence of both organisms in multiple animal models (72–74). In addition to the T6SS cluster, VirAG also induces the expression of a structural T6SS protein, hemolysin-coregulated protein (Hcp) (73). The expression of the T6SS system is tightly controlled and only expressed when the bacterium is in the host cytosol, where host glutathione reduces disulfide bonds in dimeric VirA (75). This leads to the formation of active VirA monomers, which in turn activates the RR VirG and subsequent transcription of T6SS genes (75). Interestingly, mutations in *virA* in sequential *B. pseudomallei* isolates taken from Australian CF patients were found, suggesting that modulation of this pathway is could be important for chronic infection (76). Further work is needed to determine if these "evolved" *virA* sequences make *B. pseudomallei* more virulent. The VirAG system is only found in *B. pseudomallei* and *B. mallei* and does not have a homolog in other *Burkholderia* species, including members of the BCC (43).

As mentioned above, BPSL0127 and BPSL0128 in *B. pseudomallei* encode a TCS with homology to the EsaSR system in the BCC. This system was identified by using a transposon mutant library to screen for genes required for resistance to predation the phagocytic amoeba *Dictyostelium discoideum*. Genes that mediate resistance to predation also contribute to survival and replication in macrophage cells (52). Disruption of the gene encoding the histidine kinase resulted in mutants that were less able to survive in macrophages, and mice infected with this mutant had decreased survival compared to mice infected with bacteria without the disruption (52). The authors speculated that the decreased virulence in *B. pseudomallei* BPSL0127 mutants was a result of altered gene expression, but the regulon has not been described (52). It is not clear if the EsaSR and BPSL0127/0128 systems have different functions in their respective species or if they have the same function in both species, but the phenotypes were not observed since they were not tested in the published studies. Neither *esaS* nor BPSL0127, encoding the HK, were essential in their respective species. However, the gene encoding the RR, *esaR*, and BPSL0128 are essential for growth in their respective species (77). Further investigation is needed to understand the role of the EsaSR and BPSL0127/BPSL0128 systems in both the BCC and *B. pseudomallei*. In most cases of melioidosis, survivors either clear the initial infection or have a relapse of symptoms. In a study of 707 melioidosis survivors, one patient was chronically colonized with *B. pseudomallei* without symptoms for 12 years (78). Two isolates from this patient that were isolated 139 months apart were sequenced to identify genetic changes that could contribute to long-term persistence. The RR encoded by BPSL0128 contained a non-

synonymous mutation that was predicted to be deleterious to the function of the protein in the later isolate (78). The effects of this specific mutation remain unknown, as does the contribution of the other observed mutations, but these findings suggest a role for this TCS in human infections.

Another TCS, the MrgRS system, was investigated based on its similarity to the RcsBC system of *Escherichia coli* that regulates capsule synthesis (79). Both the RR MrgR and the sensory kinase MrgS were found in many *B. pseudomallei* strains, but MrgS was not found in *B. mallei* strains. MrgRS was expressed poorly at 25°C compared to expression at 37°C or 42°C, suggesting that this pathway may be a temperature-sensing mechanism. Interestingly, antibodies against the response regulator MrgR were detected in convalescent-phase sera pooled from melioidosis patients, suggesting that this protein is immunogenic. The role of MrgRS in virulence may also include antibiotic resistance, since another group found that *mrgS* when expressed from a plasmid was able to confer high-level colistin resistance to a susceptible *Pseudomonas aeruginosa* strain (80). Clearly, more work is needed to understand the role the MrgRS system plays in virulence and/or antibiotic resistance.

The BprRS system was identified in a signature-tagged transposon mutagenesis screen using an acute murine pulmonary melioidosis model as having attenuated *in vivo* growth in *B. pseudomallei* (81). Disruption of either the sensor histidine kinase gene (*bprS*) or the RR gene (*bprR*) led to attenuation of virulence. However, surprisingly, deletion of both genes together did not have any decrease in virulence. Interestingly, when the transcriptomes of the single and double mutants were analyzed, only 7 genes were differentially expressed in the double mutant, whereas nearly 100 genes were differentially expressed in each of the single mutants. While these results were not confirmed by complementation, this finding suggest the prospect of extensive cross talk of the BrpRS system with other signaling pathways (81). The genes encoding the BprRS system are conserved across the *Burkholderia* genus, but the function of this system has not been studied in these other species. The *B. pseudomallei* BprRS system shares 52% protein identity with the *P. aeruginosa* ParSR system, which is involved in multidrug resistance in response to cationic peptides (82).

The RR encoded by *bfmR* with the sensor kinase BPSL2025 is a TCS that is only found in *B. pseudomallei*, where it is important for virulence. The sensor kinase, encoded by BPSL2025, was identified to be upregulated during infection of hamsters (83). Disruption of Bpsl2025 led to a significant decrease of virulence in the hamster model (83). When the gene encoding the RR, *bfmR*, was disrupted, there was a decrease in biofilm formation that correlated with a decreased expression of fimbriae chaperone-usher assembly genes (84). This *bfmR* mutant was also less able to grow in low-iron medium and was less able to survive within macrophages. Additionally, expression of *bfmR* was upregulated when wild-type *B. pseudomallei* cultures were grown in iron-limited conditions. Despite being less able to survive within macrophages, *bfmR* mutants had no deficiency in their ability to cause disease in mice (85). More investigation is needed to understand this system, specifically looking at the full range of phenotypes it mediates. The genes encoding this system are found only in *B. pseudomallei* and *B. mallei* and do not have homologs in other *Burkholderia* species, including members of the BCC (43).

The IrlRS TCS in *B. pseudomallei* was identified in a screen to find genes required to invade human lung epithelial cells (86). This system has homology to TCS in other organisms that mediate resistance to heavy metals, and *B. pseudomallei irlRS* mutants had increased sensitivity to both cadmium and zinc, but not to other heavy metals as seen with *irlRS* mutants in other bacterial species. There was no difference in the ability of *irlRS* mutants to survive within macrophages or cause disease in infant diabetic rat or in Syrian hamster models compared to the parental strain, suggesting that this TCS plays a limited role in disease in these acute infection models. This pathway is conserved across the *Burkholderia* genus, though its role in other species has not been evaluated.

The *B. pseudomallei* NarXL system was identified based on its homology to a nitrate-sensing system in *P. aeruginosa* (87). When the gene encoding either the sensor

TABLE 2 TCS in *B. pseudomallei*

System	Description	Reference(s)
virAG	Regulates T6SS; required for full virulence; activated by host glutathione within the cytosol	72–76
BPSL0127 and BPSL0128	Required for survival in macrophages and murine infection models	52
mrgRS	Potential temp-sensing mechanism; also regulates antibiotic resistance	79, 80
bprRS	Required for virulence in a murine model; significant cross talk with other signaling pathways	81
bfmR and BPSL2025	HK is upregulated during infection and required for virulence. RR is involved in biofilm formation by regulation of fimbriae chaperone-usher assembly genes; required for persistence in macrophages *in vitro*, but not required for virulence in murine models.	83–85
irlRS	Involved in heavy metal resistance, not required for virulence in macrophages or animal models	86
narXL	Regulates nitrate reductase system; required for nitrate inhibition of biofilm formation	87

kinase NarX or the response regulator NarL was disrupted, nitrate was unable to inhibit biofilm formation. The intracellular levels of the second messenger c-di-GMP decreased when exposed to nitrate, and the authors hypothesized that NarXL may be a sensory system that regulates the expression of several genes involved in c-di-GMP metabolism. Based on what is known about homologs of this system in *P. aeruginosa*, the authors also predicted that NarXL regulates the expression of the adjacent nitrate reductase encoded by the *narGHJI-1* operon. The role of this system in infection models was not evaluated, although its importance may be more related to the bacterium's ability to survive within the environment.

Identification of genes that are regulated by TCS is critical for understanding the role a TCS plays in the virulence and biology of an organism. One study used a protein-binding microarray to identify the DNA binding motifs of 4 RR in *Burkholderia thailandensis* (88). *B. thailandensis* is often used as a model organism for *B. pseudomallei*, since *B. pseudomallei* is a biosafety level 3 (BSL3) pathogen. The four RRs analyzed were NarL, RisA (encoded by BTH_I2094, which is the homolog of OmpR described in *B. multivorans*), KdpE (which in *Escherichia coli* regulates a potassium uptake system to regulate homeostasis), and an uncharacterized RR. Only NarL had been studied for its role in *B. pseudomallei* virulence, and RisA(OmpR) had been described in *B. multivorans* virulence, while neither of the other two RRs have been studied in any *Burkholderia* species. Several of the predicted DNA binding motifs were confirmed using gel shift assays, and, interestingly, the expression of other TCS were found to be regulated by the tested RRs. TCS regulating other TCS demonstrate an interplay between different TCS, making the elucidation of these signaling pathways even more complicated. Identification of targets of the RRs using this technique is labor intensive and requires *in vitro* activation of the RR using beryllofluoride, which binds to the RR in a fashion that mimics phosphorylation (89). Other approaches to identify TCS targets such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) are also labor intensive but can potentially be done *in vivo* if the TCS-activating signal is known.

OTHER *BURKHOLDERIA* TCS

TCS in *Burkholderia* species that are not typically human pathogens are even less studied. One TCS in *B. glumae* is the system encoded by *pidSR*, which is required for the production of pigment in *B. glumae* when grown on Casamino acid-peptone-glucose agar (90). *B. glumae* strains lacking *pidSR* were less virulent than the parental strains in two plant infection models. Interestingly, *pidSR* genes are conserved across the genus, including in species that cause infections in humans, and the predicted amino acid sequences share more than 80% identity. Despite this conservation, the function of the PidSR system remains unstudied in other pathogenic species.

Burkholderia plantarii causes a disease in rice seedlings by the production of the phytotoxin tropolone (91). A systematic approach to identify TCS that are involved in

tropolone production generated mutations in the genes encoding 55 histidine kinases and 72 RRs (91). Tropolone production was lost when *troK* (encoding an HK) or either *troR1* or *troR2* (both encoding RRs adjacent to *troK*) was deleted (91). Loss of tropolone production resulted in loss of virulence in a rice model (91). All three components are conserved across multiple species of *Burkholderia* that are pathogenic to humans. This systematic approach represents a massive undertaking to identify TCS involved in a phenotype, but by generating such a library of TCS mutants, it is possible to identify other TCS involved in other phenotypes, provided that the screen can accommodate the large number of constructs.

TCS AS THERAPEUTIC TARGETS

TCS have been tested as therapeutic targets for multiple pathogenic bacteria (44, 92, 93), although there has not yet been a published report describing inhibition of a TCS in *Burkholderia* as a therapeutic approach. Much of the published work on TCS inhibitors has focused on the WalKR system in *Staphylococcus aureus* and other Gram-positive pathogens, as well as other TCS in *Escherichia coli*, *Mycobacterium tuberculosis*, *and P. aeruginosa* (44, 92, 93). TCS that are essential for growth and TCS that are not essential for growth have been targeted. By inhibiting essential TCS, the growth of the bacteria will be inhibited, but there also is a strong selective pressure for resistance to arise. The rationale for targeting nonessential TCS that regulate virulence is that through inhibition of these pathways the bacteria will become less virulent and less able cause disease (93). To date, there have been no clinical trials using TCS inhibitors, and identification of inhibitors has been plagued by finding compounds that nonspecifically inactivate proteins in general (44, 92, 93). High-throughput screens to identify inhibitors can be improved by a better understanding of the underlying biology of the TCS.

Several studies have identified essential genes in either the BCC or *B. pseudomallei*, and within the lists of essential genes were components of TCS (77, 94). Interestingly, many of these TCS that were identified to be essential have not been evaluated for their function in *Burkholderia*. A better understanding of these TCS will allow their potential use as therapeutic targets. Therapies that target TCS will have to overcome the same challenges that *Burkholderia* pose to conventional antibiotics—namely, the outer membrane permeability barrier and the efflux pumps (95). Unfortunately, less is known about the mechanisms of antibiotic resistance compared to those in more commonly studied pathogens such as *P. aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, so more work will be needed to overcome these challenges (95).

PROSPECTS

TCS are a critical mechanism that allows bacteria to sense their environment and respond to it. *Burkholderia* species possess multiple TCS that regulate virulence, making these TCS potential therapeutic targets. Much progress has been made to identify and describe a number of *Burkholderia* TCS, but only a small fraction of the total number of TCS have been studied. One large limitation to the field is that a given TCS is typically only studied in one species or strain. Many, but not all, of the TCS are conserved across the genus, but it is unclear if they all play the same role in all species. For the EsaSR and BPSL0127/0128 systems that have been studied in two different species, it is unclear if they play the same role in both, since the phenotypes that were studied were different. Studies sequencing large number of sequential *Burkholderia* isolates have identified some TCS as being under positive selective pressure during chronic infection, suggesting that modulation of these pathways is critical for *Burkholderia* adaption to the host. In general, it is not clear what signal(s) activate most TCS, and understanding this aspect is critical for understanding the role each TCS plays in *Burkholderia* biology and virulence. Future work should focus on understanding the signals, as well as how these TCS interact with each other and with other gene regulation pathways and whether modulation of TCS function by small molecules can impact virulence. A greater

knowledge of *Burkholderia* TCS will require a multidisciplinary approach and will further the understanding of this poorly studied group of pathogens.

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