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A novel contact-independent T6SS that maintains redox homeostasis via Zn^{2+} and Mn^{2+} acquisition is conserved in the *Burkholderia pseudomallei* complex

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ABSTRACT

The *Burkholderia pseudomallei* complex consists of six phylogenetically related Gram-negative bacterial species that include environmental saprophytes and mammalian pathogens. These microbes possess multiple type VI secretion systems (T6SS) that provide a fitness advantage in diverse niches by translocating effector molecules into prokaryotic and eukaryotic cells in a contact-dependent manner. Several recent studies have elucidated the regulation and function of T6SS-2, a novel contact-independent member of the T6SS family. Expression of the T6SS-2 gene cluster is repressed by OxyR, Zur and TctR and is activated by GvmR and reactive oxygen species (ROS). The last two genes of the T6SS-2 gene cluster encode a zincophore (TseZ) and a manganeseophore (TseM) that are exported into the extracellular milieu in a contact-independent fashion when microbes encounter oxidative stress. TseZ and TseM bind Zn^{2+} and Mn^{2+}, respectively, and deliver them to bacteria where they provide protection against the lethal effects of ROS. The TonB-dependent transporters that interact with TseZ and TseM, and actively transport Zn^{2+} and Mn^{2+} across the outer membrane, have also been identified. Finally, T6SS-2 provides a contact-independent growth advantage in nutrient limited environments and is critical for virulence in *Galleria mellonella* larvae, but is dispensable for virulence in rodent models of infection.

1. Introduction

Burkholderia pseudomallei and *Burkholderia mallei*, the etiologic agents of melioidosis and glanders, are Tier 1 biological select agents due to their enhanced infectivity by the aerosol route of infection, the absence of licensed vaccines and the intensive therapeutic regimen required for patient treatment (Galyov et al., 2010; Wiersinga et al., 2018). The number of melioidosis cases around the world is predicted to be > 165,000 per year with ~ 89,000 deaths (Limmathurotsakul et al., 2016). Melioidosis patients develop a variety of clinical presentations, but sepsis, pneumonia and/or localized abscesses are the most common. *B. pseudomallei* is present in soil and water in many tropical and subtropical regions and humans and animals can be infected by inhalation, ingestion or skin inoculation of contaminated environmental sources. It is a facultative intracellular pathogen that possesses a large arsenal of virulence factors (Wiersinga et al., 2018).

B. mallei is a host-adapted clone of *B. pseudomallei* that evolved by insertion sequence-mediated genome reduction (Nierman et al., 2004). Glanders is a disease of equines that is occasionally transmitted to humans. The disease in animals can be acute or chronic, with donkeys and mules developing the acute form and horses developing the chronic form. *B. mallei* cannot persist in the environment and has been eradicated from the western world by the development of an effective skin test (mallein) and the slaughter of infected animals. Laboratory-acquired human infections have been documented and often result in an acute glanders infection (Howe and Miller, 1947; Srinivasan et al., 2001).

The *B. pseudomallei* complex also contains four *Burkholderia* species that exhibit phenotypic, biochemical, antigenic and/or genetic similarities to *B. pseudomallei* and *B. mallei* (Fig. 1). These species are generally regarded as nonpathogenic, or weakly pathogenic, and have been isolated from soil and water sources around the world. *B. thailandensis* is an environmental saprophyte previously referred to as a *B. pseudomallei*-like organism due to the presence of conserved biochemical, morphological and antigenic profiles (Brett et al., 1998). This organism has been used extensively as a biological safety level-2 (BSL-2) surrogate for studying orthologous genes in the highly pathogenic members of the complex. The remaining members of the *B. pseudomallei* complex include *B. oklahomensis* (Glass et al., 2006), *B. humptydooensis* (Tuanyok et al., 2017) and *B. singularis* (Vandamme et al., 2017).

Type VI secretion systems (T6SS) are membrane-spanning nanomachines that resemble inverted contractile bacteriophage tails (Alteri

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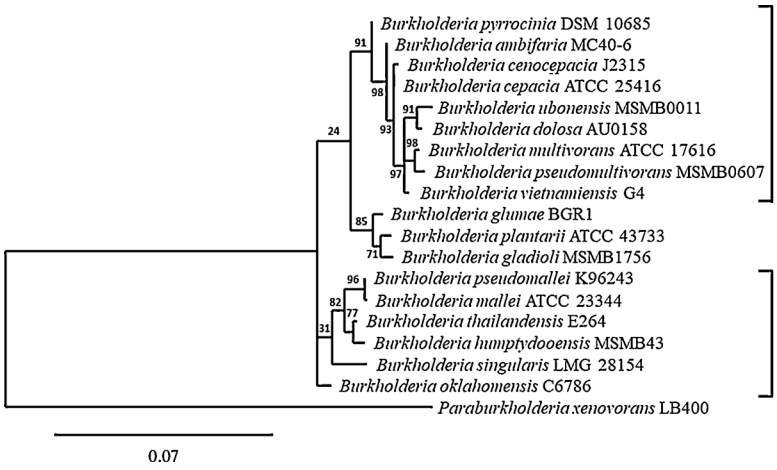

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA sequences of members of the *B. cepacia* complex, the *B. pseudomallei* complex and closely-related *Burkholderia* representatives. Phylogeny.fr was used to build the tree in "One Click" mode and utilizes MUSCLE for multiple alignment, Gblocks for automatic alignment curation, PhyML for tree building and TreeDyn for tree rendering (Dereeper et al., 2008). The sequence of *Paraburkholderia xenovorans* LB400 was used as an outgroup. The scale bar indicates the number of substitutions per site. Bootstrap support values are shown as % as indicated at nodes.

and Mobley, 2016). They inject effector molecules into target cells in a contact-dependent manner and provide Gram-negative bacteria with a survival advantage in the environment or in the host. All members of the *B. pseudomallei* complex possess multiple T6SS and research conducted using *B. pseudomallei*, *B. mallei* and *B. thailandensis* suggests that each T6SS may perform a distinct function in diverse environmental or host niches (Burtnick et al., 2010; Schwarz et al., 2010; Burtnick et al., 2011). Two distinct *Burkholderia* T6SS nomenclature schemes were proposed by Schell et al. (2007) and Shalom et al. (2007), and the former is used exclusively in this communication. Until recently little was known about the regulation and function of T6SS-2, termed T6SS-4 by Shalom et al. (2007), in the *B. pseudomallei* complex. This review summarizes recently published literature describing this novel contact-independent T6SS.

2. Metal acquisition by contact-independent T6SS helps combat reactive oxygen species (ROS)

ROS are a collection of partially reduced oxygen (O_2) species, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($OH^{\cdot}$), whose formation is unavoidable in an oxygen-rich environment (Dixon and Stockwell, 2014; Imlay, 2019). They are potentially toxic to cells due to their ability to damage DNA, proteins and lipids (Fig. 2). The formation of these species is facilitated by the reactivity of intracellular Fe^{2+} with H_2O_2 in the so-called Fenton reaction. A growing number of environmental stresses that promote the production of ROS in bacteria are also being identified (Fig. 2), including bactericidal antibiotics (Dwyer et al., 2015), peptidoglycan recognition proteins (PGRPs) (Kashyap et al., 2014) and lethal attacks by competing bacteria and bacteriophages (Dong et al., 2015).

Bacteria sense and respond to oxidative stress through regulatory proteins like OxyR, SoxR and SoxS (Chiang and Schellhorn, 2012; Seo et al., 2015). The genes under control of these transcription factors often include antioxidant enzymes, such as superoxide dismutase, catalase and alkyl hydroperoxide reductase, which catalyze the elimination of ROS and help maintain redox homeostasis (Staerck et al., 2017). Zinc (Zn^{2+}) and manganese (Mn^{2+}) transporters are also often under control of oxidative stress transcriptional regulators (Seo et al., 2015) and these metal ions play a role in oxidative stress protection by serving as cofactors or structural components of antioxidant enzymes and by forming low molecular weight antioxidant complexes (Aguirre and Culotta, 2012; Oteiza, 2012; Lisher and Giedroc, 2013). Low molecular weight Mn complexes, such as Mn-phosphate, Mn-citrate and Mn-carbonate, function to dismutate O_2^- and detoxify H_2O_2. A seminal work by the Shen laboratory in 2015 identified a contact-independent T6SS in *Yersinia pseudotuberculosis* that was integral for the acquisition of Zn^{2+} from the extracellular milieu during conditions of oxidative stress (Wang et al., 2015). The expression of *Y. pseudotuberculosis* T6SS-4 was induced by oxidative stress and was under the control of OxyR. Mutations in structural components of the T6SS-4 resulted in strains that accumulated high levels of ROS and were sensitive to H_2O_2-mediated killing, indicating that this secretion system contributes to oxidative stress resistance. Unexpectedly, T6SS-4 exported a Zn^{2+}-binding protein (YezP) that facilitated bacterial acquisition of this metal ion to mitigate potential damage due to hydroxyl radicals (Wang et al., 2015). While this was the first description of a contact-independent T6SS involved in metal ion uptake, a subsequent study identified a *Pseudomonas aeruginosa* T6SS-exported effector (TseF) that played a key role in the acquisition of iron from the surrounding environment (Lin et al., 2017). The discovery of "metal transporting T6SS" in *Yersinia* and *Pseudomonas* renewed interest in the previously uncharacterized T6SS-2 gene cluster in the *B. pseudomallei* complex (Si et al., 2017a, b; Duong et al., 2018; Losada et al., 2018).

3. Transcriptional regulation of the T6SS-2 gene cluster in the *B. pseudomallei* complex

The T6SS-2 gene cluster is conserved in all members of the *B. pseudomallei* complex (Table 1). Previous studies demonstrated that the T6SS-2 gene cluster in *B. pseudomallei* was optimally transcribed in minimal medium (Ooi et al., 2013) and poorly expressed in rich medium (Burtnick et al., 2011), but the regulatory factor(s) mediating the expression of this cluster were unknown. Si et al. (2017) found that a *B. thailandensis oxyR* mutant exhibited increased expression of the T6SS-2 gene cluster and enhanced resistance to ROS as compared to the wild-type parental strain. Similarly, the OxyR-controlled genes alkyl hydroperoxide reductase (*ahpC*) and catalase (*katG*) were also overexpressed in the Δ*oxyR* background (Si et al., 2017a). The T6SS-2 genes were derepressed in wild-type *B. thailandensis* when exposed to the oxidizing agent cumene hydroperoxide (CPH), indicating that this secretion system is "turned on" under conditions of oxidative stress. The T6SS-2 promoter region contains a putative OxyR binding site and His-tagged OxyR specifically bound to this sequence in electrophoretic mobility shift assays (EMSA) (Si et al., 2017a). This study was the first to demonstrate that the T6SS-2 genes are under direct negative control by the oxidative stress regulator OxyR. The results suggest that OxyR undergoes a conformational change during oxidative stress and can no longer bind tightly to the operator sequence of the T6SS-2 promoter and block transcription initiation by RNA polymerase σ^{70} (Fig. 2)

Zn^{2+} is an essential trace metal involved in many biological processes, but it can also be toxic and intracellular concentrations of this metal ion must be tightly regulated (Gilston et al., 2014). The *B. thailandensis* genome harbors five distinct T6SS, but only T6SS-2 is repressed by exogenously added 60 μM Zn^{2+} (Si et al., 2017b). The

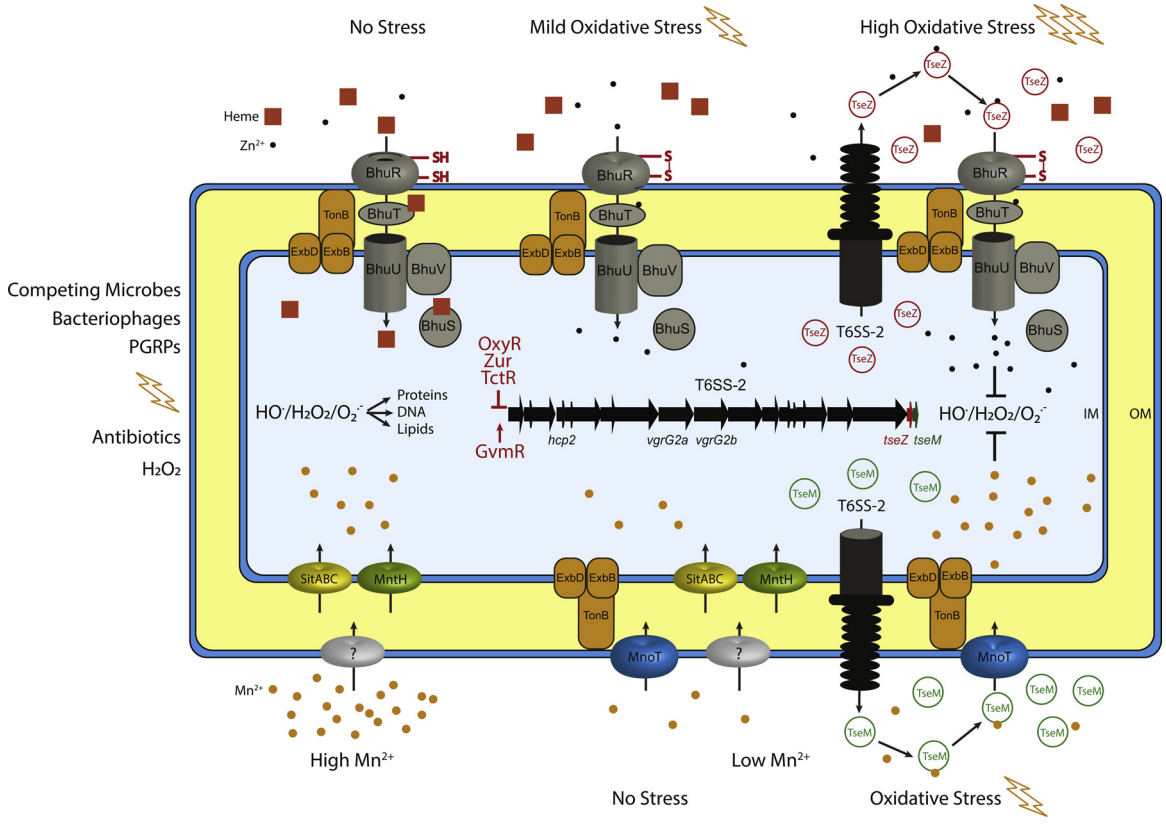

Fig. 2. Schematic representation of the regulation and function of the T6SS-2 gene cluster in the *B. pseudomallei* complex. A representative member of the *B. pseudomallei* complex is shown as a rectangle with thin blue outer (OM) and inner membranes (IM) and a yellow periplasmic space. The cytosol of the bacterium is light blue and the T6SS-2 genes are represented as black arrows oriented in the direction of transcription. The T6SS-2 structural apparatus is depicted as an outward-oriented black bacteriophage tail that spans the IM and the OM. The last two genes of the cluster are colored red (*tseZ*) and green (*tseM*) and they encode a T6SS-2-exported zincophore (red circle) and manganeseophore (green circle), respectively. The names of transcriptional regulators that repress (⊥) or activate (↑) the expression of the T6SS-2 gene cluster are shown upstream in red font. Heme is represented as a dark orange square, Zn^{2+} is a small black dot and Mn^{2+} is a solid yellow circle. The TonB system is shown in orange, the dual-function heme-Zn^{2+} BhuRSTUV transporter is dark gray, and proteins involved in Mn^{2+} transport are blue (MnoT), yellow (SitABC), green (MntH) and light gray (a putative constitutively-produced OM channel). The sulfhydryl groups of Cys^{692} and Cys^{697} (red font) on BhuR are linked by an intramolecular disulfide bond under conditions of oxidative stress (R-S-S-R), but not in its absence (-SH). A number of exogenous stimuli, including competing microbes, bacteriophages, PGRPs, antibiotics and H_2O_2, can lead to oxidative stress (orange lightning bolts) and the formation of cytosolic ROS ($HO^{\cdot}$, H_2O_2, and $O_2^{\cdot -}$) that can damage DNA, proteins and lipids. The Zn^{2+} and Mn^{2+} acquisition mediated by T6SS-2, TseZ, TseM, BhuR and MnoT under conditions of oxidative stress help to combat ROS (⊤/⊥) and prevent excessive protein, DNA and lipid damage.

Table 1
Structural, functional and regulatory genes associated with the T6SS-2 in members of the *B. pseudomallei* complex.

Gene(s)	#K96243	E264	ATCC 23,344	C6786	MSMB43	LMG 28,154
T6SS-2	BPSS0515-BPSS0532	BTH_II1902-BTH_II1885	*BMAA0438-BMAA0454	BG90_RS29925-BG90_RS29840	BW21_RS08010-	BSIN_RS04655-BSIN_RS04575
tseZ	BPSS0533	BTH_II1884	BMAA0455	BG90_RS29835	BW21_RS08100	BSIN_RS04570
tseM	BPSS0534	BTH_II1883	BMAA0456	BG90_RS29830	BW21_RS08105	BSIN_RS04565
oxyR	BPSL2866	BTH_I1281	BMA2390	BG90_RS10930	BW21_RS17685	BSIN_RS13845
zur	BPSL0825	BTH_I0691	BMA0329	BG90_RS03955	BW21_RS29135	BSIN_RS11670
tctR	BPSL3431	BTH_I3344	BMA2918	BG90_RS07825	BW21_RS14525	BSIN_RS22080
gvmR	BPSL0117	BTH_I0124	BMA0138	BG90_RS07300	BW21_RS13935	BSIN_RS05810
tonB	BPSS0368	BTH_II2024	BMAA1801	BG90_RS30680	BW21_RS02385	BSIN_RS17865
exbB	BPSS0367	BTH_II2025	BMAA1802	BG90_RS30685	BW21_RS02390	BSIN_RS17860
exbD	BPSS0366	BTH_II2026	BMAA1803	BG90_RS30690	BW21_RS02395	BSIN_RS17855
sitABC	BPSL0824-BPSL0822	BTH_I0690-BTH_I0688	BMA0328-BMA0326	BG90_RS03960-BG90_RS03970	BW21_RS29140-BW21_RS29150	BSIN_RS11665-BSIN_RS11655
bhuRSTUV	BPSS0244-BPSS0240	BTH_II2139-BTH_II2143	BMAA1826-BMAA1830	BG90_RS18860-BG90_RS18885	BW21_RS02975-BW21_RS02995	BSIN_RS22660-BSIN_RS22680
mntH	BPSL1554	BTH_I2687	BMA0860	BG90_RS16235	BW21_RS22910	BSIN_RS21425
mnoT	BPSL2553	BTH_I1598	BMA0477	BG90_RS12505	BW21_RS19285	BSIN_RS09770

B. pseudomallei K96243; *B. thailandensis* E264; *B. mallei* ATCC 23,344; *B. oklahomensis* C6786; *B. humptydooensis* MSMB43; *B. singularis* LMG 28,154.

* The first two genes of the T6SS-2 cluster are truncated in ATCC 23,344 by IS*407*A. *B. mallei* strains 2002721276, KC 1092, BMZ, Kweiyang #4, BMY, BMK and ATCC 10,399 also contain this mutation, but *B. mallei* strains 2002734299, A193, Strain 11, Ivan, Bahrain 1, BURK081, 102, Budapest, BMQ, 092700E, SR092700I, India 86-587-2, 2002721280, SAVP1, NCTC 10,247 and PRL-20 possess intact copies of the first two genes of the T6SS-2 cluster.

promoter region of T6SS-2 contains a predicted Zur box, a regulatory sequence often found upstream of genes involved in Zn^{2+} acquisition and utilization. The Zn^{2+}-binding protein Zur binds to the Zur box to repress transcription under conditions of excess Zn^{2+}, but transcription is derepressed when the intracellular Zn^{2+} concentration drops and can no longer serve as a co-repressor with Zur (Gilston et al., 2014). The Zur box and the OxyR-binding site both overlap the *B. thailandensis* T6SS-2 -10 promoter element and presumably prevent recognition by RNA polymerase σ^{70} (Si et al., 2017a, b). Thus, Zur serves to repress expression of T6SS-2 during nutrient replete conditions provided there is little or no oxidative stress (Fig. 2).

Losada et al. (2018) recently devised a genetic screen to identify a repressor of the *B. pseudomallei* T6SS-2 gene cluster in rich medium. The *hcp2* gene, encoding a critical T6SS-2 structural component, was replaced with a promoterless kanamycin resistance (Km^r) gene to serve as a selectable marker for expression of the T6SS-2 genes. This strain was mutagenized with a gentamicin resistance (Gm^r) transposon and 8 distinct Km^r Gm^r mutants were isolated that harbored transposons within a gene encoding a MarR family transcriptional regulator (Losada et al., 2018). This gene was termed *tctR* for type VI secretion system cluster two regulator. Inactivation of *tctR* resulted in a 50-fold increase in the expression of a *hcp2-lacZ* fusion and transcriptional profiling revealed that all but one of the T6SS-2 genes were upregulated in the Δ*tctR* background, indicating that TctR is a repressor of the T6SS-2 gene cluster (Fig. 2). Unlike OxyR and Zur, no TctR binding site was identified upstream of the T6SS-2 genes. This study also found that subinhibitory antibiotics and nutrient limitation activated expression of the T6SS-2 genes (Losada et al., 2018). Previous studies have demonstrated that bactericidal antibiotics inherently result in the formation of ROS that contribute to an antibiotic's lethality (Kohanski et al., 2007; Dwyer et al., 2014). As mentioned above, the T6SS-2 genes are activated by oxidative stress and the ROS generated by subinhibitory antibiotics are likely responsible for the increased gene expression. The increased expression of the *B. pseudomallei* T6SS-2 gene cluster under nutrient limited conditions (Ooi et al., 2013) was confirmed in the study by Losada et al. (2018) and may be due to relatively low Zn^{2+} concentrations in minimal medium resulting in Zur derepression.

A novel LysR type transcriptional regulator, GvmR, was recently described in *B. pseudomallei* that is involved in regulating genes involved in diverse virulence and metabolic processes, including protein secretion, amino acid synthesis, glyoxylate shunt, iron-sulfur cluster assembly, secondary metabolite biosynthesis, pyruvate metabolism, ATP synthesis, and porin synthesis (Duong et al., 2018). Transcriptional profiling experiments conducted with *B. pseudomallei* wild-type and Δ*gvmR* strains found 331 differentially regulated genes, with 141 genes downregulated and 190 genes upregulated in the Δ*gvmR* background. Eleven of the T6SS-2 genes were significantly downregulated in the Δ*gvmR* strain, suggesting that GvmR is a positive regulator of this gene cluster (Fig. 2). In addition, GvmR was required for optimal growth and expression of the T6SS-2 gene cluster under nutrient limited conditions (Duong et al., 2018).

4. Acquisition of Mn^{2+} under oxidative stress is mediated by T6SS-2, TseM and MnoT

Mn^{2+} is an essential micronutrient that possesses inherent antioxidant properties (Aguirre and Culotta, 2012; Lisher and Giedroc, 2013). Import of Mn^{2+} across the outer membrane (OM) has not been studied in the *B. pseudomallei* complex, but the inner membrane (IM) transporter MntH has been identified in *B. pseudomallei* (Shalom et al., 2007). MntH is a member of the natural resistance-associated macrophage protein (NRAMP) family and is a H^+-stimulated Mn^{2+} transporter (Kehres et al., 2000). The *B. pseudomallei mntH* gene was identified in an *in vivo* expression technology (IVET) screen as being induced inside of macrophages where it likely imports Mn^{2+} to combat ROS (Shalom et al., 2007). Homologues of the SitABCD Mn^{2+} transporter (Kehres et al., 2002) are conserved in the *B. pseudomallei* complex (Table 1), but the activity of this ATP-binding cassette (ABC) family IM transporter has yet to be examined (Fig. 2).

B. thailandensis T6SS-2 mutants exhibited survival rates that were significantly less than wild-type under a variety of oxidative stress conditions (H_2O_2, $CdCl_2$, diamide and CHP) and they accumulated elevated levels of intracellular ROS under these conditions (Si et al., 2017a). Exogenously added Mn^{2+} provided the wild-type strain with increased survival in the presence of CHP, but provided little benefit to a T6SS-2 mutant. In addition, the uptake of Mn^{2+} by a T6SS-2 mutant was significantly less than wild-type under oxidative stress conditions. These studies indicated that T6SS-2 was important for resistance to oxidative stress *via* the acquisition of Mn^{2+}. The protein encoded by the last gene of the T6SS-2 gene cluster, termed TseM for type VI secretion system effector for Mn^{2+} binding, was found to be a Mn^{2+}–binding protein exported by *B. thailandensis* in a T6SS-2-dependent manner (Si et al., 2017a).

In an attempt to identify a potential OM receptor for TseM, a glutathione-S-transferase (GST) pull-down assay was conducted using GST-TseM and a crude lysate of CHP-treated *B. thailandensis* (Si et al., 2017a). A putative TonB-dependent transporter (TBDT) was identified as a specific TseM binding partner in the assay. TBDTs are OM proteins that transport ferric chelates such as siderophores, transferrin, and heme, but they also transport vitamin B_{12}, nickel chelates, and some carbohydrates (Noinaj et al., 2010). A complex of three IM proteins, TonB, ExbB and ExbD, interact with TBDTs to transduce energy derived from the proton motive force to facilitate active transport of the substrates across the OM. The *B. thailandensis* TseM receptor was termed MnoT for Mn^{2+}–specific outer membrane transporter (Fig. 2). The paper by Si et al. (2017a) was the first to describe a TBDT that specifically transports Mn^{2+} across the OM. MnoT, TonB, ExbB and ExbD are all conserved in members of the *B. pseudomallei* complex (Table 1). The *mnoT* gene was repressed by OxyR and high levels of extracellular Mn^{2+}, but was activated under conditions of oxidative stress and in the presence of low concentrations of exogenous Mn^{2+}. Fig. 2 summarizes these experiments and shows that under conditions of oxidative stress and limited nutrient availability, T6SS-2 exports TseM in a contact independent manner and it specifically binds Mn^{2+} in the extracellular milieu. TseM then binds to MnoT which actively transports Mn^{2+} across the OM to the periplasmic space. The subsequent transport of Mn^{2+} across the IM is likely carried out by MntH and/or SitABC (Fig. 2). Cytosolic Mn^{2+} helps maintain redox homeostasis by serving as a cofactor for antioxidant enzymes, such as superoxide dismutase SodA and catalase KatN, and by forming low molecular weight Mn complexes that eliminate ROS nonenzymatically (Lisher and Giedroc, 2013).

5. Acquisition of Zn^{2+} under oxidative stress is mediated by T6SS-2, TseZ and BhuR

Zn^{2+} contributes to bacterial redox homeostasis by a variety of mechanisms, including serving a cofactor for Cu^{2+}/Zn^{2+} superoxide dismutase (Vanaporn et al., 2011; Oteiza, 2012). Passive import of metal ions across the OM can occur through nonspecific porins when extracellular concentrations are relatively high, but energy-dependent transporters with inherent specificity are required for import when the metal ion content in the extracellular milieu is low (Porcheron et al., 2013). The transport of Zn^{2+} has not been experimentally characterized in the *B. pseudomallei* complex. The *B. thailandensis* T6SS-2 gene cluster was repressed by Zur when intracellular Zn^{2+} concentrations were high, but was derepressed when Zn^{2+} levels were low (Si et al., 2017b). The gene immediately upstream of *tseM, tseZ* (Fig. 2), encodes a zinc-finger binding protein that is 39% identical to *Y. pseudotuberculosis* YezP (Wang et al., 2015). This protein, termed TseZ for type VI secretion system effector for Zn^{2+} binding, was shown to be a Zn^{2+}-binding effector exported by T6SS-2 in a contact-independent fashion under

conditions of low Zn^{2+} and oxidative stress (Si et al., 2017b). A *tseZ* mutant exhibited elevated levels of intracellular ROS and displayed a decreased survival rate as compared to wild-type when exposed to CHP (Si et al., 2017b). The CHP survival rate of a *tseZ* mutant could be rescued by exogenous recombinant TseZ, demonstrating that TseZ is a zincophore responsible for delivering antioxidant Zn^{2+} to *B. thailandensis* to combat ROS (Fig. 2).

GST-TseZ and a crude lysate of CHP-treated *B. thailandensis* were used in a GST pull-down assay to identify a putative TseZ receptor on the surface of *B. thailandensis* (Si et al., 2017b). TseZ specifically interacted with a TonB-dependent receptor, BhuR, exhibiting homology to the *Y. pestis* heme uptake system receptor HmuR (Fig. 2). The *Yersinia* HmuRSTUV system transports heme from the extracellular milieu to the bacterial cytoplasm by utilizing an OM TBDT (HmuR), a periplasmic heme-binding protein (HmuT), an IM ABC transporter (HmuUV) and a cytoplasmic protein that cleaves the protoporphyrin ring to release iron from heme (HmuS) (Thompson et al., 1999; Huang and Wilks, 2017; Onzuka et al., 2017). The *Burkholderia* heme uptake system (BhuRSTUV) was first identified in *B. pseudomallei* by employing IVET to find genes induced inside of host macrophages (Shalom et al., 2007). The *bhuT* gene was identified as being induced inside of RAW264.7 macrophages, but a Δ*bhuT* strain did not display a defect in intracellular macrophage survival and was able to utilize heme as an iron source. By comparison, Kvitko et al. (2012) showed that the *B. pseudomallei* BhuRSTUV system, referred to as HmuRSTUV, was essential for heme uptake (Kvitko et al., 2012). In the Shalom et al (2007) study, the Δ*bhuT* strain retained a functional BhuR protein for heme transport across the OM, but an alternative system may exist for transport across the CM. A *B. thailandensis* Δ*bhuR* mutant also displayed a severe growth defect on plates containing heme as the sole iron source (Si et al., 2017b). The BhuRSTUV system is conserved in the *B. pseudomallei* complex (Table 1) and a consensus Fur binding site overlaps the putative *bhuR* promoter and the *bhu* operon is induced by low iron (Shalom et al., 2007; Si et al., 2017b). Taken together, these studies suggest that the BhuRSTUV system in the *B. pseudomallei* complex is involved in heme acquisition in niches containing limited iron and little or no oxidative stress (Fig. 2).

The direct interaction of TseZ with BhuR suggested that the BhuRSTUV system might also be involved in the acquisition of Zn^{2+}. Si et al. (2017b) found that a *B. thailandensis* Δ*bhuR* mutant was severely impaired in Zn^{2+} uptake under conditions of oxidative stress, but not under normal conditions. In addition, the Δ*bhuR* mutant was more sensitive to CHP-mediated killing than wild-type. The expression of *bhuR* was repressed by high levels of Zn^{2+} and induced by CHP, further supporting a role for the BhuRSTUV system in acquisition of Zn^{2+} and resistance to oxidative stress. The *B. thailandensis* BhuR protein contained two surface-exposed cysteine residues, Cys^{692} and Cys^{697}, that were linked by an intramolecular disulfide bond in the presence of CHP, but not in its absence (Si et al., 2017b). The results indicated that BhuR acts as a redox sensor, and a dual transporter, that specifically transports heme under conditions of low oxidative stress and Zn^{2+} under conditions of high oxidative stress (Fig. 2). The formation of the disulfide bond in BhuR during oxidative stress results in a conformational change that alters substrate specificity and allows binding to the T6SS-2 effector TseZ. The subsequent transport of Zn^{2+} chelated by TseZ into the cell helps promote survival by reducing intracellular levels of ROS (Fig. 2).

5.1. T6SS-2 provides a survival advantage in interbacterial competition assays and a virulence benefit in Galleria mellonella larvae

The importance of metal acquisition by T6SS-2 in environmental survival and virulence was assessed using interbacterial competition assays, the *G. mellonella* infection model and rodent models of infection (Schwarz et al., 2010; Burtnick et al., 2011; Si et al., 2017a, b). Interbacterial competition assays were conducted by co-culturing *B. thailandensis* wild-type and mutant strains with *Escherichia coli* in manganese–limited M9 liquid medium containing CHP. Wild-type *B. thailandensis* displayed a competitive advantage over *E. coli*, but the advantage was eliminated in *B. thailandensis* strains harboring ΔT6SS-2, Δ*tseM* and Δm*noT* mutations (Si et al., 2017a). Expression of *mnoT* in *E. coli* allowed intracellular accumulation of Mn^{2+} when provided with exogenous TseM and it increased the competitive ability against wild-type *B. thailandensis*. Competition assays were also conducted using *B. thailandensis* wild-type and mutant strains co-cultured with *E. coli*, *Pantoea alhagi* or *Staphylococcus aureus* in zinc-limited M9 liquid medium supplemented with CHP. Wild-type *B. thailandensis* exhibited a competitive advantage against all three bacterial species, but the competitive edge was lost in *B. thailandensis* ΔT6SS-2, Δ*tseZ* and Δ*bhuR* mutants (Si et al., 2017b). The *bhuR* gene was expressed in *E. coli* and *P. alhagi* and it promoted the intracellular accumulation of Zn^{2+} in the presence of exogenously-added TseZ and increased the competitive fitness of both species against wild-type *B. thailandensis*. The competitive assays were performed in broth culture and demonstrated that the competitive advantage afforded by T6SS-2 was mediated in a contact-independent manner rather than a contact-dependent manner that is characteristic of many bacterial T6SS (Alteri and Mobley, 2016). The results suggest that the metal transporting activity of T6SS-2 in members of the *B. pseudomallei* complex may play a critical contact-independent role in survival in polymicrobial communities with limited nutrient availability (Fig. 2).

Larvae of the greater wax moth *Galleria mellonella* represent an alternative model of infection and they have been used to identify pathogen virulence factors and study the innate immune response (Wand et al., 2011; Ramarao et al., 2012). *B. thailandensis* wild-type and ΔT6SS-2, Δ*tseM* and Δm*noT* mutants were used to infect *G. mellonella* larvae and greater than 70% of the larvae infected with the wild-type strain died, but less than 30% of the larvae infected with the mutants died (Si et al., 2017a). Similarly, less than 35% of the larvae infected with the *B. thailandensis* Δ*tseZ* and Δ*bhuR* mutants died (Si et al., 2017b). These studies indicate that Mn^{2+} and Zn^{2+} acquisition by T6SS-2 is important for virulence in the wax moth larvae model of infection.

Mammals employ “nutritional immunity” against invading bacterial pathogens by sequestering essential transition metals required for growth and survival (Hood et al., 2012; Damo et al., 2013). Calprotectin (CP) is a calcium-activated antimicrobial peptide that is released by neutrophils and functions by sequestering Mn^{2+} and Zn^{2+} from pathogenic bacteria *in vivo*. Bacteria may overcome CP sequestration by exporting specific Mn^{2+} and Zn^{2+} chelators with high affinity for these metals (Hood et al., 2012; Liu et al., 2012). Previous animal studies revealed that elevated levels of CP are present in the sera of nonhuman primates infected with *B. pseudomallei* and *B. mallei* (Glaros et al., 2015; Natesan et al., 2017). The export of TseM and TseZ by T6SS-2 might be expected to counter the CP-mediated chelation of Mn^{2+} and Zn^{2+} (Fig. 2). Surprisingly, there was no significant difference in the ability of *B. pseudomallei* wild-type and a T6SS-2 mutant to cause disease in the Syrian hamster model of melioidosis (Burtnick et al., 2011). Similarly, there was no difference in the virulence of *B. thailandensis* wild-type and a T6SS-2 mutant in the mouse model of lethal aerosol infection (Schwarz et al., 2010). The results indicate that T6SS-2 is important for virulence in an insect model of infection, but plays little or no role in pathogenesis in mammalian models of infection. By comparison, T6SS-1 is critical for virulence in insect and mammalian models of infection (Burtnick et al., 2010, 2011; Fisher et al., 2012).

6. Conclusion and future perspective

Up until recently, T6SS were described as complex nanomachines that resemble inverted contractile bacteriophage tails and function by injecting effector molecules into eukaryotic host cells or bacterial competitors in a contact-dependent manner (Alteri and Mobley, 2016). Recent reports, including those reviewed here, indicate that T6SS can

export metal chelators into the extracellular milieu in a contact-independent manner and acquire transition metals for combating ROS or for metabolic purposes (Wang et al., 2015; Lin et al., 2017; Si et al., 2017a, =b). Future studies will undoubtedly reveal additional novel T6SS effectors that promote survival in complex environmental and host niches.

T6SS-2 is conserved in the *B. pseudomallei* complex where it serves to export TseZ and TseM to the extracellular milieu in a contact-independent manner. The acquisition of Zn^{2+} and Mn^{2+} by these chelators is mediated by the OM TBDTs BhuR and MnoT. The uptake of antioxidant metals provide members of the *B. pseudomallei* complex with protection against damage to DNA, proteins and lipids caused by toxic ROS. The regulation of the T6SS-2 gene cluster is complex and involves multiple repressors (OxyR, Zur and TctR) and an activator (GvmR). These transcriptional regulators sense nutrient availability and oxidative stress and respond by increasing expression of the T6SS-2 gene cluster (Fig. 2). Activation of the T6SS-2 promotes intracellular redox homeostasis, contact-independent survival against bacterial competitors and virulence in *G. mellonella* larvae (Si et al., 2017a,b).

B. pseudomallei possesses six distinct T6SS gene clusters (Schell et al., 2007; Shalom et al., 2007), but the functional activities of T6SS-3, T6SS-4 and T6SS-5 remain uncharacterized. Similar to T6SS-2, the T6SS-4 gene cluster was activated by oxidative stress conditions (Jitprasutwit et al., 2014). Transcription of the T6SS-2, T6SS-3 and T6SS-4 gene clusters were also up-regulated by subinhibitory antibiotics (Losada et al., 2018), compounds known to promote the formation of toxic ROS (Kohanski et al., 2007; Dwyer et al., 2014). These results suggest that the T6SS-3 and T6SS-4 clusters may play a role in resistance to oxidative stress, but further studies are necessary to understand the regulatory circuits that control their expression and characterize their secreted effector molecules.

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