

Characterization of secreted sphingosine-1-phosphate (S1P) lyases required for virulence and intracellular survival of *Burkholderia pseudomallei*.

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Summary

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, plays a critical role in the orchestration of immune responses. S1P levels within the mammalian host are tightly regulated, in part through the activity of S1P lyase (S1PL) which catalyses its irreversible degradation. Herein we describe the identification and characterization of secreted S1PL orthologues encoded by the facultative intracellular bacteria *Burkholderia pseudomallei* and *Burkholderia thailandensis*. These bacterial orthologues exhibited S1PL enzymatic activity, functionally complemented an S1PL-deficient yeast strain, and conferred resistance to the antimicrobial sphingolipid *D-erythro*-sphingosine. We report that secretion of these bacterial S1PLs is pH-dependent, and is observed during intracellular infection. S1PL-deficient mutants displayed impaired intracellular replication in murine macrophages (associated with an inability to evade the maturing phagosome) and were significantly attenuated in murine and larval infection models. Furthermore, treatment of *Burkholderia*-infected macrophages with either S1P or a selective agonist of S1P receptor 1 enhanced bacterial colocalisation with LAMP-1 and reduced their intracellular survival. In summary, our studies confirm bacterial-encoded S1PL as a critical virulence determinant of *B. pseudomallei* and *B. thailandensis*, further highlighting the pivotal role of S1P in host-pathogen interactions. In addition, our data suggest that S1P pathway modulators have potential for the treatment of intracellular infection.

Introduction

Intracellular bacterial infections, including those caused by *Mycobacterium tuberculosis*, *Salmonella typhi* and *Burkholderia pseudomallei*, pose a major global health burden affecting tens of millions of people annually. The intracellular location provides protection against immune defences and antimicrobial therapy, with the result that such infections are often difficult to eradicate, and are frequently persistent or recurrent in nature. New therapeutic strategies are urgently required, the rational design of which demands a full appreciation of the mechanisms employed by intracellular bacteria to subvert the host cells in which they reside. Herein we focus on one such subversion strategy – the targeting of the host sphingosine-1-phosphate (S1P) axis.

S1P is a bioactive sphingolipid metabolite implicated in numerous biological processes (Alvarez *et al.*, 2007). In response to diverse stimuli, plasma membrane sphingolipids (e.g. sphingomyelin) can be readily metabolized to ceramide, and subsequently to sphingosine. Two sphingosine kinases (SphKs) phosphorylate sphingosine to generate S1P, levels of which are tightly regulated by balancing SphK activity with the activity of enzymes responsible for S1P dephosphorylation (S1P phosphatases; S1PP) or irreversible S1P degradation (S1P lyase; S1PL). The S1P pathway has been described as a “cellular rheostat that determines cell survival or death” (Alvarez *et al.*, 2007), as whilst S1P promotes cell survival, its precursors (ceramide and sphingosine) and one of its degradation products (hexadecenal) promote apoptosis (Kumar *et al.*, 2011). Moreover, the role of S1P extends beyond the regulation of cell survival. S1P activity has been extensively studied in the context of immune and inflammatory responses, where it plays roles in cellular differentiation and polarization, vascular integrity, trafficking and activity of immune cells, and cytokine/chemokine profiles (for review, see Spiegel and Milstien, 2011; Weigert *et al.*, 2009). S1P exerts such pleiotropic effects as it can function as an intracellular second

messenger as well as acting in an autocrine and/or paracrine manner to activate five distinct G protein-coupled cell surface receptors (S1PR₁₋₅) (Alvarez *et al.*, 2007).

In the context of the innate immune response, S1P is a critical regulator of macrophage function, influencing phagosome-lysosome fusion by triggering the mobilisation of intracellular Ca²⁺ (Blom *et al.*, 2005). Intracellular pathogens that preferentially target macrophages employ diverse strategies to circumvent their bactericidal activity, and disruption of S1P-mediated phagosome maturation is one such strategy. This is exemplified by *M. tuberculosis*, which inhibits the activation and phagosomal translocation of SphK, thus blocking the S1P-dependent increase in cytosolic Ca²⁺ and impairing phagosome maturation (Malik *et al.*, 2003; Thompson *et al.*, 2005). Similarly, *Shigella flexneri* has recently been reported to suppress the host inflammatory response by lowering S1P levels through the two branches of the S1P pathway; promoting the transcriptional upregulation of host genes encoding the S1P lyase and phosphatase enzymes and the parallel downregulation of SphK-encoding genes (Kim *et al.*, 2014). The molecular events that underlie SphK inhibition by *M. tuberculosis* and the host S1P pathway modulation by *Shigella* remain to be defined.

To date, the only documented bacterial effector proven to target the host S1P pathway is an S1PL orthologue (LegS2) of *Legionella pneumophila* that is secreted by the Dot/Icm Type IV secretion system (Degtyar *et al.*, 2009). This *Legionella* S1PL orthologue contributes to the restriction of *L. pneumophila* and the restraining of autophagy within infected macrophages, and also contributes to virulence within a murine pulmonary infection model (Abu Khweek *et al.*, 2016; Rolando *et al.*, 2016). In the course of our studies, we observed that *Burkholderia pseudomallei* and *Burkholderia thailandensis*, two closely-related facultative intracellular bacterial species, each encode two putative S1PL orthologues that are phylogenetically distinct from LegS2 of *L. pneumophila*. *B. pseudomallei* is the causative agent of melioidosis, an endemic disease in Southeast Asia and Northern Australia. Recent estimates put the global

burden of melioidosis at 165,000 cases per year worldwide, with mortality rates of up to 50 % (Limmathurotsakul *et al.*, 2016). Central to the pathogenesis of *B. pseudomallei* is its ability to invade, survive and replicate in both phagocytic and non-phagocytic cells (Jones *et al.*, 1996). Whilst *B. thailandensis* is highly attenuated relative to *B. pseudomallei*, it displays a similar intracellular phenotype characterized by endosomal escape, intracellular replication and actin-based motility (Wand *et al.*, 2011), and as such is a valuable model for studying the intracellular lifestyle of *B. pseudomallei*.

Herein we report the characterization of the *Burkholderia* S1PL orthologues, demonstrating that they indeed possess S1PL activity and that they play a critical role in virulence that is distinct from that of LegS2 of *Legionella*. In doing so, our studies provide new insight into intracellular survival strategies employed by *B. pseudomallei* and further highlight the critical role of the S1P axis at the host-pathogen interface. In addition, through treatment of *Burkholderia*-infected macrophages with S1P receptor agonists, we demonstrate that specific modulators of the S1PR₁-mediated pathway may represent a therapeutic strategy for the treatment of intracellular infections.

Results

Identification of Burkholderia-encoded sphingosine-1-phosphate lyases (S1PL)

In the course of studies investigating bacterial enzymes involved in sphingolipid biosynthesis and metabolism (Raman *et al.*, 2010), we identified putative S1PL-encoding genes within the genomes of *B. pseudomallei* and *B. thailandensis*. Using the *Burkholderia* genome database (Winsor *et al.*, 2008) and *B. pseudomallei* K96243 and *B. thailandensis* E264 as representative strains, we identified two putative S1PLs in each species – encoded by *BPSS2021* and *BPSS2025* in *B. pseudomallei* K96243, and *BTH_II0309* and *BTH_II0311* in

B. thailandensis E264. Within each organism, genes encoding the two putative S1PLs are not co-located within an operon but are located within the same genomic region, with a high degree of synteny between the corresponding regions of each species (Fig. 1). The S1PL-encoding genes of *B. thailandensis* and *B. pseudomallei*, and the corresponding proteins, show 84-92 % identity both within and between species. Furthermore, the *Burkholderia* proteins show approximately 37-39 % amino acid identity to the well-characterized human and yeast S1PL orthologues. Notably, all amino acids reported to be essential for the activity of the yeast S1PL orthologue (Dpl1) (Mukhopadhyay *et al.*, 2008) are conserved in the *Burkholderia* S1PLs (Fig. S1). The *Burkholderia* proteins show only 32 % amino acid identity to the S1PL orthologue of *L. pneumophila* (LegS2), and the S1PL phylogenetic tree depicted in Fig. S2 emphasizes the diversity amongst S1PLs of prokaryotic and eukaryotic origin. Whilst LegS2 of *L. pneumophila* branches with eukaryotic S1PLs, consistent with its proposed protozoan origin (Degtyar *et al.*, 2009), the S1PLs of *B. pseudomallei* and *B. thailandensis* branch with those of *Symbiobacterium*, *Myxococcus* and *Stigmatella* (with which they exhibit approximately 45 % amino acid identity).

The two S1PL-encoding genes are conserved across all available *B. pseudomallei* and *B. thailandensis* genomes (Winsor *et al.*, 2008), but are absent from the closely-related *Burkholderia mallei*. Within annotated *B. pseudomallei* and *B. thailandensis* genomes, there is inconsistent annotation of the S1PLs with regards the predicted position of the N-terminus (Fig. S3). S1PLs are pyridoxal 5'-phosphate (PLP)-dependent enzymes, and our initial studies of recombinant *Burkholderia* S1PLs revealed that the proteins only bound the PLP co-factor when cloned from the conserved MDLEEG sequence depicted in Fig. S3 (data not shown). Consequently, we adopted that conserved sequence as the N-terminus for all the studies described herein. Using that N-terminus, each S1PL-encoding gene of *Burkholderia* is predicted to encode a protein of approximately 52 kDa. However, the recombinant

Burkholderia S1PLs purify as homodimers when expressed in *E. coli* (data not shown), and native PAGE analysis of *B. thailandensis* expressing His-tagged BTH_II0309 or BTH_II0311 reveals the presence of oligomeric complexes (Fig. S4). This is consistent with the oligomeric nature of the yeast S1PL orthologue (Dpl1), which occurs as a dimer or multiple of dimers (Mukhopadhyay *et al.*, 2008).

The S1PL activity of the recombinant *Burkholderia* proteins was confirmed by measuring the formation of (2E)-hexadecenal from S1P by electrospray mass spectrometry (ESI-MS), based on a method from Berdyshev *et al.* (2011). The S1PL activity of the *Burkholderia* orthologues (exemplified by recombinant BTH_II0309) is demonstrated in Fig. 2, where BTH_II0309 catalysed the degradation of S1P with the resulting formation of hexadecenal. In addition, full biochemical characterization of the recombinant *B. pseudomallei* S1PLs was performed, confirming that both BPSS2021 and BPSS2025 are active and display similar K_M values. These biochemical studies, together with the determination of the crystal structure of PLP-bound BPSS2021, will be described elsewhere (McLean *et al.*, Unpublished data).

Having confirmed the lyase activity of the *Burkholderia* S1PLs, we sought to determine whether they were functionally equivalent to their well-characterized yeast orthologues. The S1PL-deficient mutant of *Saccharomyces cerevisiae* ($\Delta dpl1$) is hypersensitive to D-erythro-sphingosine (Saba *et al.*, 1997), so we assessed whether the *Burkholderia* orthologues could restore D-erythro-sphingosine resistance to a $\Delta dpl1$ mutant. As shown in Fig. 3, resistance was partially restored by BTH_II0309 or BTH_II0311 individually, and fully restored by co-expression of both. In addition to confirming that the bacterial S1PLs are functionally equivalent to eukaryotic orthologues, these observations also suggest that optimal S1PL enzymatic activity requires both *Burkholderia* proteins. Analogous to the phenotype of the $\Delta dpl1$ *S. cerevisiae* mutant, S1PL-deficient *B. thailandensis* mutants (described below) also

displayed hypersensitivity to D-erythro-sphingosine, a phenotype that was fully restored by complementation (Fig. S5).

Secretion of B. thailandensis S1PLs is pH-dependent, and is observed during intracellular infection

Any role for the *Burkholderia* S1PLs in virulence and/or immune evasion would be dependent on their secretion. *B. thailandensis* strains constitutively expressing FLAG-tagged BTH_II0309 or BTH_II0311 were constructed, enabling detection of FLAG-tagged proteins in cell pellets and culture supernatants by anti-FLAG immunoblotting. When cultured in LB adjusted to pH 4.5 or pH 7, the FLAG-tagged S1PLs were detected in whole-cell lysates regardless of the pH (Fig. 4A). In contrast, the S1PLs were only detected in the culture supernatants when cultured at pH 4.5. To exclude the possibility of S1PL secretion being an artefact arising from their constitutive overexpression whilst under acid-related stress, we confirmed that a non-secreted protein (GFP) when expressed in *B. thailandensis* under the same conditions was not detectable in culture supernatant by Western blot analysis (Fig. S6). Together, these results support the pH-dependent secretion of the *Burkholderia* S1PLs.

A critical virulence factor of *B. pseudomallei* is the Bsa Type III secretion system that facilitates escape from endocytic vacuoles and intracellular survival (Stevens *et al.*, 2002). In *B. thailandensis*, the activity of the orthologous system is stimulated by acidic pH (Jitprasutwit *et al.*, 2010). To assess whether pH-dependent secretion of the *B. thailandensis* S1PLs is facilitated by the Bsa system, we inactivated *bsaZ* (BTH_II0839), a structural component of the Bsa secretion apparatus (Stevens *et al.*, 2002). We did not detect secretion of the known T3SS effector BopE when the resulting Bsa-deficient mutant was cultured at acidic pH, confirming that the Bsa system was functionally inactive (Fig. S7A). In contrast,

we still observed pH-dependent secretion of the *B. thailandensis* S1PLs (Fig. S7B), indicating that their secretion is independent of the Bsa system.

In an attempt to define the intracellular localisation of secreted *Burkholderia* S1PLs, we infected macrophages with the FLAG-tagged S1PL-expressing strains described above and assessed the localisation of the FLAG-tagged proteins 2 hours post infection (hpi). Although the strains constitutively express the S1PLs, different patterns of anti-FLAG reactivity were evident (Fig 4B), consistent with their secretion being influenced by environmental stimuli. In some infected macrophages, the FLAG-tagged S1PLs were entirely restricted to the bacterial cells themselves, indicative of non-secretion. In contrast, other infected macrophages exhibited diffuse anti-FLAG reactivity indicative of S1PL secretion. Attempts to define the localisation of secreted S1PLs within infected macrophages were inconclusive, as the proteins showed only limited colocalisation with each of the cellular compartments studied (ER, mitochondria, and cytoplasm; Fig. S8).

Burkholderia-encoded S1PLs are required for intracellular survival

To assess the role of the *Burkholderia*-encoded S1PLs in virulence, we created S1PL-deficient mutants of both *B. thailandensis* E264 and *B. pseudomallei* K96243. The majority of our studies used *B. thailandensis* as our model organism. Owing to the genetic intractability of *B. thailandensis*, it was not possible to construct double mutants lacking both *BTH_II0309* and *BTH_II0311*, thus single mutants were constructed by insertional inactivation. Selected experiments were also performed with *B. pseudomallei* in order to verify the *B. thailandensis*-based observations. In addition, as *B. pseudomallei* is more genetically-tractable, this enabled generation of a double mutant through the deletion and/or inactivation of both *BPSS2021* and *BPSS2025*. *B. thailandensis* and *B. pseudomallei* mutants

were validated by PCR analysis, with the *B. pseudomallei* mutants further validated by whole genome sequencing.

Given the importance of intracellular survival to *B. pseudomallei* pathogenesis and the documented role of S1P in macrophage activity and phagosome maturation, we focused on defining the contribution of the *Burkholderia* S1PLs to intracellular survival and replication. Murine macrophages were infected with wildtype or S1PL-deficient *B. thailandensis* or *B. pseudomallei* strains and intracellular replication was assessed over 6 hours. Both wildtype *B. thailandensis* and wildtype *B. pseudomallei* exhibited significant intracellular replication over this period (Fig. 5A & 5B respectively). In contrast, the corresponding S1PL-deficient mutants showed no apparent replication, with the number of viable bacteria significantly reduced relative to the respective wildtype strains by 6 hpi (Fig. 5A & 5B). Complementation of the single *BPSS2025* mutant partially restored intracellular survival (Fig. S9). Notably, the single and double mutants of *B. pseudomallei* displayed a comparable phenotype (Fig. 5B).

To gain insight into the mechanisms responsible for the impaired intracellular replication of the S1PL-deficient mutants, we assessed the localisation of RFP-expressing *B. thailandensis* strains (wildtype and S1PL-deficient mutants) with the lysosomal marker LAMP-1 at the same timepoints. LAMP-1, which is required for fusion of lysosomes with phagosomes (Huynh *et al.*, 2007), is recruited during the late stage of phagosome maturation. Colocalisation of bacteria with LAMP-1 therefore indicates bacteria that have failed to escape from the maturing phagosome. The impaired intracellular replication of the S1PL-deficient mutants was associated with their increased localisation with LAMP-1, as demonstrated by representative micrographs (Fig. 5C) and quantitative analysis of LAMP-1 colocalisation (Fig. 5D). Collectively, these results indicate that the *Burkholderia* S1PLs play a role in intracellular replication and promote the avoidance of the maturing phagosome.

Burkholderia S1PLs are required for virulence in Galleria and murine models of infection.

Initially, the role of the S1PLs in virulence was assessed using the *B. thailandensis* strains in the *Galleria mellonella* model of infection. Due to the high degree of functional homology between the innate immune system of insects and mammals, numerous studies have successfully applied the *Galleria* infection model to assess bacterial virulence. With particular relevance to the studies described herein, the *Galleria* model has been validated for several *Burkholderia* species, including *B. thailandensis* (Wand *et al.*, 2011).

Larvae were challenged with approximately 25 CFU of the relevant *B. thailandensis* strains and larval survival assessed at 40 hpi. For each independent experiment, multiple inoculations were performed for each strain and exact CFU counts determined for each inoculum to ensure comparisons between strains were robust. As shown in Fig. 6A, inactivation of the S1PL-encoding genes in *B. thailandensis* was associated with significant virulence attenuation, with infected larvae showing increased survival relative to larvae inoculated with wildtype *B. thailandensis*. Virulence of the individual mutants was fully restored by complementation (Fig. 6B & 6C). When studying the complemented strains alongside the mutants, the *BTH_II0309* and *BTH_II0311* strains were studied independently from each other, so as to accommodate the multiple inoculations per strain. This explains the differing larval survival rates observed when comparing Fig. 6A-C, as survival is influenced by minor variations in inoculum (± 20 CFU) and batch-to-batch variation in *Galleria*. When studied alongside each other, there is no difference in the virulence of the two mutants, with both showing comparable attenuation (Fig. 6A).

On the basis of the virulence attenuation observed in the *Galleria* model coupled with the failure of S1PL-deficient *Burkholderia* to replicate within murine macrophages, we assessed the virulence of the *B. pseudomallei* Δ BPSS2025 mutant in a murine infection model. Balb/c

mice were inoculated with *B. pseudomallei* via the intraperitoneal route and survival was monitored for five weeks. Mice infected with 3.8×10^4 CFU of wildtype *B. pseudomallei* all died within 17 days (median survival 12 days). In contrast, mice challenged with a slightly higher inoculum of $\Delta BPSS2025$ (5.6×10^4 CFU) exhibited 80 % survival at the end of the 5 week study period (Fig. 7; $p = 0.0064$). Determined LD₅₀ values for *B. pseudomallei* wildtype and $\Delta BPSS2025$ were < 3790 CFU and 170,500 CFU respectively, confirming the significant attenuation of the S1PL-deficient mutant. Complementation of the $\Delta BPSS2025$ mutant failed to restore virulence in the murine model (determined LD₅₀ = 178,668 CFU). However, plating of tissue homogenates from mice chronically-infected with the complemented strain revealed that all recovered bacteria were fully sensitive to tetracycline, suggesting that the lack of complementation is a consequence of plasmid instability during infection.

Exogenous S1P receptor agonists enhance the bactericidal activity of Burkholderia-infected macrophages

Given the clear role of the *B. thailandensis* and *B. pseudomallei* S1PLs in promoting virulence and intracellular survival, we sought to explore whether treatment of infected macrophages with S1P or an appropriate S1PR agonist could circumvent the activity of the secreted bacterial S1PLs, promoting the activity of the S1P pathway and thus aiding bacterial killing. The expression patterns of the five S1PRs that mediate the extracellular activities of S1P vary across different cells & tissues. Murine macrophages reportedly express just S1PR₁ and S1PR₂ (Hughes *et al.*, 2008), and whilst there are no reported mono-selective S1PR₂ agonists, several S1PR₁-selective agonists have been described. Therefore, in the present study, we chose to assess the impact of S1P pathway modulation on the bactericidal activity

of infected macrophages using both S1P (the natural agonist of both S1PR₁ and S1PR₂) and the S1PR₁-selective agonist, CYM-5442 (Gonzalez-Cabrera *et al.*, 2008).

Murine macrophages were infected with wildtype *B. thailandensis* or *B. pseudomallei* (MOI 10) for two hours prior to treatment with S1P or CYM-5442 (neither of which possess direct antimicrobial activity; data not shown), and the intracellular survival of bacteria was assessed 2 and 6 hours post-treatment. Following 6 hours treatment, S1P and CYM-5442 both elicited a comparable reduction in bacterial survival within *B. thailandensis*- and *B. pseudomallei*-infected macrophages (Fig. 8A & 8B respectively). No significant reduction was observed 2 hours post treatment (data not shown). This reduction in viable intracellular bacteria following S1P/CYM-5442 treatment was associated with an increase in bacterial colocalisation with LAMP-1 (Fig. 8C & 8D), consistent with the attenuated intracellular phenotype of the S1PL-deficient mutants. Based on these results, we conclude that S1P pathway modulation is an attractive therapeutic strategy for the treatment of intracellular bacterial infections, with beneficial bactericidal activity attributable to the specific activation of the S1PR₁ pathway.

Discussion

Our studies described herein highlight a previously-unrecognised strategy employed by *B. pseudomallei* to subvert host cell function and promote intracellular survival and virulence. Together with previous reports focusing on *M. tuberculosis* (Malik *et al.*, 2003; Thompson *et al.*, 2005), *S. flexneri* (Kim *et al.*, 2014) and *L. pneumophila* (Abu Khweek *et al.*, 2016; Degtyar *et al.*, 2009; Rolando *et al.*, 2016), our studies further highlight the host S1P axis as an emerging field in bacterial pathogenesis and host-microbe interactions, with bacteria employing different strategies to subvert the S1P pathway. In the context of macrophage activity, S1P can influence endosome-endosome and phagosome-lysosome fusion via distinct

mechanisms, including mobilisation of intracellular calcium (Blom *et al.*, 2005) and the stimulation of actin assembly (Anes *et al.*, 2003; Kuehnel *et al.*, 2009). Data presented herein highlight the role of the *Burkholderia*-encoded S1PLs in virulence and intracellular survival, the latter attributable to their role in promoting the evasion of the maturing phagosome. It remains to be established whether this S1PL-mediated phagosome avoidance arises through an actual delay to S1P-mediated phagosome maturation via disruption of host cell S1P levels, or through promoting bacterial escape from the phagocytic vacuole via an undetermined mechanism. Regardless of the mechanism(s) involved, we propose the secreted S1PLs act in concert with documented *Burkholderia* intracellular survival strategies, including the Bsa Type III secretion system that is known to facilitate escape from endocytic vacuoles (Stevens *et al.*, 2002). Whilst our data suggest that the *Burkholderia* S1PLs are not effectors of the Bsa system, S1PL secretion is similarly regulated in a pH-dependent manner and can be observed during macrophage infection.

Strikingly, the *Burkholderia*-encoded S1PLs described herein appear phylogenetically distinct from the *Legionella* orthologue, which to date is the only other prokaryotic S1PL to be implicated in pathogenesis (Abu Khweek *et al.*, 2016; Rolando *et al.*, 2016). Furthermore, whilst the S1PLs of *B. thailandensis* and *B. pseudomallei* are required for intracellular replication, the S1PL of *Legionella* contributes to the restriction of intracellular growth, with S1PL-deficient *L. pneumophila* mutants showing enhanced intracellular replication (Abu Khweek *et al.*, 2016; Rolando *et al.*, 2016). Although the molecular mechanisms underlying these contrasting intracellular phenotypes remain to be elucidated, they may reflect the differing intracellular lifestyles of these organisms, as whilst *B. pseudomallei* and *B. thailandensis* both escape from endosomes and replicate freely within the cytosol, *L. pneumophila* intracellular replication occurs within the confines of the *Legionella*-containing vacuole (LCV). Further comparative studies are required to ascertain how these

Burkholderia- and *Legionella*-encoded S1PLs elicit their differing effects, taking into account the timing of their secretion, their targeting within infected host cells and their interplay with other bacterial effectors. As highlighted previously, S1P acts as both an intracellular second messenger and as an autocrine/paracrine ligand for five distinct S1P receptors (S1PRs). Consequently, when considering the precise mechanisms of the secreted S1PLs of *Legionella* and *Burkholderia* species during intracellular infection, there exists both S1PR-dependent and S1PR-independent scenarios to explain their biological activity. Depletion of the intracellular S1P pool by the bacterial lyases may impair both the intracellular activity of S1P and the autocrine signalling via S1PRs (where S1P synthesized within the cell is exported to activate its own receptors). Notably, and with particular relevance to the present study, both intracellular and extracellular signalling pathways have been shown to converge on calcium mobilisation from the ER (Li *et al.*, 2015; Ghosh *et al.*, 1994; Ghosh *et al.*, 1990), a process that underpins phagosome maturation. This convergence of S1PR-dependent and independent signalling pathways on calcium mobilisation likely explains the concordance of phenotypes observed in the present study, where macrophages infected with S1PL-deficient mutants showed the same phenotype as macrophages treated with exogenous S1P (namely, enhanced bacterial colocalisation with LAMP-1 and reduced intracellular survival; Fig. 5 & 8).

Previous functional and structural studies of S1PL orthologues from other species (notably of yeast and the bacterial species, *Symbiobacterium thermophilum*; Bourquin *et al.*, 2010) provide us with valuable clues as to the structure and catalytic activity of the *Burkholderia* proteins. However, the *Burkholderia* S1PLs merit further study in their own right as they display several unusual features when considered in the context of the PLP-dependent enzyme superfamily (reviewed by Eliot and Kirsch, 2004), namely the presence of two copies of highly homologous genes, their extracellular location and their potential higher order structure. To our knowledge, there are no other instances of organisms possessing two

distinct S1PL-encoding genes, as is evident in *B. thailandensis* and *B. pseudomallei*. Data from previously published transcriptomic studies of *B. pseudomallei* have revealed that both S1PL-encoding genes are expressed under wide-ranging culture conditions (Ooi *et al.*, 2013) and during intracellular infection of macrophages (Chieng *et al.*, 2012). Whilst we have observed that each of the recombinant homodimeric *Burkholderia* S1PLs possess enzymatic activity individually, the higher degree of sphingosine resistance conferred by co-expression of *BTH_I10309* and *BTH_I10311* in *S. cerevisiae* $\Delta dpl1$ (Fig. 3) suggests that a hetero-oligomer may be required for optimal enzymatic activity. In such a scenario, inactivation or deletion of one of the S1PL-encoding genes would be sufficient to disrupt enzymatic activity, potentially explaining why each of the individual *B. thailandensis* mutants characterized herein display a comparable phenotype (Fig. 5 & 6), and why the single and double mutants in *B. pseudomallei* are also phenotypically indistinguishable (Fig. 5). Whilst we have observed oligomeric *Burkholderia* S1PL (Fig. S4), to date we have been unable to determine whether the observed oligomers are hetero- or homo-oligomeric, or a combination of both.

The targeting of the *Burkholderia* S1PLs following their secretion within the infected host cell is equally enigmatic. Eukaryotic S1PL is predominantly localised to the endoplasmic reticulum (ER) membrane where the S1P substrate is abundant (Ikeda *et al.*, 2004), whilst the S1PL orthologue of *L. pneumophila* (LegS2) has been reported to localise to both the mitochondria and the ER (Degtyar *et al.*, 2009; Rolando *et al.*, 2016), with the suggestion that it may localise to the contact sites between the two organelles (Rolando *et al.*, 2016). In our studies, the *Burkholderia* S1PLs did not show conclusive localisation to any of the cellular compartments studied (Fig. S8), nor does their sequence provide any clues as to their intracellular targeting. It should be stressed that the biosynthesis of S1P is very dynamic – both temporally and spatially. For example, whilst sphingosine kinase 1 (SphK1) is primarily a cytosolic protein in unstimulated mammalian cells, it rapidly translocates to the plasma

membrane, intracellular organelles (including the phagosome; Thompson *et al.*, 2005) and cytoskeleton in response to diverse cellular stimuli (for review, see Siow and Wattenberg, 2011). Consequently, future high resolution mapping of the temporal and spatial secretion of *Burkholderia* S1PLs during infection of host cells, in conjunction with sphingolipidomic analyses, will be fundamental to providing mechanistic insight into their precise role in pathogenesis and host S1P pathway disruption.

S1P has been widely implicated in human health and disease, and the therapeutic opportunities around S1P pathway modulation are numerous. Agonists, antagonists and inhibitors of the S1PRs and/or enzymes of the S1P biosynthetic/catabolic pathway are at various stages of clinical development for the treatment of diverse conditions including cancer, neurodegenerative disorders and inflammatory diseases (for review, see Proia and Hla, 2015). Clinical experience with the S1PR agonist FTY720 (GilenyaTM), approved as the first oral treatment for multiple sclerosis (Brinkmann *et al.*, 2010), proves that it is possible to specifically target the S1P pathway without serious adverse effects. The pivotal role played by S1P in immune function has also prompted studies of S1PR agonists as novel therapeutics for the treatment of fungal, viral and bacterial infections. In our own study, we demonstrated that exogenous S1PR agonists (S1P and CYM-5442) enhance the bactericidal activity of *Burkholderia*-infected macrophages (Fig. 8), an effect that has also been observed following S1P-treatment of *Mycobacterium*-infected macrophages (Garg *et al.*, 2004). Thus, S1PR-dependent processes play a pivotal role in regulating macrophage activity and their ability to control intracellular pathogens. S1PR agonists have also shown efficacy in infection models via other distinct mechanisms, for example through the reduction of infection-associated immunopathology in models of influenza (Teijaro *et al.*, 2014; Walsh *et al.*, 2011), lipopolysaccharide-mediated acute lung injury (Peng *et al.*, 2004), *Bordetella pertussis* infection (Skerry *et al.*, 2015) and *M. tuberculosis* infection (Garg *et al.*, 2004). Furthermore,

through its action on immune cell trafficking, the S1PR agonist FTY720 reduces the dissemination of *Francisella tularensis* from the airways in a murine infection model (Bar-Haim *et al.*, 2008). Collectively, these studies demonstrate that by harnessing the immunomodulatory activity of the S1P pathway, S1PR agonists have considerable potential as novel anti-infectives.

In summary, we describe the characterization of secreted S1PLs of *B. pseudomallei* and *B. thailandensis* that are required for intracellular replication and virulence, providing novel insight into the mechanisms by which these facultative intracellular organisms can evade host cell function. Our studies further highlight the S1P axis as a pivotal regulator of host-pathogen interactions and as a potential therapeutic target for the treatment of infectious diseases. Future studies should capitalize on the availability of existing S1P-targeted compounds and the associated clinical knowledge, with the aim of repurposing the latest generation of selective S1PR agonists as novel immunomodulatory-based anti-infectives.

Experimental Procedures

Bacterial strains, cells lines and culture conditions

Bacterial strains and plasmids are listed in Supplementary Information (Table S1). Bacteria were routinely cultured with aeration in Luria broth (LB) at 37 °C. As required, antibiotics were used at the following concentrations: kanamycin 25 µg ml⁻¹; gentamicin 50 µg ml⁻¹; trimethoprim 100 µg ml⁻¹; tetracycline 100 µg ml⁻¹. J774A.1 murine macrophages were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare) supplemented with 10 % foetal bovine serum (Gibco) at 37 °C in 5 % CO₂.

Recombinant Expression of Burkholderia S1PLs.

S1PL-encoding genes from *B. thailandensis* E264 and *B. pseudomallei* K96243 were PCR-amplified and cloned into pET-28a (Novagen, Madison, WI). The protein was expressed with a twenty residue N-terminal tag containing a six-histidine motif to allow isolation by immobilised metal affinity chromatography. Expression plasmids were transformed into the *E. coli* strain BL21 (DE3) for protein expression. Typically, cells were grown at 37 °C to an optical density (600 nm) of 0.6, prior to induction of protein expression with 0.1 mM isopropyl- β -D-thiogalactoside (IPTG) for 4 hours at 30 °C. Cells were harvested by centrifugation, lysed by sonication, and purified using HisTrap affinity and size exclusion chromatography (Superdex S200) on an AKTA FPLC system (GE Healthcare). Eluted protein was flash-frozen and stored at -80 °C in 20 mM HEPES/150 mM NaCl/10 % glycerol (pH 7.5). Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL) and all proteins were determined to be >90 % pure by Protein-PAGE.

Assay of S1PL activity by ESI-MS

The lyase activity of recombinant *Burkholderia* S1PLs was confirmed by measuring the formation of (2E)-hexadecenal (HEX) as a semicarbazone derivative by electrospray ionisation mass spectrometry (ESI-MS) based on a method from (Berdyshev *et al.*, 2011). Briefly an enzyme stock solution was prepared by dialysing 10 μ M S1PL in 20 mM HEPES (pH 7.5), 400 mM NaCl, and 100 μ M PLP. A 2.6 mM S1P stock was prepared in 0.1 % Triton X-100 with additional sonication. Using these stocks an enzyme assay was prepared to a final volume of 500 μ l containing 6 μ M S1PL and 0.26 mM S1P substrate in a reaction buffer containing 20 mM HEPES, 400 mM NaCl and 0.1 mM PLP. Control assays lacking S1PL and S1P substrate were prepared accordingly and all samples were incubated for 20 minutes at 37 °C. Methanol was added to stop the reaction and the HEX semicarbazone (HEX-SC) derivative was generated by heating the reaction at 40 °C for 2 h with 0.2 ml of 5

mM semicarbazide hydrochloride in methanol containing 5 % formic acid. A HEX standard (prepared as described previously; (Liu *et al.*, 2010)) was treated in the same way. The resultant semicarbazone was analysed by direct infusion MS on a micro-TOF2 (Bruker) in ESI-positive mode. Ion spray voltage was held at 4.5 kV at a temperature of 200 °C with a Hexapole RF of 70 V peak-to-peak and Skimmer 2 potential of 20 V.

Sphingosine antimicrobial assay

The antimicrobial activity of *D-erythro*-sphingosine C-18 (Cayman chemical; MI, USA) was assessed as described previously (Possemiers *et al.*, 2005), with minor modifications. Briefly, standardised bacterial suspensions (10^6 CFU ml⁻¹) derived from overnight cultures were incubated for 2 hours at 37 °C with agitation in a saline solution supplemented with *D-erythro*-sphingosine (25 µM, dissolved in ethanol). After incubation, serial dilutions were plated onto LB agar plates for enumeration of CFU. Survival was determined by comparison to ethanol only controls.

Complementation of Saccharomyces cerevisiae Δdpl1

BTH_II0309 and *BTH_II0311* genes were codon-optimised for *S. cerevisiae* expression by gene synthesis (Invitrogen) and cloned into the Gateway pDONR221 vector (Life Technologies) to create pENTR::*BTH_II0309* and pENTR::*BTH_II0311* respectively. The *BTH_II0309* and *BTH_II0311* sequences were subsequently cloned from the pENTR vectors into pAG413GPD-ccdB and pAG415GPD-ccdB yeast destination vectors respectively using LR Clonase recombination (Invitrogen), generating the expression plasmids pDEST413::*BTH_II0309* and pDEST415::*BTH_II0311*. A similar process was used to construct pDEST413::*dpl1*, expressing the *S. cerevisiae* S1PL orthologue. The *S. cerevisiae* BY4741 (parental strain) and YDR294C ($\Delta dpl1$) strains were transformed with the

appropriate plasmids as described previously (Agatep *et al.*, 1998). Transformants were selected on the basis of histidine (pAG413GPD-ccdB) and/or leucine (pAG415GPD-ccdB) prototrophy and were verified by PCR.

Saccharomyces cerevisiae sphingosine resistance assay

S. cerevisiae strains were grown in selective minimal liquid culture medium at 30 °C overnight with agitation. Serial dilutions of standardized culture were spotted onto YNB agar with or without supplementation with 10 µM *D-erythro*-sphingosine C-18. Plates were incubated at 30 °C for 48-72 h prior to visual assessment of growth.

Detection of FLAG-tagged SIPLs in bacterial lysates and culture supernatant

B. thailandensis constitutively expressing either FLAG-tagged BTH_II0309 or FLAG-tagged BTH_II311 were grown to mid-log phase in LB broth adjusted to either pH 7 or pH 4.5 and cells harvested by centrifugation. Bacterial cells were lysed in NuPAGE LDS Sample Buffer (Novex), whilst culture supernatants were filtered and proteins precipitated with 10 % (v/v) trichloroacetic acid. Proteins recovered from the whole cell lysates and culture supernatants were separated on 4-12 % NuPAGE SDS-PAGE (Novex) gel and transferred to nitrocellulose membrane. BTH_II0309-FLAG and BTH_II0311-FLAG were detected by Western blot using 1:1000 mouse anti-FLAG (Invitrogen) and 1:15000 goat anti-mouse LI-COR® IRDye 800cw. The protein bands were visualised with the LI-COR Odyssey scanner and software.

Construction of mutants and complemented strains

SIPL-deficient mutants of *B. thailandensis* E264 and *B. pseudomallei* K96243 were generated via either insertional inactivation using pGPQTp (Flannagan *et al.*, 2007) or unmarked gene deletions using pDM4 and *sacB*-based counterselection (Logue *et al.*, 2009).

Complementation of the mutants was achieved using the previously described pDA17 vector (Flannagan *et al.*, 2008), that drives constitutive expression from a *dhfr* promoter. All plasmids (for mutagenesis and complementation) were mobilised into *B. thailandensis* or *B. pseudomallei* by triparental mating using the helper plasmid, pRK2013 (Figurski and Helinski, 1979). All mutants and complemented strains were confirmed by appropriate PCR validation, with the *B. pseudomallei* deletion mutant (Δ BPSS2025) further validated by whole genome sequencing.

Phagocytic uptake and intracellular survival assays

Bacterial uptake and subsequent intracellular survival in macrophages was assessed using a modified kanamycin protection assay as described previously (Wand *et al.*, 2011). In summary, J774A.1 macrophages were incubated with relevant *B. thailandensis* or *B. pseudomallei* strains (MOI 10) for 2 h to allow bacterial internalization, after which the medium was replaced with fresh L15 supplemented with kanamycin (1000 $\mu\text{g ml}^{-1}$) to kill extracellular bacteria. Viable intracellular bacteria were enumerated at 2 and 6 hpi by plating serial dilutions of macrophage lysates.

Immunofluorescence microscopy for bacteria/LAMP-1 colocalisation studies

J774A.1 macrophages seeded onto 12 mm diameter glass coverslips were infected (MOI 10) with wildtype *B. thailandensis* and the *BTH_II0309* and *BTH_II0311* mutants transformed with the reporter plasmid pBHR4-*groS*-RFP (Wand *et al.*, 2011). After treatment with kanamycin (1000 $\mu\text{g ml}^{-1}$) to kill extracellular bacteria, monolayers were washed twice with PBS and fixed with paraformaldehyde (4 % w/v in PBS; 15 min, room temperature). Cells were permeabilized with saponin (0.5 % v/v in PBS; 1 h) and non-specific binding sites blocked with 1 % (v/v) bovine serum albumin (BSA) in PBS. LAMP-1 was stained with

1:100 rabbit polyclonal antibody (Abcam) and the bound antibody was detected with 1:400 goat anti-rabbit IgG-Alexa 488 (Molecular probes). The stained cells were washed three times with PBS, mounted onto slides using Vectashield (Vector Laboratories) and visualized using a Zeiss Axioplan epifluorescent microscope equipped with an oil immersion lens (Zeiss 100× A-plan). Image analysis and processing was performed using Fiji (Schindelin *et al.*, 2012). A minimum of 100 bacteria were counted per strain/condition for each experiment. Colocalisation between bacteria and LAMP-1 was considered when bacteria were surrounded by a ring-like LAMP-1-enriched structure, indicating that the membrane of the *Burkholderia*-containing phagosome is positive for LAMP-1.

S1P and S1PR₁ agonist treatment of macrophages

S1P (Cayman Chemical; MI, USA) and the water-soluble S1PR₁ selective agonist CYM-5442 (Tocris Biosciences; Bristol, UK) were added to infected J774A.1 monolayers after a 2 h infection period with wildtype *B. thailandensis* E264 or *B. pseudomallei* K96243 (MOI 10). S1P was added at a final concentration of 5 μM, a concentration previously shown to elicit a strong anti-mycobacterial response in infected macrophages (Garg *et al.*, 2004). CYM-5442 was added at a final concentration of 0.5 μM, a concentration known to activate the S1PR₁-dependent pathways *in vitro* (Gonzalez-Cabrera *et al.*, 2008). Colocalisation of bacteria with LAMP-1 and/or intracellular survival within treated and untreated macrophages was then assessed as described above.

Fluorescent staining of FLAG-tagged SIPLs in infected macrophages

J774A.1 cells were infected with *B. thailandensis* expressing either FLAG-tagged BTH_II0309 or FLAG-tagged BTH_II311 (MOI 50). Following killing of extracellular bacteria (1000 μg ml⁻¹ kanamycin, 2 hours), infected cells were fixed with paraformaldehyde

and permeabilized with saponin as described above. Non-specific binding sites were blocked with Image-iT® FX signal enhancer (Life technologies) for 30 min at room temperature, prior to overnight incubation (4 °C) with mouse anti-FLAG (Invitrogen) and rabbit anti-Calnexin (Abcam) primary antibodies (each diluted 1:250 in 10 % normal goat serum / 0.1 % PBS-Tween 20). Bound primary antibodies were subsequently detected with 1:400 dilutions of goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (Molecular probes). Following washing, the stained cells were mounted and visualized as described above.

Galleria mellonella infection model

Virulence of relevant *B. thailandensis* strains was assessed using the *G. mellonella* infection model as described previously (Wand *et al.*, 2011), with an inoculum of approximately 25 CFU per larvae. For each independent experiment, inoculations were performed with multiple inoculum dilutions for each strain and exact CFU counts for each inoculum were determined to ensure comparisons between strains were robust (as minor variation from the intended 25 CFU inoculum impacted on larval survival). Larval survival was assessed 40 hours post-infection (hpi).

BALB/c murine infection model

Investigations involving animals were carried out according to the requirements of the UK Animal (Scientific Procedures) Act 1986 under project licence PPL 30/3026. This project licence was approved following an ethical review by Dstl's Animal Welfare and Ethical Review Body. Studies were performed using female BALB/cAnNCrl mice (BALB/c; Charles River UK) implanted with a sub-cutaneous Pico transponder (Uno BV, Netherlands) to allow individual mice to be tracked through the study. On arrival into containment level 3 animal facilities, mice were randomly allocated into cages of five animals and acclimatised to their

new surroundings for five days before any procedures were performed. Animal husbandry practices and environmental conditions during study were as described previously (Scott *et al.*, 2016). Challenges were performed with *B. pseudomallei* K96243 prepared as described previously (Scott *et al.*, 2016) and delivered via the intraperitoneal route. Mice received one of three different inocula per strain (five mice per strain per inoculum), corresponding to approximately 3.8×10^3 – 3.8×10^5 CFU *B. pseudomallei* K96243 and 5.6×10^4 – 5.6×10^6 CFU *B. pseudomallei* Δ BPSS2025. Mice were checked at least twice daily following challenge and clinical signs for each mouse recorded for five weeks post-infection. Humane end-points were used throughout these studies to minimise suffering, with culls performed via cervical dislocation at the end-point. At the end of the study, animals were culled and organs removed for enumeration of bacterial burden (lungs, liver, spleen). These were mashed through 40 μ m sieves into PBS, serially diluted and plated onto L-agar.

Statistical analysis

Kaplan–Meier survival curves were generated using GraphPad PRISM v6 and analysed using a log rank (Mantel-Cox) test within PRISM. The LD₅₀ for each strain was determined using the method of Reed and Muench (Reed and Muench, 1938). For all other assays, statistical analyses were performed by one-way ANOVA with Tukey post hoc testing (for multiple comparisons) or Student’s t-test (for pairwise comparisons). In all cases, $p < 0.05$ was deemed statistically significant.

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Author contributions

Conceptualization, ARB, DJ Campopiano and MS-T; Methodology & Investigation, RC, CJM, AES, JL, AK and DJ Clarke; Writing – Original Draft, RC and ARB; Writing – Review & Editing, RC, CJM, AES, ARB, DJ Campopiano and MS-T.

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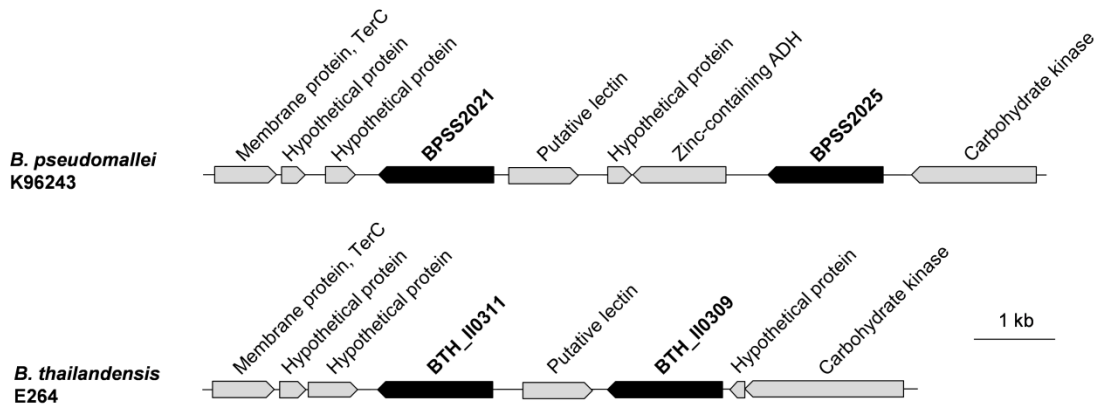


Fig. 1. S1PL-encoding genomic regions of *B. pseudomallei* K96243 and *B. thailandensis* E264. Each organism possesses two S1PL-encoding genes (highlighted in black) located within the same genomic region. A high degree of synteny exists between the S1PL-encoding regions of each species. For ease of comparison, the *B. thailandensis* genomic region is depicted in reverse orientation.

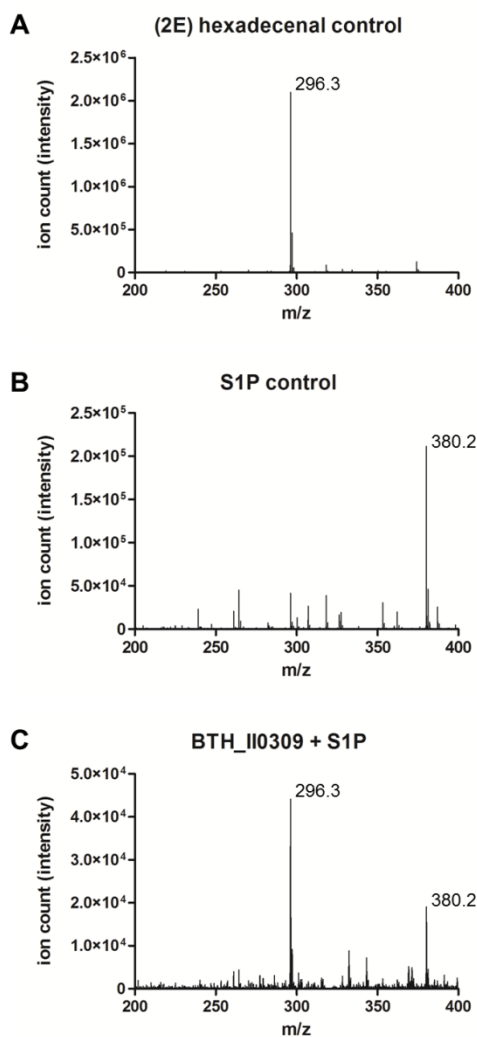


Fig. 2. Demonstration of lyase activity of recombinant *Burkholderia* S1PL by ESI-MS.

The lyase activity of recombinant *Burkholderia* S1PLs (exemplified here by BTH_II0309) was confirmed by using ESI-MS to assess the formation of (2E)-hexadecenal (HEX) as a semicarbazone derivative (HEX-SC) from S1P. (A) HEX standard reaction showing an ion consistent with HEX-SC ($[M+H]^+$, $C_{17}H_{34}N_3O$, predicted $m/z = 296.3$). (B) No enzyme control, showing no HEX-SC formation and an ion consistent with the mass of the S1P substrate ($[M+H]^+$, $C_{18}H_{39}NO_5P = 380.2$). (C) Incubation of S1P with recombinant BTH_II0309 results in the degradation of S1P and formation of HEX-SC (evidenced by coexistence of ions with $m/z = 296.3$ and 380.2).

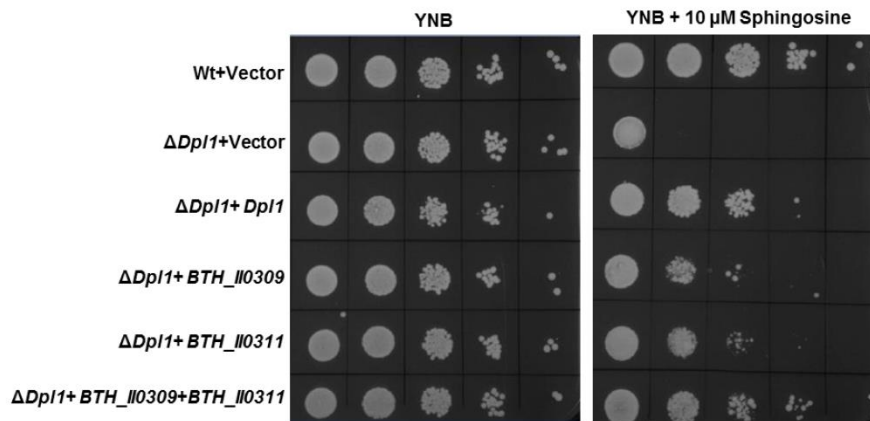


Fig. 3. The S1PLs of *B. thailandensis* functionally complement S1PL-deficient yeast. *Burkholderia* S1PLs codon-optimized for expression in yeast were synthesized and cloned into S1PL-deficient *S. cerevisiae* $\Delta dpl1$. Serial dilutions of transformants were spotted onto YNB agar \pm 10 μ M D-erythro-sphingosine. The results are representative of three independent experiments following 48-72 h incubation, and confirm that the *Burkholderia* proteins functionally complement the yeast mutant.

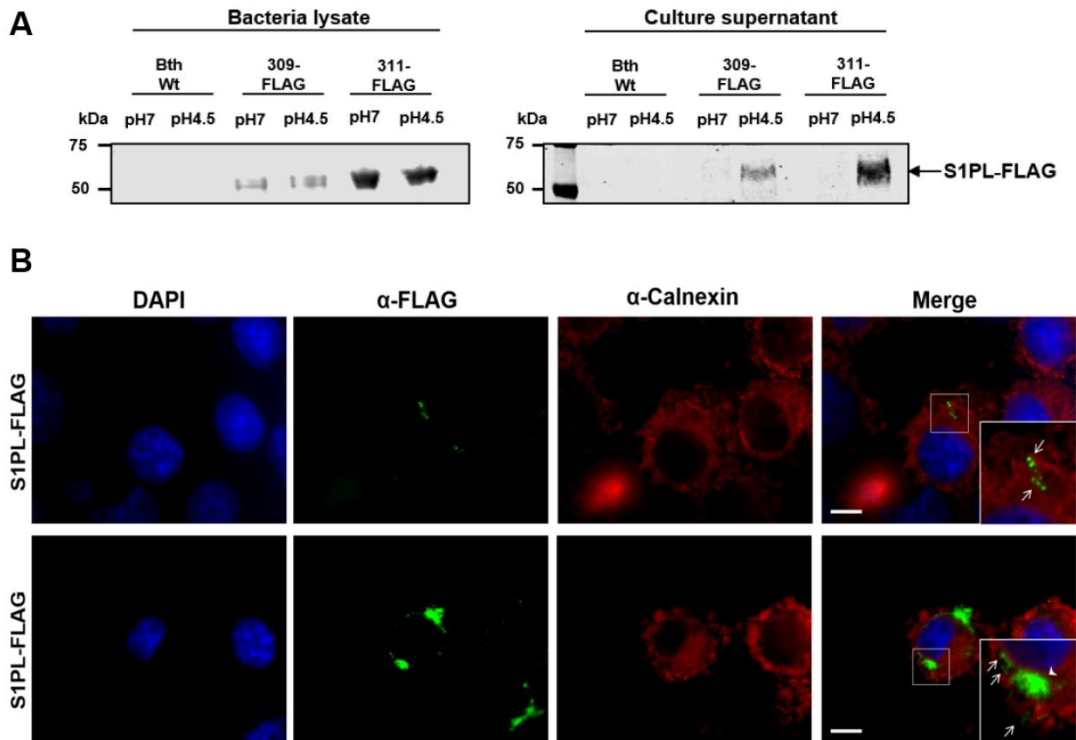


Fig. 4. Secretion of *B. thailandensis* S1PLs is pH-dependent, and can be observed during intracellular infection. (A) Wildtype *B. thailandensis* and *B. thailandensis* constitutively expressing FLAG-tagged S1PL (BTH_II0309 or BTH_II0311) were cultured at pH 7 or pH 4.5. Proteins from whole-cell lysates and culture supernatants were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-FLAG antibody. The S1PL proteins were only detected in culture supernatant from *B. thailandensis* cultured at pH 4.5. No anti-FLAG reactivity was observed for the wildtype *B. thailandensis*, thus excluding cross-reactivity with endogenous proteins. (B) J774A.1 cells were infected with *B. thailandensis* expressing BTH_II0309-FLAG or BTH_II0311-FLAG (MOI 50). The endoplasmic reticulum and S1PL-FLAG proteins were stained with anti-Calnexin and anti-FLAG antibodies respectively (in conjunction with appropriate secondary antibodies; refer to Materials and Methods), and cell nuclei were stained with DAPI. Top panels show FLAG-tagged S1PL detection restricted to bacterial cells, whilst bottom panels show staining indicative of S1PL secretion. Right insets represent magnified images of boxed areas. White arrows indicate intracellular bacteria, whilst the arrow head indicates secreted S1PL. No

FLAG reactivity was detected in macrophages infected with wildtype *B. thailandensis* (not shown). Scale bar represents 10 μm .

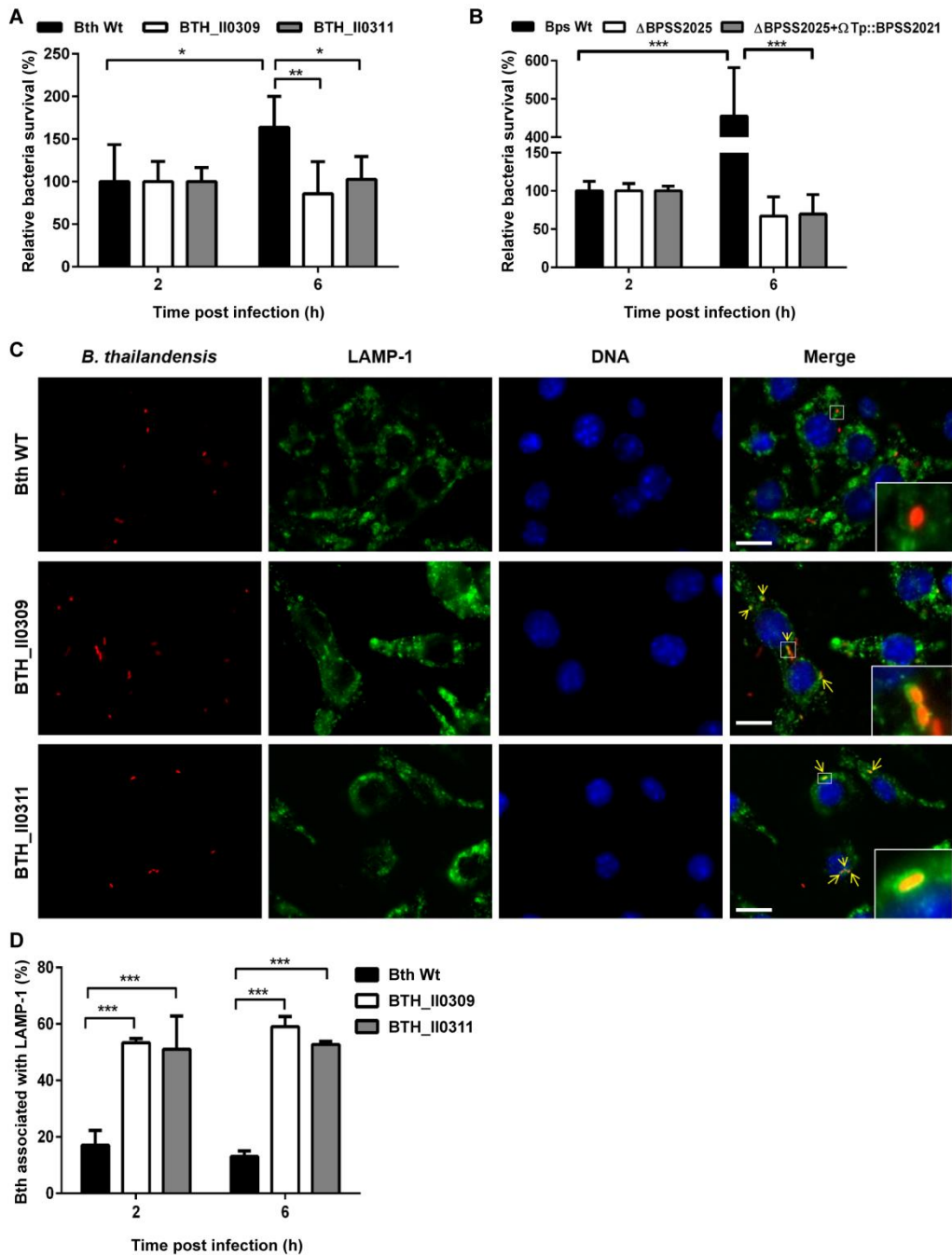


Fig. 5. S1PL-deficient *Burkholderia* exhibit impaired intracellular replication and increased colocalisation with LAMP-1-containing vesicles in macrophages. (A & B) J774A.1 macrophages were infected with relevant strains of *B. thailandensis* (A) or *B. pseudomallei* (B). Viable intracellular bacteria were quantified at 2 and 6 hpi by spotting serial dilutions of macrophage lysates. For each strain, bacterial survival was normalised to the CFU counts observed at 2 hpi and presented as relative bacteria survival (%) so as to

visualise intracellular replication between 2 and 6 hpi. For both *B. thailandensis* and *B. pseudomallei*, the S1PL-deficient mutants showed impaired intracellular replication relative to the respective wildtype. Notably, the two individual S1PL mutants of *B. thailandensis* exhibited a comparable phenotype (A), as did the single and double mutants of *B. pseudomallei* (B). (C & D) Assessment of the colocalisation of RFP-expressing *B. thailandensis* strains with the lysosomal marker LAMP-1 demonstrated that S1PL-deficient mutants exhibit increased LAMP-1 colocalisation. In representative micrographs (C), right insets represent magnified images of boxed areas and the yellow arrows indicate bacterial colocalisation with LAMP-1. Scale bar represents 10 μ m. For quantitative analysis of bacterial colocalisation with LAMP-1 (D), positive or negative colocalisation was assessed for 100 intracellular bacteria per strain per independent experiment to achieve a percentage colocalisation. Data shown represent the mean \pm SEM of three independent experiments. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

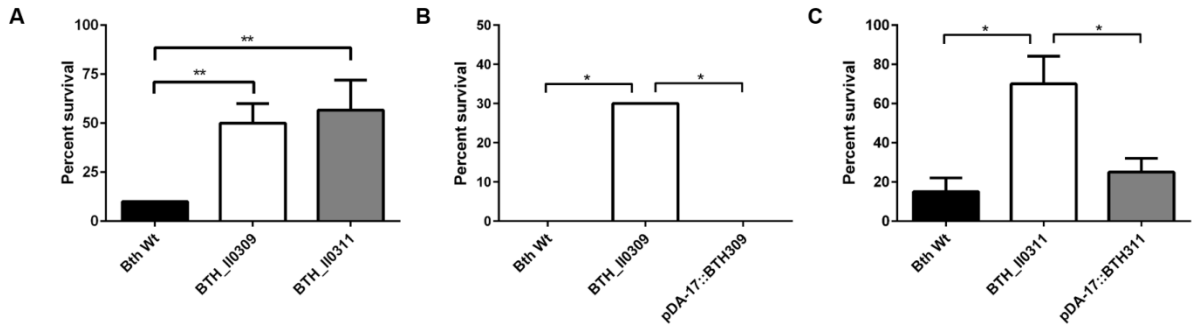


Fig. 6. *B. thailandensis* S1PLs are required for virulence in a larval infection model.

Virulence of relevant *B. thailandensis* strains was assessed using the *Galleria mellonella* infection model using an inoculum of approx. 25 CFU and assessing percentage larval survival at 40 hpi. (A) Relative to the wildtype, both the individual *BTH_II0309* and *BTH_II0311* mutants exhibit comparable attenuated virulence, with delayed larval killing. (B & C) Complementation fully restored virulence to the *BTH_II0309* and *BTH_II0311* mutants. When studying the complemented strains alongside the mutants, the *BTH_II0309* and *BTH_II0311* strains were studied independently from each other, so as to accommodate multiple inoculations per strain (refer to main text for details). This is reflected in the differing larval survival observed between panels B & C, which show analysis of *BTH_II0309* and *BTH_II0311* strains respectively. When studied alongside each other, there is no difference in the virulence of the two mutants (panel A). Results represent the mean \pm SEM of three independent experiments (10 larvae per strain, per experiment). * $p < 0.05$, ** $p < 0.005$.

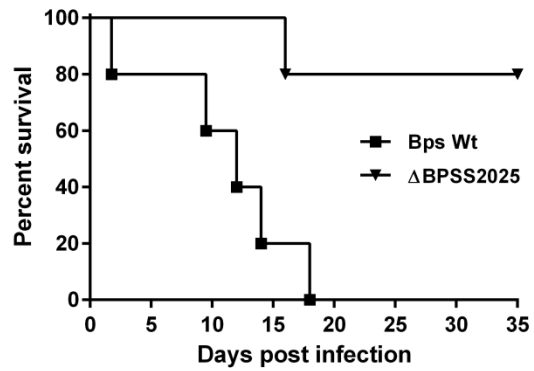


Fig. 7. *B. pseudomallei* S1PL contributes to virulence in a murine model of infection.

Virulence of *B. pseudomallei* Δ BPSS2025 was assessed using an intraperitoneal infection model in BALB/c mice. Data show the survival (over a five week period) of mice inoculated with approximately 4×10^4 CFU of either wildtype or Δ BPSS2025 *B. pseudomallei*, demonstrating significant attenuation of the BPSS2025-deficient mutant ($p = 0.0064$).

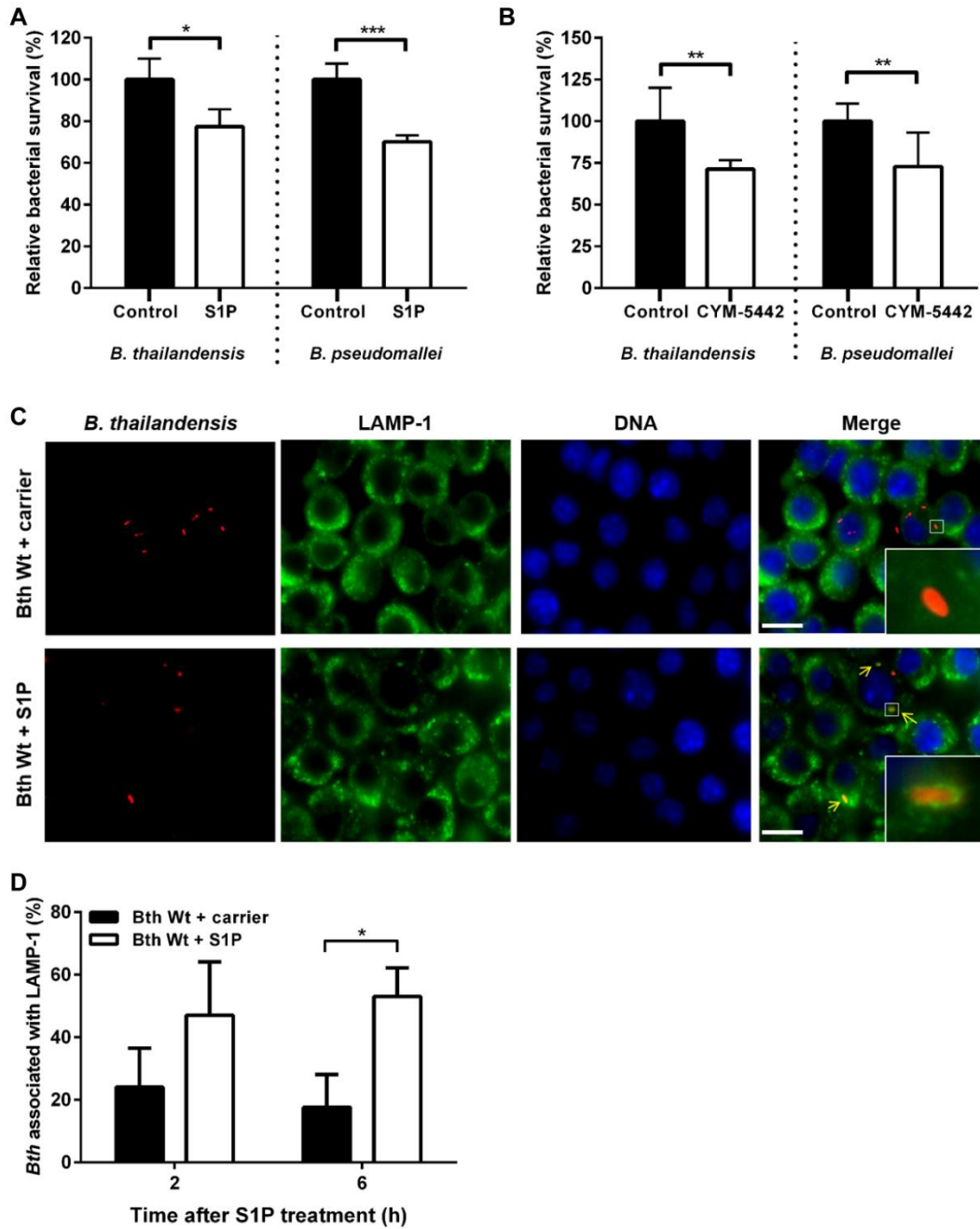


Fig. 8. Treatment of *Burkholderia*-infected macrophages with S1P or an S1PR₁ agonist enhances bacterial killing. (A & B) J774A.1 macrophages were infected with wildtype *B. thailandensis* (A) or *B. pseudomallei* (B) for 2 h, prior to treating with S1P or CYM-5442 (alongside relevant carrier only controls). Viable intracellular bacteria were quantified after 6 hours and percentage survival calculated relative to controls. For both species, S1P and CYM-5442 treatments resulted in comparable reductions in surviving intracellular bacteria. (C & D) Assessment of the colocalisation of RFP-expressing *B. thailandensis* strains with the

lysosomal marker LAMP-1 in infected macrophages with or without S1P treatment. S1P-treatment enhanced bacterial colocalisation with LAMP-1, as indicated in representative micrographs (C) and via quantitative analysis (relative to carrier only control; D). (C) Right insets represent magnified images of boxed areas, with the yellow arrows indicating bacterial colocalisation with LAMP-1. Scale bar represents 10 μ m. (D) For quantitative analysis, positive or negative colocalisation was assessed for 100 intracellular bacteria per strain per independent experiment to achieve a percentage colocalisation. In panels A, B & D, data represent mean \pm SEM of three independent experiments. * $p < 0.01$; ** $p < 0.001$.

Characterization of secreted sphingosine-1-phosphate (S1P) lyases required for virulence and intracellular survival of *Burkholderia pseudomallei*.

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SUPPORTING INFORMATION. Supporting Figures S1-S9 and Table S1.



Figure S1. Amino acid alignment of representative *Burkholderia* and eukaryotic S1PLs. Sequence alignment of BPSS2025, BPSS2021, BTH_II0309, BTH_II0311, human & yeast S1PL. Asterisks indicate amino acid residues that are important for the enzymatic activity of the yeast protein (Mukhopadhyay *et al.*, 2008), all of which are conserved in the *Burkholderia* orthologues. Accession numbers: Human S1PL, CAA09590; Yeast S1PL, AHY75267. Alignment generated using Vector NTI Advance 11 (Invitrogen), with blue, green and yellow shading indicating similarities (“conserved”, “block of similar”, and “identical” respectively).

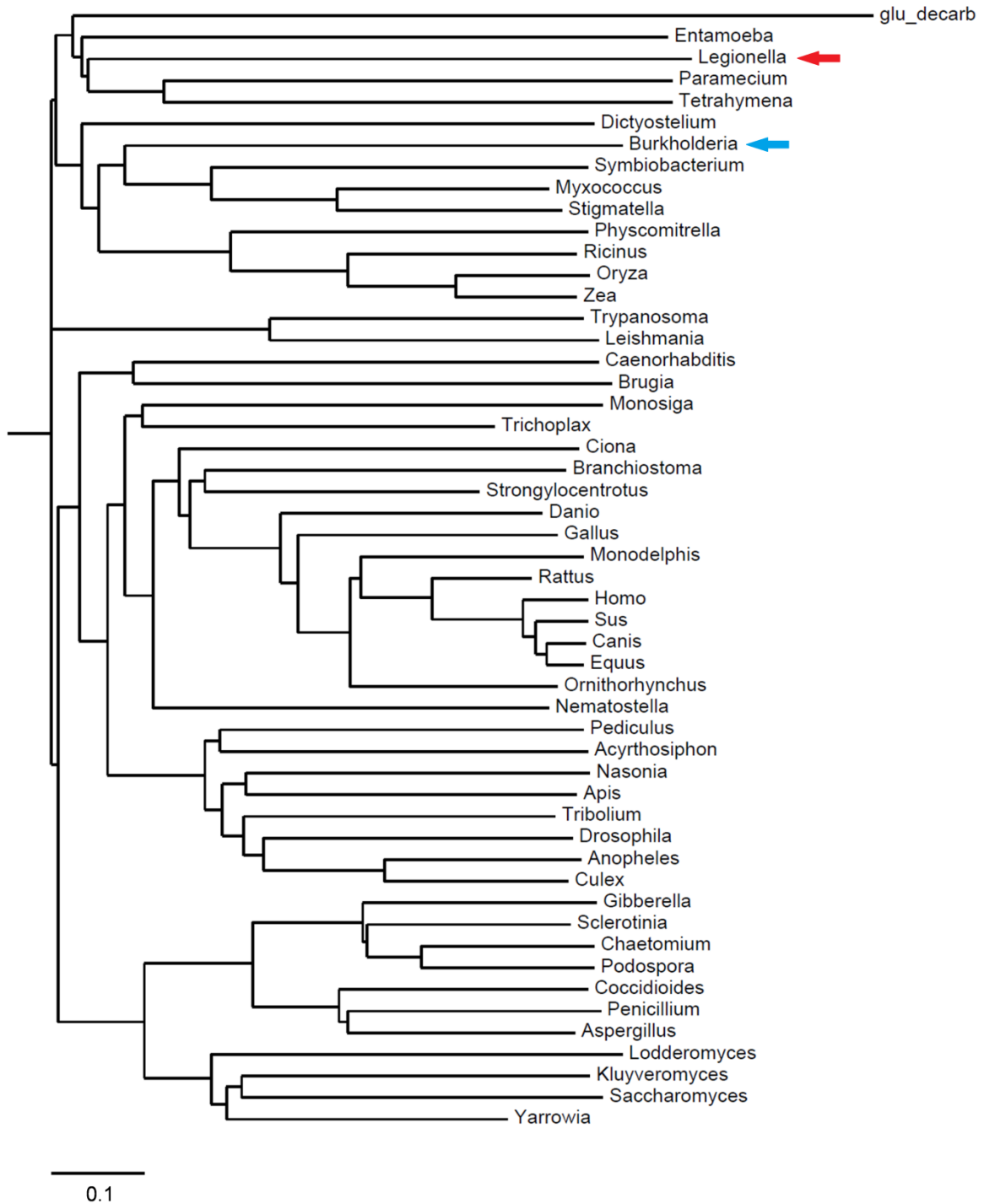


Figure S2. Phylogenetic tree of representative prokaryotic and eukaryotic S1PL orthologues. Representative protein sequences were aligned using the MUSCLE programme (Edgar, 2004), and the subsequent tree visualized using Phylodendron (<http://iubio.bio.indiana.edu/treeapp/treeprint-form.html>). Glutamate decarboxylase from *Francisella* was used as an outgroup (*glu_decarb*). *Legionella* and *Burkholderia* S1PL orthologues are highlighted with red and blue arrows respectively.

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BPSS2021      (1) -----MRSIAARSSHAGALEQFGMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BURPS1106A_A2756 (1) -----MRSIAARSSHAGALEQFGMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BURPS668_A2912 (1) -----MRSIAARSSHAGALEQFGMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BTH_II0311    (1) -----MRSIAARSSHAGALEQFGMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BTH_II0309    (1) -----MRSIAARSSHAGALEQFGMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BPSS2025      (1) MRVACFVDFMQPHPGNIRTHIGVPTDVGTAKENVMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BURPS1106A_A2761 (1) -----MQPHPGNIRTHIGVPTDVGTAKENVMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR
BURPS668_A2918 (1) -----MPTDVGTAKEENVMDLEEGVRLYPYAEFGALHEFPERGMPFRRLLEE LRSMAVREDR

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Figure S3. Alignment of the N-terminal region of representative *Burkholderia* S1PLs. Alignment of the N-terminal region of selected *Burkholderia* orthologues drawn from the *Burkholderia* genome database (Winsor *et al.*, 2008) reveal inconsistencies in the prediction of the N-terminus. Only when cloned from the conserved MDLEEG sequence did the *Burkholderia* S1PL orthologues exhibit the anticipated enzymatic activity. Sequences depicted: BPSS2021 & BPSS2025 (*B. pseudomallei* K96243), BTH_II0309 & BTH_II0311 (*B. thailandensis* E264), A2756 & A2761 (*B. pseudomallei* 1106a) and A2912 & A2918 (*B. pseudomallei* 668). Alignment generated using Vector NTI Advance 11 (Invitrogen), with blue, green and yellow shading indicating similarities (“conserved”, “block of similar”, and “identical” respectively).

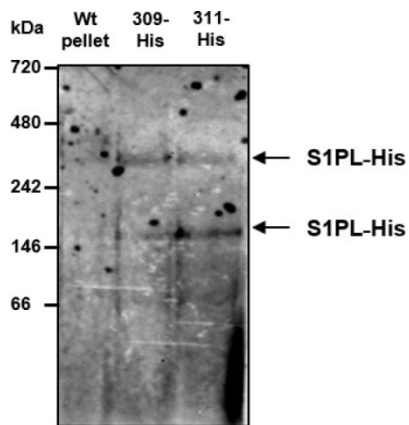


Figure S4. Western blot analysis of native His-tagged BTH_I10309 and BTH_I10311 indicates oligomeric complexes. Using *B. thailandensis* E264 as a parental strain, sequence encoding a 6x His-tag was integrated at the 3' end of either *BTH_I10309* or *BTH_I10311* using the suicide vector pGPΩTp to introduce a C-terminal 6His-tag. Cells from 1 litre mid-log phase bacterial cultures were harvested by centrifugation and disrupted by sonication. The proteins from the bacterial pellets were applied to a His-Selective Nickel affinity gel (Sigma) column and washed with equilibration buffer. Bound His-tagged proteins were eluted from the column under native conditions using 500 mM imidazole elution buffer and separated on a 4-16 % NativePAGE (Novex) gel electrophoresis (6 µg of total protein loaded per lane). Proteins were transferred to a PVDF membrane and detected using 1:500 mouse anti-His polyclonal antibody (Novagen) and 1:1500 goat anti-mouse LI-COR® IRDye 800cw. Whilst the individual proteins are both approximately 52 kDa, higher molecular weight oligomeric complexes were observed for both proteins.

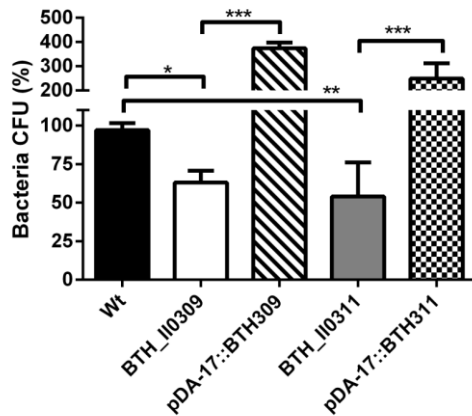


Figure S5. The S1PLs of *B. thailandensis* contribute to D-erythro-sphingosine resistance. Relevant *B. thailandensis* strains were assessed for sphingosine susceptibility in a saline solution supplemented with 25 μ M of D-erythro-sphingosine, with percentage bacterial survival calculated relative to a saline only control for each strain. Each of the individual S1PL-deficient mutants (BTH_II0309 and BTH_II0311) exhibited hypersensitivity to D-erythro-sphingosine relative to wildtype (Wt) *B. thailandensis* E264, a phenotype that was fully restored upon complementation (pDA17::BTH309/BTH311). Results shown represent the mean +SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

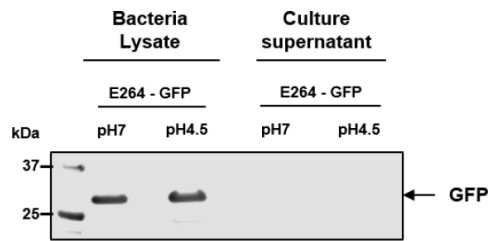


Figure S6. Constitutively-expressed GFP protein is only detectable in bacterial lysates. To ensure that the observed pH-dependent secretion of S1PL was not an artefact arising from overexpression whilst under acid-related stress, proteins from whole-cell lysates and culture supernatant (8 μ g of total protein loaded per lane) of *B. thailandensis* expressing pDA17::GFP grown in LB pH 7 or pH 4.5 were separated by 4-12 % SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-GFP antibody. Irrespective of the pH, GFP was only detected in lysates from cell pellets.

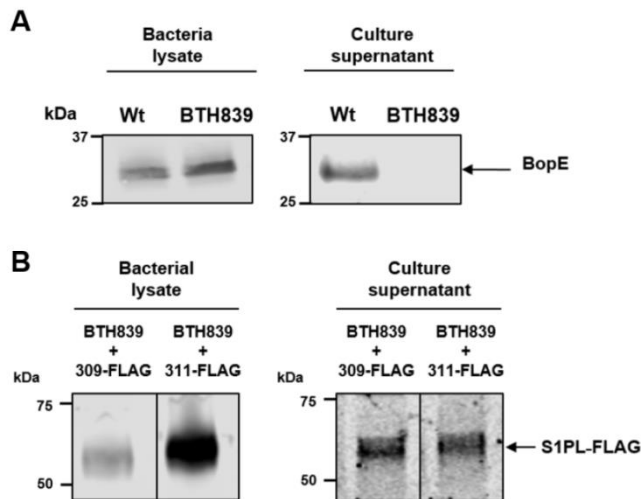


Figure S7. The pH-dependent secretion of the *Burkholderia* S1PLs is independent of the Bsa type III secretion system. A *B. thailandensis* Bsa T3SS mutant was constructed by insertional inactivation of *bsaZ* (BTH_II0839) in order to assess the impact on the acid-induced S1PL secretion. **(A)** When cultured at pH 4.5, secretion of the known T3SS effector BopE was abolished in the *bsaZ* mutant (BTH839), confirming the inactivation of the Bsa secretion system. **(B)** FLAG-tagged BTH_II0309 or BTH_II0311 were expressed in the *bsaZ* mutant cultured at pH 4.5. Secretion of both proteins was still detected, despite the inactivation of the Bsa system.

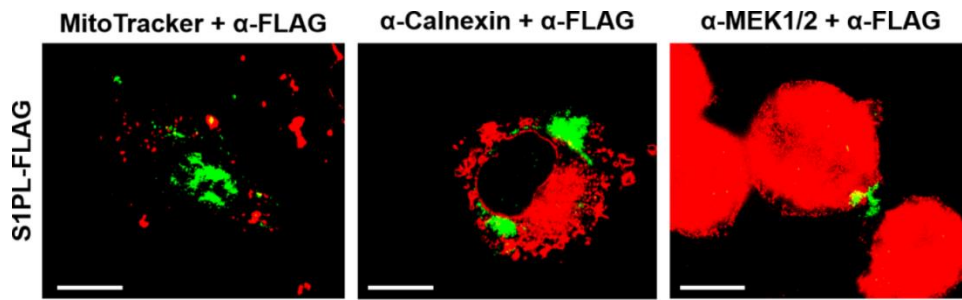


Figure S8. Investigating the localisation of secreted *B. thailandensis* S1PLs during macrophage infection. J774A.1 cells were infected (MOI 50, 2 hours) with *B. thailandensis* expressing either BTH_II0309-FLAG or BTH_II0311-FLAG. Following killing of extracellular bacteria with kanamycin ($1000 \mu\text{g ml}^{-1}$, 2 hours), staining of cellular compartments and anti-FLAG detection was performed. Mitochondria were stained with MitoTracker Red-CMXRos, ER with anti-Calnexin, and the cytosolic compartment stained with anti-MEK1/2. The *B. thailandensis* S1PL-FLAG proteins were detected with anti-FLAG. For colocalisation presentation clarity, images were convoluted and processed with the (default) threshold algorithm of the Fiji software (Schindelin *et al.*, 2012). Colocalisation is shown in yellow. Scale bars represent $10 \mu\text{m}$.

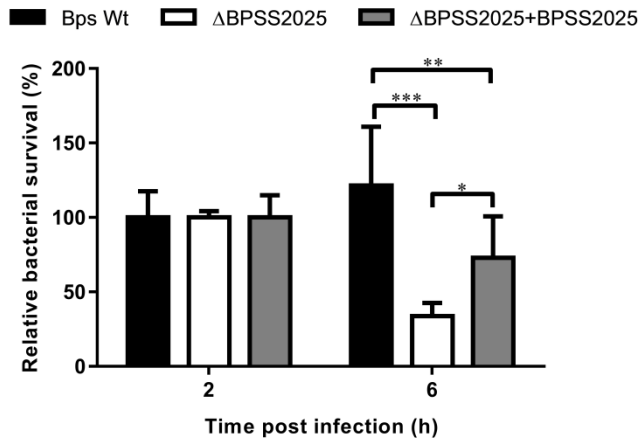


Figure S9. BPSS2025 S1PL is required for intracellular replication of *B. pseudomallei*. J774A.1 macrophages were infected with relevant strains of *B. pseudomallei* and viable intracellular bacteria were quantified at 2 and 6 hpi by spotting serial dilutions of macrophage lysates. For each strain, bacterial survival was normalised to the CFU counts observed at 2 hpi and presented as relative bacteria survival (%) so as to visualise intracellular replication between 2 and 6 hpi. The S1PL-deficient mutant (Δ BPSS2025) showed impaired intracellular replication relative to the wildtype, a phenotype that was partially restored by complementation. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Table S1. List of strains and plasmids used in this study.

Strains or plasmids	Description	Reference / Source
<i>Burkholderia thailandensis</i>		
Bth Wt	Wildtype <i>B. thailandensis</i> E264	(Brett <i>et al.</i> , 1998)
<i>BTH_II0309</i>	E264, pGPΩTp:: <i>BTH_II0309</i> , Tp ^R	This study
<i>BTH_II0311</i>	E264, pGPΩTp:: <i>BTH_II0311</i> , Tp ^R	This study
pDA17:: <i>BTH_II0309</i>	E264, pGPΩTp:: <i>BTH_II0309</i> , pDA17:: <i>BTH_II0309</i> , Tp ^R Tet ^R	This study
pDA17:: <i>BTH_II0311</i>	E264, pGPΩTp:: <i>BTH_II0309</i> , pDA17:: <i>BTH_II0311</i> , Tp ^R Tet ^R	This study
E264-RFP	E264, pBHR4- <i>groS-rfp</i> , Cm ^R	This study
<i>BTH_II0309</i> -RFP	<i>BTH_II0309</i> , pBHR4- <i>groS-rfp</i> , Tp ^R , Cm ^R	This study
<i>BTH_II0311</i> -RFP	<i>BTH_II0311</i> , pBHR4- <i>groS-rfp</i> , Tp ^R , Cm ^R	This study
<i>BTH_II0309</i> -FLAG	E264, pDA17:: <i>BTH_II0309</i> -C-terminal FLAG tag, Tet ^R	This study
<i>BTH_II0311</i> -FLAG	E264, pDA17:: <i>BTH_II0311</i> -C-terminal FLAG tag, Tet ^R	This study
<i>Burkholderia pseudomallei</i>		
Bps Wt	Wildtype <i>B. pseudomallei</i> K96243	(Holden <i>et al.</i> , 2004)
<i>BPSS2025</i>	K96243, Δ <i>BPSS2025</i> (pDM4 mutagenesis)	This study
<i>BPSS2025+2021</i>	K96243, Δ <i>BPSS2025</i> , pGPΩTp:: <i>BPSS2021</i> , Tp ^R	This study
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (<i>Str</i> ^R) <i>endA1 nupG</i> λ ⁻	Invitrogen
GT115	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1 rspL</i> (<i>StrA</i>) <i>endA1</i> Δ <i>dcm uidA</i> (Δ <i>MluI</i>):: <i>pir-116</i> Δ <i>sbcC-sbcD</i>	Invitrogen
<i>Saccharomyces cerevisiae</i>		
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	K. Haynes, Exeter
YDR294C	BY4741, Δ <i>dpl1</i>	K. Haynes, Exeter
Wt+ vector	BY4741, pDEST413	This study
Δ <i>dpl1</i> + vector	YDR294C, pDEST413	This study
Δ <i>dpl1</i> + <i>dpl1</i>	YDR294C, pDEST413:: <i>dpl1</i>	This study
Δ <i>dpl1</i> + <i>BTH_II0309</i>	YDR294C, pDEST413:: <i>BTH_II0309</i>	This study
Δ <i>dpl1</i> + <i>BTH_II0311</i>	YDR294C, pDEST415:: <i>BTH_II0311</i>	This study
Δ <i>dpl1</i> + <i>BTH_II0309</i> + <i>BTH_II0311</i>	YDR294C, pDEST413:: <i>BTH_II0309</i> , pDEST415:: <i>BTH_II0311</i>	This study
Plasmids		
pGPΩTp	<i>ori</i> _{R6K} , ΩTp ^R cassette, <i>mob</i> ⁺	(Flannagan <i>et al.</i> , 2007)
pDM4	<i>ori</i> _{R6K} , <i>sacB</i> Cm ^R	(Logue <i>et al.</i> , 2009)
pRK2013	<i>ori</i> _{colE1} , RK2 derivative, Kan ^R , <i>mob</i> ⁺ <i>tra</i> ⁺	(Figurski and Helinski, 1979)
pDA17	<i>ori</i> _{pBBR1} , Tet ^R , <i>mob</i> ⁺ , <i>P_{dhfr}</i>	(Flannagan <i>et al.</i> , 2008)
pDONR™221	Gateway vector, Kan ^R	K. Haynes, Exeter
pBHR4- <i>groS</i> -RFP	Broad-host range vector containing far-red fluorescent protein TurboFP635, Cm ^R	(Wand <i>et al.</i> , 2011)
pDEST413	Gateway vector, Amp ^R , HIS3, CEN ORI <i>ccdB</i>	K. Haynes, Exeter
pDEST415	Gateway vector, Amp ^R , LEU2, CEN ORI <i>ccdB</i>	K. Haynes, Exeter
pDEST413:: <i>dpl1</i>	Gateway vector, Amp ^R , HIS3, CEN ORI <i>ccdB</i> , <i>dpl1</i>	This study
pDEST413:: <i>BTH_II0309</i>	Gateway vector, Amp ^R , HIS3, CEN ORI <i>ccdB</i> , <i>BTH_II0309</i>	This study
pDEST415:: <i>BTH_II0311</i>	Gateway vector, Amp ^R , LEU2, CEN ORI <i>ccdB</i> , <i>BTH_II0311</i>	This study

Resistance determinants: Tet^R, Tetracycline; Tp^R, Trimethoprim; Cm^R, Chloramphenicol; Amp^R, Ampicillin; Kan^R, Kanamycin.

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