Objective Central to the pathogenesis of many bacterial pathogens is their ability to survive and replicate within mammalian cells, and disrupt cellular pathways within the host cell. This project focuses on one potential strategy employed by such intracellular bacteria – bacterial-encoded sphingosine-1-phosphate lyases (S1PLs). S1PLs irreversibly degrade sphingosine-1-phosphate (S1P), a bioactive metabolite of sphingolipid metabolism within mammalian cells. S1P promotes mammalian cell survival (through control of programmed cell death), proliferation and migration, and also plays an essential role in diverse aspects of immune function, particularly immune cell trafficking [1]. By depleting S1P pools within mammalian cells, S1PL can perturb these various functions. Additionally, degradation of S1P by S1PL produces hexadecenal, which itself can cause wide-ranging effects on mammalian cells, including cytoskeletal reorganisation. Mammalian S1PLs are directly implicated in numerous physiological & pathological processes, and consequently S1PL inhibitors have been developed and are in clinical trials [2]. S1PLs of intracellular bacteria represent an exciting novel therapeutic target, exploiting existing knowledge of eukaryotic S1PLs & their inhibitors.

To date, two prokaryotic S1PLs have been described [3; 4], most recently within Legionella pneumophila. The Legionella S1PL is secreted by the bacterium and is predominantly targeted to the host cell mitochondria [4]. Analysis of available genome sequences of Burkholderia pseudomallei, an intracellular pathogen that causes the disease melioidosis, reveals the presence of two distinct paralogs of S1PL. The catalytic regions of these putative S1PLs are well conserved; all amino acids previously reported to be critical for activity of human, yeast and/or Legionella S1PL activity are entirely conserved in both B. pseudomallei paralogs. Transcriptomic analysis of B. pseudomallei has shown that both S1PLs (genes BPSS2021 & BPSS2025) are expressed, and that their expression appears to be co-ordinately regulated in response to defined stresses, despite the fact that they are not linked within an operon. To date, working with Dr Dominic Campopiano of the University of Edinburgh’s School of Chemistry, we have cloned, expressed and purified one of the S1PLs from B. pseudomallei (BPSS2021), and have demonstrated that it does indeed possess S1PL activity.

We have also identified S1PL homologues in other intracellular bacteria, including Listeria spp. and Mycobacterium spp., suggesting that degradation of S1P in mammalian host cells may be a strategy of such intracellular pathogens for subverting host cells. Using Burkholderia as a model organism, this project will aim to define the role of bacterial-encoded S1PLs during infection, assessing both the importance of S1PL activity to the bacterium and the consequences of bacterial S1PL activity on the mammalian cell. Together, these studies will assess bacterial S1PLs as therapeutic targets for intracellular pathogens.

Research Programme

1. Assessing the role of Burkholderia S1PLs in virulence & bacterial physiology

Unmarked deletion mutants of the two S1PL paralogs (single & double mutants) will be made using the method based on the homing endonuclease I-SceI [5]. The resulting mutants (and corresponding complemented strains) will be assessed for a range of standard phenotypes, including virulence in the Galleria mellonella and macrophage infection models, assays for bacterial cell membrane integrity & permeability, growth in minimal media and survival following nutrient deprivation. Nutrient deprivation and growth in minimal media have been shown to result in differential expression of both putative S1PLs in B. pseudomallei (unpublished data).

2. Investigating the Burkholderia S1PL activity & localization

The Burkholderia S1PLs will be used to complement an s1pl-deficient mutant of Saccharomyces cerevisiae to confirm whether one or other or both Burkholderia paralogs are functionally equivalent to eukaryotic S1PLs.

Additionally, whilst the Legionella & Burkholderia S1PL homologues (as annotated within available genome sequences) are predicted to be cytoplasmic, experimental investigation of the Legionella
S1PL shows it to be secreted [4]. Furthermore, study of the predicted amino acid sequences of the *Burkholderia* S1PLs has revealed some ambiguity as to the precise location of the start of the protein, with the result that the protein may include an N-terminal signal sequence. The localization of both *Burkholderia* paralogs will be determined by tagging each encoded protein with an appropriate tag (6His or GFP), and using relevant detection methods to localize the protein.

### 3. Assessing the impact of *Burkholderia* S1PLs on host cells during infection

Wild-type and S1PL-deficient mutants (single and double) of *Burkholderia* will be used to infect macrophages, enabling assessment of phenotypes including multi-nucleate giant cell formation, cytoskeletal organization and cytotoxicity. Additionally, following infection, the concentration of mammalian S1P will be monitored using a commercially-available S1P ELISA assay (Echelon Biosciences) to determine if the pool of cellular S1P is depleted during infection. *Burkholderia* encoding tagged S1PLs (6His or GFP) will also be used in macrophage infection assays, to explore S1PL secretion *in vivo*, and establish where the proteins localize to (if secreted).

### 4. The impact of S1P pathway modulators on *Burkholderia* infection

Macrophage and *G. mellonella* infection models will be repeated in the presence of available S1PL inhibitors and/or S1P receptor agonists (either through inclusion in culture media, or via co-injection), to determine how disruption of the S1P pathway alters the course of infection.

**Benefits of proposed technology**

This proposal uses established methods to investigate a novel aspect of the pathogenesis of intracellular bacteria that offers real potential for therapeutic intervention. The design of the study will ensure that the role of bacterial S1PL in infection is investigated from both ‘viewpoints’ (pathogen & host). This detailed investigation of S1PLs in *Burkholderia* will represent a novel extension to the existing S1PL literature.

**Challenges**

There is a risk that disruption of the two *Burkholderia* S1PLs (singly or in combination) will not result in a detectable phenotype. However, by employing a wide range of assays, we will mitigate that risk. Additionally, tagging of S1PL to enable its localization may impact on protein activity, but different approaches are available (e.g. 6His/GFP, C-terminal/N-terminal) to ensure success. All constructs will be fully validated to ensure enzyme activity is retained.

**Maturity of Technology**

The techniques to be employed are mature, and many of them are used extensively within our laboratory and/or within our in-house Bioimaging Facility (where the microscopy will be performed). What is novel is the synthesis of these techniques into a single programme to address the role of S1P/S1PL in bacterial pathogenesis.

**Major goals/milestones by fiscal year**

- **Year 1**: Task 1. Construction & phenotypic investigation of strains (single/double mutants & complemented). Task 2(a). Complementation of *S. cerevisiae* mutant. **Year 2**: Task 2(b). Construction of tagged S1PL constructs (6His / GFP) and verification to confirm S1PL activity is retained. Localization of protein. Task 3. Optimization of ELISA & confocal methods, and initial investigation. **Year 3**: Task 3. Continued investigation of the impact of *Burkholderia* S1PLs on mammalian host cells. Task 4. The impact of S1P pathway modulators on *Burkholderia* infection.

**References:**