Burkholderia pseudomallei kynurenine formamidase plays a role in the regulation of phenotypes associated with chronic disease

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ABSTRACT

Kynurenine formamidase (KynB) forms part of the kynurenine pathway which metabolises tryptophan to anthranilic acid. This metabolite can be used for downstream production of HAQ signalling molecules that control virulence in *Pseudomonas aeruginosa.* Here we investigate the role of *kynB* in the production of H(M)AQs and virulence potential of *B. pseudomallei* K96243, the causative agent of melioidosis. Deletion of *kynB* resulted in reduced H(M)AQ production, increased biofilm formation, decreased swarming and increased tolerance to ciprofloxacin. Addition of spent media isolated from wild type *B. pseudomallei* or adding exogenous anthranilic acid could complement some of these phenotypes. This study suggests the kynurenine pathway is a critical source of anthranilic acid and signalling molecules that regulate phenotypes which are associated with chronic disease.

Burkholderia pseudomallei is a motile, Gram-negative, environmental bacterium that resides in soil and stagnant water as a saprophyte in south east Asia and northern Australia [1]. *B. pseudomallei* is an opportunistic human pathogen and through inhalation or entry via skin abrasions, causes the infectious disease melioidosis [2]. Melioidosis has a mortality rate of up to 44% and is the third most frequent cause of death from infections disease in North East Thailand, despite antibiotic intervention [3]. The bacteria can also establish an asymptomatic latent infection with symptoms occurring years post exposure, or manifest as a chronic recurring infection with up to 15% of patients relapsing following completion of therapy [4]. Due to its intrinsic resistance to many antibiotics, a high mortality rate, chronic disease manifestations and infection by the airborne route *B. pseudomallei* is also considered a potential bioterrorism agent [5,6].

Quorum sensing systems are involved in cell-to-cell signalling and through secondary metabolites control and regulate many genes associated with virulence. Increasingly, these systems are attractive targets for novel therapeutics [7,8]. Mutation of quorum sensing apparatus in *B. pseudomallei* results in an increased time to death in mice and hamster models [9,10]. *B. pseudomallei* possesses 3 *luxI* and 5 *luxR* quorum sensing genes and synthesizes a range of signalling molecules, including N-octanoyl-homoserine lactone, N-decanoyl-homoserine lactone, N-(3-hydroxyoctanoyl)-L-homoserine lactone, N-(3-hydroxydecanoyl)-L-homoserine lactone and N-(3-oxotetradecanoyl)-L-homoserine [9]. *B. pseudomallei* also contain the genes *hhqABCDE (hmqABCDE)* which are homologous to *pqsABCDE* from *Pseudomonas aeruginosa.* In *P. aeruginosa,* these genes code for a biosynthetic pathway required for the synthesis of 2-alkyl-4(1H)-quinolones (HAQs), 2,4-dihydroxyquinoline (DHQ) and 2-aminoacetophenone (2-AA) [11-13]. Inactivation of HAQ synthesis through deletion of *pqsE*, or *pqsA* decreases *P. aeruginosa* virulence in a mouse acute infection suggesting this is a good target for novel antimicrobial therapeutics [14].

Previous analysis of *B. pseudomallei* culture supernatants revealed that the predominant HAQs in *B. pseudomallei* are HMAQs. These are HAQs that contain an unsaturated aliphatic side chain that is typically methylated at the 3 position by a methyltransferase encoded by *hmqG* [15]. HAQ synthesis requires anthranilic acid. In *P. aeruginosa,* this metabolite is derived via three distinct pathways. Two of the pathways encoded by *phnAB* or *trpEG* convert chorismic acid to anthranililc acid, while a third, the kynurenine pathway, converts tryptophan to anthranilic acid [16,17]. *B. pseudomallei* has no apparent *phnAB* homologs but does possess the TrpEG and kynurenine pathways. The kynurenine pathway consists of three enzymes encoded by *kynA* (tryptophan-2,3-dioxygenase), *kynB* (kynurenine formamidase), and *kynU* (kynureninase) [18]. The kynurenine pathway has previously been shown as a critical source of anthranilic acid for the production of PQS in *P. aeuroginosa* and the production of HMAQs in *B. thailandensis* and *Burkholderia ambifaria* through the incorporation of radiolabelled tryptophan [15,17]. Subsequently this pathway seems likely to be key in the

production of 4-quinolones (and many other secondary metabolites) in *B. pseudomallei* and be an important source of anthranillic acid for signalling during human infections. [17].

In this study we set out to investigate the role of the kynurenine pathway in *B. pseudomallei*. We show that deletion of *kynB* results in the loss of HAQ/HMAQs and affects phenotypes which are associated with chronic disease caused by other bacteria.

MATERIALS AND METHODS

Bacterial strains, growth conditions and chemicals

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria–Bertani (LB) broth at 37°C with shaking (200 rpm) or on LB agar plates at 37°C. Where appropriate, media was supplemented with chloramphenicol 25 µg/ml for selection of the pBHR or pDM4 plasmids. For growth in M9 media with tryptophan or L-kynurenine, these carbon sources were added at a final concentration of 5mM and cultures were grown for 48 hours.

Construction and complementation of the *B. pseudomallei* K96243 Δ*kynB* mutant

PCR was used to amplify both 500bp upstream of the *kynB* gene using primers KynB_1 (5'-GTATCCCGGGAAATCGGAGTAGTGCTTCAG-3') and KynB_2 5'-GCATGCGTCTTCAGAATTCCATGCCGTTGTCGAAGAAACT-3') and 500bp downstream using KynB_3 (5'-CGACAACGGCATGGAATTCTGAAGACGCATGCGCGTGCCC-3' and KynB_4 (5'-CCGTACTAGTGTCGATGAGGCGCAGTTCGT-3') The PCR product also contained regions of sequence that were specific for the start and end of the *kynB* gene in order for homologous recombination to occur. The upstream and downstream PCR products were then digested with *Eco*RI and ligated together to create the knockout cassette. This was then cloned into pDM4 [29] via the *Xma*I and *Spe*I restriction sites. The resulting plasmid was transformed into *E. coli* DH5α λpir cells and then conjugated into *B. pseudomallei* K96243 to create a merodiploid strain with the aid of the helper strain *E. coli* pKR2013. Colonies were plated onto LB agar supplemented with 10% (w/v) sucrose to drive *sacB* expression in pDM4 and promote recombination. Δ*kynB* mutants were confirmed by PCR and sequencing of the resulting DNA fragment. For construction of pBHR-*kynB*, the *kynB* gene was PCR amplified using primers kynB_comp_fwd (5'-GGCTCACTAGTATGGACACGATCTGGGAC-3') and kynB_comp_rv 5'-GCCATGATATCCACGCGCATGCGTCTTC-3') and cloned into *Spe*I and *Eco*RV sites of pBHR4.

Biofilm assay

Cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were grown on tryptic soy agar (TSA) plates at 37°C for 16 hour and then inoculated into PBS standardising to OD_{590}nm 1.0. Cultures were then diluted 1:2000 in fresh tryptic soy broth (TSB) or filter sterilised spent media taken from an overnight culture of *B. pseudomallei* K96243 grown in TSB. 150µl aliquots were added to a 96-well peg lidded plate (12 wells for each culture) and incubated at 37°C. After 24 hours the peg lid was transferred into fresh or spent TSB media for a further 24 hours. Peg lids were washed in PBS before drying at 65°C for 30 minutes. Peg grown biofilms were stained with crystal violet and the optical density measured at 490nm following 3 x PBS and an ethanol treatment to release the stain for quantification.

Swarming assay

Overnight cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were diluted to OD_{590}nm 1.0 in either LB or filter

sterilised spent media taken from an overnight culture of *B. pseudomallei* K96243 grown in LB. 2 µl samples were then spotted onto the centre of swarming plates (5 g/l bacto agar, 8 g/l nutrient broth N°2, 0.5%(w/v) glucose). Plates were then incubated for 24 hours at 37ºC and the distance swarmed measured with a ruler.

Persister assay

Overnight cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were standardised to OD_{590}nm 0.2 in LB or filter sterilised spent media taken from an overnight culture of *B. pseudomallei* K96243 grown in LB. 500 µl of culture was then mixed with 500 µl of 400 µg/ml (200 x MIC) ciprofloxacin in LB in a well of 24 well plate and incubated for 24 hours at 37ºC. Where appropriate anthranilic acid was also added to the persister assays at the indicated concentrations. After incubation, cells were washed in LB and plated onto LB agar or LB agar supplemented with chloramphenicol for enumeration.

Siderophore activity

Overnight cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were harvested by centrifugation. 3 µl of the supernantant was then spotted in triplicate onto CAS agar plates and incubated for 24 hours at 37ºC. Removal of iron from the agar was seen with a blue to orange colour change. Siderophore activity was measured by determining the area of this orange halo.

Protease activity

B. pseudomallei K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were grown in LB for 24 hours and then 1.5 ml aliquots were harvested by centrifugation. 500 µl of the supernantant added to 500 µl of 5mg/ml azocasesin. The samples were then incubated in a heat block for 30 mins at 37ºC. 1 ml of 3.2% TCA was then added and samples centrifuged at 13,000 rpm for 5 minutes. A 500 µl was then added to 500 µl of 0.5M NaOH. The optical density was then recorded at OD_{450}nm. Protease activity was obsserved as red to yellow colour change.

Metabolite extraction

5 ml overnight cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* were standardised to OD_{590}nm 2.0. Samples were then aliquoted into 1 ml fractions and cells were harvested by centrifugation for 7 minutes at 13,000 rpm. The supernatant was transferred to a fresh tube and 1 ml of acidified ethyl acetate (0.01% acetic acid) was added and mixed vigorously for 1 min. The organic upper layer was then transferred to a fresh tube. The lower solvent layer was then re-extracted as above and the two organic phases were combined. The cell pellet was re-suspended in 1 ml of methanol and incubated at room temperature for 10 minutes to lyse the cells. The samples were then centrifuged at 13,000 rpm for 7 minutes to remove cell debris and the supernatant transferred to a fresh tube. Both ethyl acetate (supernatant) and methanol extracted (cells) samples were then air dried to completeness and stored at -20ºC until mass spec analysis.

Mass spectrometry analysis

Dried samples were re-dissolved in 100 µl of methanol. Undissolved material was removed by centrifugation and the clarified sample transferred to an LC vial prior to analysis. 10µl injections of each sample were used.

The HPLC system used was a Waters 2795 separations module. The column oven was maintained at 35°C. The HPLC Column used was a Phenomenex Gemini C18 column (3.0 µm, 100 x 3.0 mm) with an appropriate guard column. Mobile phase A was water +0.1% (v/v)

formic acid, and mobile phase B methanol +0.1% (v/v) formic acid. The flow rate throughout the chromatographic separation was 450 µl/min. The binary gradient began initially at 10% B and ran isocratically for the first 1 min before increasing linearly to 99% B in 9.5 min. The gradient was then maintained at 99% B for 5 min. A decrease to 10% B occurred over 1 min, and stayed at this composition for 4 min. Total run time per sample was 20 min.

The MS system used was a Micromass Quattro Ultima triple quadrupole mass spectrometer equipped with an electro spray ionisation (ESI) interface. Instrument control, data collection and analysis were conducted using Masslynx software. The electrospray settings were: cone gas: 160 L/hr, desolvation gas: 760 L/hr, source temperature: 150°C, desolvation temperature: 350°C.

MS analysis was conducted under positive electrospray conditions (+ES) with the MS operating in precursor ion scan mode. The scan range was *m/z* 80-450 with the collision energy being ramped between 20-35 eV during each scan. Precursor scans of *m/z*= 173.1 were used for detecting methyl-AQs, with precursors of *m/z*=159.1 used for detecting AQs.

C57BL/6 infection study

Groups of 8 or 10 female C57BL/6 mice (6-8 week-old; Harlan Laboratories, Bicester, Oxon, UK) were used throughout the studies. All animal experiments were performed in accordance with the guidelines of the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee at the London School of Hygiene and Tropical Medicine. For each infection, aliquots were thawed from frozen bacteria stocks and diluted in pyrogen-free saline (PFS). Prior to intranasal (i.n.) infection, mice were anesthetized intraperitoneally with ketamine (50mg/kg; Ketaset; Fort Dodge Animal, Iowa, USA) and xylazine (10 mg/kg; Rompur; Bayer, Leverkusen, Germany) diluted in PFS. Challenge was performed administering a total volume of 50 µl i.n. containing approximately 200 or 2500 colony forming units of *B. pseudomallei* K96243 wild type or isogenic Δ*kynB* mutant. Infection dose was confirmed as described elsewhere [19]. Control uninfected mice received 50 µl of PFS.

RESULTS

kynB (BPSL0848) encodes a functional kynurenine formamidase

To confirm that BPSL0848 encodes a functional kynurenine formamidase, *B. pseudomallei* K96243, *B. pseudomallei* Δ*kynB* and *B. pseudomallei* Δ*kynB*/pBHR-*kynB* strains were grown in M9 media supplemented with either typtophan or kynurenine as the sole carbon source. When the strains were inoculated into M9 with tryptophan, (the substrate for the kynurenine pathway), the wild type and complemented strains were able to grow, whereas no growth was seen with the Δ*kynB* mutant (Fig 1a). In contrast, when the strains were inoculated into M9 with kynurenine (the product for kynurenine formamidase) the medium was growth permissive for all strains (Fig 1b).

Deletion of Δ*kynB* resulted in the production of fewer HAQs

Overnight cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* were harvested by centrifugation and the supernatant and cell pellets were separated and metabolites were solvent extracted. Samples were then analysed by LC-MS looking for families of saturated, unsaturated and saturated methyl HAQs (Table 2). The data revealed that wild type *B. pseudomallei* K96243 and the complement strain contained 3 saturated methyl HAQs, 2 saturated HAQs and 2 unsaturated HAQ types. 2 out of the 3 methyl HAQs were absent in the Δ*kynB* mutant and the third had a

reduced peak area. The 2 saturated HAQs were also absent from the Δ*kynB* mutant as was one unsaturated HAQ. The other unsaturated HAQ had a reduced peak area in the mutant.

Deletion of *kynB* significantly increased biofilm production

Since KynB converts kynurenine into anthranilic acid, a precursor for signalling molecules such as HHQ and 2-AA, we assessed the effect of deleting *kynB* on biofilm production. *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* were grown for 48 hours in TSB at 37°C. Peg lids inserted into the cultures were used for the growth of biofilms and this was quantified after crystal violet staining (Fig 2). When the wild type and complemented strains were grown in TSB the OD_{490}nm following crystal violet staining was approximately 0.1-0.15. In contrast the OD_{490}nm for *B. pseudomallei* K96243 Δ*kynB* was significantly greater at 0.30 ($p<0.0001$).

Deletion of *kynB* significantly decreased bacterial swarming

Next, the effect of deleting *kynB* on swarming motility was evaluated. Stationary phase cultures of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* were standardised to an OD_{590}nm 1.0 and then spotted onto swarming agar plates and incubated for 24 hours at 37°C. On average the wild type and complemented strains swarmed approximately 35 and 25 mm respectively from the point of inoculation (Fig 3a). In contrast the Δ*kynB* mutant strain swarmed significantly less at approximately 15 mm ($p<0.05$ or $p<0.0001$). The mutant also had a different swarming pattern, forming small branch like structures that were absent in the wild type and complemented strains (Fig 3b).

Deletion of *kynB* increased tolerance to ciprofloxacin

The effect of deleting *kynB* on antibiotic tolerance was then tested. Firstly, stationary phase cultures of *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* were standardised to an OD_{590}nm 0.1 (10^8 CFU/ml) and incubated with a range of ciprofloxacin concentrations for 24 hours to determine the MIC. The MIC of ciprofloxacin for both strains was approximately 2 µg/ml (Fig S1). Subsequently, *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* were standardised to an OD_{590}nm 0.1 (10^8 CFU/ml) and incubated with 100 x MIC (200 µg/ml) ciprofloxacin for 24 hours. On enumeration of surviving bacteria, there were approximately 10-fold more *B. pseudomallei* K96243 Δ*kynB* compared to the wild type and complement strains (Fig 4a, ~10^{-3} compared to ~10^{-4}). This difference was significant ($p<0.0001$ or $p<0.001$).

To determine whether the difference in antibiotic tolerance between *B. pseudomallei* K96243 and *B. pseudomallei* K96243 Δ*kynB* was due to a difference in the persister cell populations, these strains were treated with 100 x MIC ciprofloxacin and cell numbers determined periodically over 30 hours. A biphasic kill curve was observed for both strains and the population of antibiotic survivors after 24 and 30 hours was greater for the Δ*kynB* mutant compared to wild type (Fig 4b).

Treating *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* with 100 x MIC ceftazidime (200 µg/ml) resulted in no significant difference in survival frequencies on enumeration. All three strains showed approximately 10% survival (data not shown).

Deletion of *kynB* did not affect siderophore activity, protease activity or virulence in C57BL6 mice

Spot plating standardised overnight cultures of the *B. pseudomallei* strains onto CAS agar plates revealed no difference in siderophore activity. Likewise, a colorimetric assay to detect protease activity in spent media isolated from *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* showed no difference in absorbance (data not shown).

Infection of C57BL/6 mice with *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* resulted in similar patterns of survival. After challenge with approximately 3300 CFU of wild type or 2,200 CFU of the Δ*kynB* mutant all mice had died by 4 days post infection. After challenge with 60 CFU of wild type or 145 CFU of the Δ*kynB* mutantsimilar numbers of mice had died at the termination of the study on day 170 (data not shown).

Addition of spent media from *B. pseudomallei* K96243 can partially suppress the hyperbiofilm phenotype of Δ*kynB* but had no effect on the antibiotic tolerance or swarming phenotypes

B. pseudomallei K96243 or *B. pseudomallei* K96243 Δ*kynB* were inoculated into a 96- well plate and grown for 48 hours in either TSB or spent TSB media that had been isolated from a culture of wild type *B. pseudomallei* K96243. Peg lids inserted into the cultures were used to cultivate the biofilms. Biofilm growth was quantified by crystal violet staining and measuring the optical density. When the wild type strain was grown in either TSB or spent media the OD_{490}nm following crystal violet staining was approximately 0.1-0.15 (Fig 5a). In contrast the OD_{490}nm for *B. pseudomallei* K96243 Δ*kynB* varied depending on the growth media. In fresh TSB media the OD_{490}nm was approximately 0.35, whereas in spent media the optical density was significantly reduced to 0.25 ($p<0.0001$).

When *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* were resuspended in spent media from K96243 and spotted onto swarming agar, the bacterial strains swarmed to a similar distance as the corresponding strain in fresh media (Fig 5b). Likewise, resuspending the strains in K96243 spent media before ciprofloxacin treatment had no effect on persister frequency compared to fresh LB (Fig 5c).

Addition of exogenous anthranilic acid reduced the hyperbiofilm phenotype but increased bacterial persistence.

Next we asked whether supplementing *B. pseudomallei* Δ*kynB* with anthranilic acid (the end product of the kynABU pathway) could complement the biofilm and persister phenotypes. Firstly *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* were inoculated into a 96-well plate and grown for 48 hours in either TSB or TSB media supplemented with 0, 1 or 10 mM anthranilic acid. Addition of 1 mM anthranilic acid to the wild type strain had no effect on biofilm growth compared to a non-supplemented control, whereas addition of 10 mM was able to partially reduce biofilm growth (fig 6a). In comparison, addition of anthranilic acid to the Δ*kynB* mutant was able to significantly reduce biofilm formation in a step wise manner compared to the non-supplemented Δ*kynB* control ($p<0.0001$). 10 mM anthranilic acid was able to repress biofilm formation of the Δ*kynB* mutant to wild type levels.

Next overnight cultures of *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* were standardised to OD_{590}nm 0.1 and incubated with 100 x MIC ciprofloxacin and either 0, 1 or 10 mM anthranilic acid for 24 hours (fig 6b). Following incubation cells were washed and plated onto LB agar plates for enumeration. When cells were treated with ciprofloxacin only, persister frequencies for both wild type and Δ*kynB* were similar to previous persister assays. Addition of anthranilic acid significantly increased persister frequencies of the wild type strain in a relative manner, i.e. 10-fold more anthranilic acid resulted in approximately 10-fold more persisters. Increasing the concentration of anthranilic acid also increased persister frequencies for the Δ*kynB* mutant. Supplementation of 10 mM anthranilic acid increased both *B. pseudomallei* and *B. pseudomallei* K96243 Δ*kynB* persister frequencies to comparable levels at around 5×10^{-2}.

DISCUSSION

There are many manifestations of melioidosis in humans, ranging from acute to chronic disease and to a latent and often inapparent infection which can spontaneously re-appear as overt disease [2] . Chronic infection accounts for approximately 11% of patients and is characterised by symptoms lasting >2 months [2,20]. The treatment of disease can be difficult because of the high degree of resistance to many antimicrobial drugs. Even following apparently successful drug intervention, relapse occurs in 10-30% of cases [20]. Recurrent disease is a occurs in 6-13 % of patients. After primary exposure, sometimes accompanied with symptomatic disease, the infection becomes latent, only to appear as symptomatic disease up to several decades later [2,21]. The intrinsic resilience of *B. pseudomallei* to harsh environments such as nutrient depletion, salt concentrations and acidic environments may also have important ramifications in establishing chronic disease in humans [22,23].

As with *B. pseudomallei, P. aeruginosa* infections can also be difficult to treat. In *P. aeruginosa* the bacterium is able to switch between phenotypes associated with chronic and phenotypes associated with acute disease. During acute infection, cells express a wide variety of virulence factors including type III secretion systems, flagella and QS-regulated virulence factors such as proteases, elastases, phenazines, and toxins that allow the establishment of infection. [24-26]. In this state the bacteria are more susceptible to antibiotic treatment. Conversely, the onset of chronic disease is associated with the development of biofilm, hypermutability, conversion to mucoidy, acquisition of resistance and suppression of the expression of acute phase virulence factors and the bacteria are less susceptible to antibiotic treatment [27-29]. This switch from acute to chronic phase involves the RetS-LadS-GacSA-Rsm regulatory cascade, with RetS and LadS having opposing effects on the GacS sensor kinase [30-32].

The onset of chronic disease caused by many other bacterial species is associated with biofilm formation. For example, venous leg ulcers have been associated with *Staphylococcus aureus* biofilms [33] while biofilms play a key role in recurrent urinary tract infections caused by uropathogenic *Escherichia coli* [34]. The cells in these biofilms are resistant to antibiotics, partially as a consequence of the physical constraints on the ability of antibiotics and antimicrobials to fully penetrate and kill all the cells [35,36] and partly because biofilms are also rich in drug-tolerant persister cells [37]. Biofilms and persister cells may be important reservoirs in chronic and recurring melioidosis.

The kynurenine pathway plays a role in regulating a range of phenotypes associated with chronic disease including biofilm formation, swarming and persistence. Biofilm formation and swarming are inversely correlated in *P. aeruginosa* and this is also our finding in *B. pseudomallei* [38]. The Δ*kynB* mutant of *B. pseudomallei* produced more biofilm than wild type and swarmed less, producing branched like structures. Previous studies with *P. aeruginosa*

have shown that the *pqs* operon was important in controlling these phenotypes [39] [40] [41]. Deletion of *pqsH* decreased biofilm density, while incubation of the mutant with exogenous PQS increased biofilm density [39]. However, previous studies have shown that *B. pseudomallei* does not produce PQS [15,42] and we found no evidence that the K96243 strain produces HHQ. Our data suggests that other HAQ(s) regulate biofilm and swarming in *B. pseudomallei* K96243.

Our results also showed that the *B. pseudomallei* Δ*kynB* mutant was more tolerant to ciprofloxacin due to an increase in the persister population but there was no difference in the frequency of ceftazidime persisters. Previous studies suggest that ceftazidime reveals persisters with anaerobic-like metabolism, whereas ciprofloxacin reveals persisters with reduced metabolism [43,44]. Quorum sensing can also influence the appearance of ciprofloxacin persisters; deletion of the HAQ synthesis gene *pqsA* in *P. aeruginosa* increased ciprofloxacin tolerance, while overexpressing HAQs increased susceptibility [45].Other quorum sensing molecules have been linked to persistence in other bacteria. For example, the addition of pyocyanin, paraquat or acyl homo serine-lactone significantly increased persister numbers of logarithmic phase *P. aeruginosa* [46] while indole increased persistence in *E. coli* [47]. It therefore seems possible that increase in frequency of ciprofloxacin persister cells reflect the disruption of signalling cascades in the *kynB* mutant.

The kynurenine pathway can feed anthranilic acid into the HAQ/HMAQ quorum signalling pathway and deletion of *kynB* reduced the level of these signalling molecules in the bacterial culture supernatant.

Since the Δ*kynB* mutant produced fewer HAQ/HMAQs, we attempted to complement the mutant by addition of spent media isolated from wild type cultures or chemically complement with the kynurenine pathway product anthranilic acid. Previous assays have shown that supplementing exogenous anthranilic acid to kynurenine pathway mutants can restore the production of HAQ compounds [17]. Both spent media and anthranilic acid reduced hyper biofilm production in the Δ*kynB* mutant, with 10 mM anthranilic acid restoring production to the same level as the wild type. This suggests that both anthranilic acid and HAQ/HMAQ production are important in biofilm regulation.

Spent media from wild type cultures was unable to complement the reduced swarming or increased ciprofloxacin persister phenotypes. Failure to complement these phenotypes maybe due to HAQ/HMAQs in the supplemented spent media being below a threshold needed to elicit a response in these assays. However, anthranilic acid complementation also failed to reduce the perister frequencies of the Δ*kynB* mutant. This suggests that the persister phenotype is not due to the loss of anthranilic acid or HAQ/HMAQ production. Paradoxically, at the highest concentration of anthranilic acid tested (10 mM) the persister frequency of both wild type and Δ*kynB* were both increased to a similar level. Clearly there is complex interplay between different regulatory mechanisms controlling persistence that warrants further study.

Quorum sensing pathways are attractive targets for the design of novel therapeutics in many bacteria, as they offer an alternate approach to treat infection by targeting virulence rather than through the inhibition of bacterial growth.

Deletion of *kynB* did not affect virulence of *B. pseudomallei* in a C57BL/6 mice. Thepathogenesis of experimental melioidosis in this strain of mice is reported to shown some similarities to chronic human disease [48]. Importantly, our results indicate that the phenotypes we have investigated, and which are associated with long-term infections caused by other bacteria, do not appear to be associated with chronic disease caused by *B. pseudomallei*. However, it is important to highlight that it is not known whether the C57BL/6 model of disease reflects the long-term and often clinically insignificant form of melioidosis which can re-appear

periodically. The development of a murine model of latent infection would allow the role of KynB in long trem infections to be further investigated.

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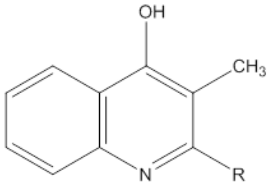
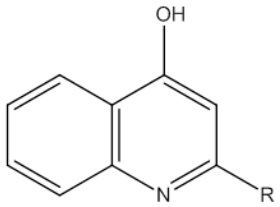
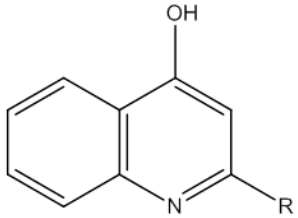
Table 1 Bacterial strains used or created in this study

Bacterial Strain	**Genotype/ comments**	**Source**
E. coli DH5α λ*pir*	ΔlacU169(ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen	Lab strain collection
E. coli DH5α (pRK2013)	ΔlacU169(ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, pRK2013 (KmR oriColE1 RK2-Mob$^+$ RK2-Tra$^+$	Lab strain collection
B. pseudomallei K96243	Clinical isolate	Lab strain collection
B. pseudomallei K96243 *ΔkynB*	K96243 derivative. Unmarked deletion *ΔkynB*	This study
Plasmid	**Comments**	**Source**
pBHR-*kynB*	camR	This study
pDM4	camR	[49]

Table 2. Peak areas of H(M)AQs following LC-MS analysis of extracted supernatant and whole bacterial cells. ND= not detected.

Saturated Methyl AQs			Supernatant Extracts			Cell Extracts		
	R	[M+H]⁺	K96	ΔKynB	Comp	K96	ΔKynB	Comp
	C_5H_{11}	230	ND	ND	ND	ND	ND	ND
	C_6H_{13}	244	ND	ND	ND	ND	ND	ND
	C_7H_{15}	258	7862	ND	6343	6058	ND	2144
	C_8H_{17}	272	ND	ND	ND	1133	ND	ND
	C_9H_{19}	286	38934	1230	21153	65947	727	37570
	$C_{10}H_{21}$	300	4353	ND	1988	12076	ND	6404

Saturated AQs			Supernatant Extracts			Cell Extracts		
	R	[M+H]⁺	K96	ΔKynB	Comp	K96	ΔKynB	Comp
	C_5H_{11}	216	ND	ND	ND	ND	ND	ND
	C_6H_{13}	230	ND	ND	ND	ND	ND	ND
	C_7H_{15}	244	ND	ND	ND	ND	ND	ND
	C_8H_{17}	258	ND	ND	ND	ND	ND	ND
	C_9H_{19}	272	2411	ND	6996	3344	ND	1732
	$C_{10}H_{21}$	286	2659	ND	4202	ND	ND	ND
	$C_{11}H_{23}$	300	ND	ND	ND	ND	ND	ND

Unsaturated AQs			Supernatant Extracts			Cell Extracts		
	R	[M+H]⁺	K96	ΔKynB	Comp	K96	ΔKynB	Comp
	C_5H_9	214	ND	ND	ND	ND	ND	ND
	C_6H_{11}	228	ND	ND	ND	ND	ND	ND
	C_7H_{13}	242	ND	ND	1345	ND	ND	ND

C_8H_{15}	256	ND	ND	ND	ND	ND	ND
C_9H_{17}	270	22564	1250	43537	21279	464	10201
$C_{10}H_{19}$	284	2438	ND	2477	5035	ND	1748
$C_{11}H_{21}$	298	ND	ND	ND	ND	ND	ND

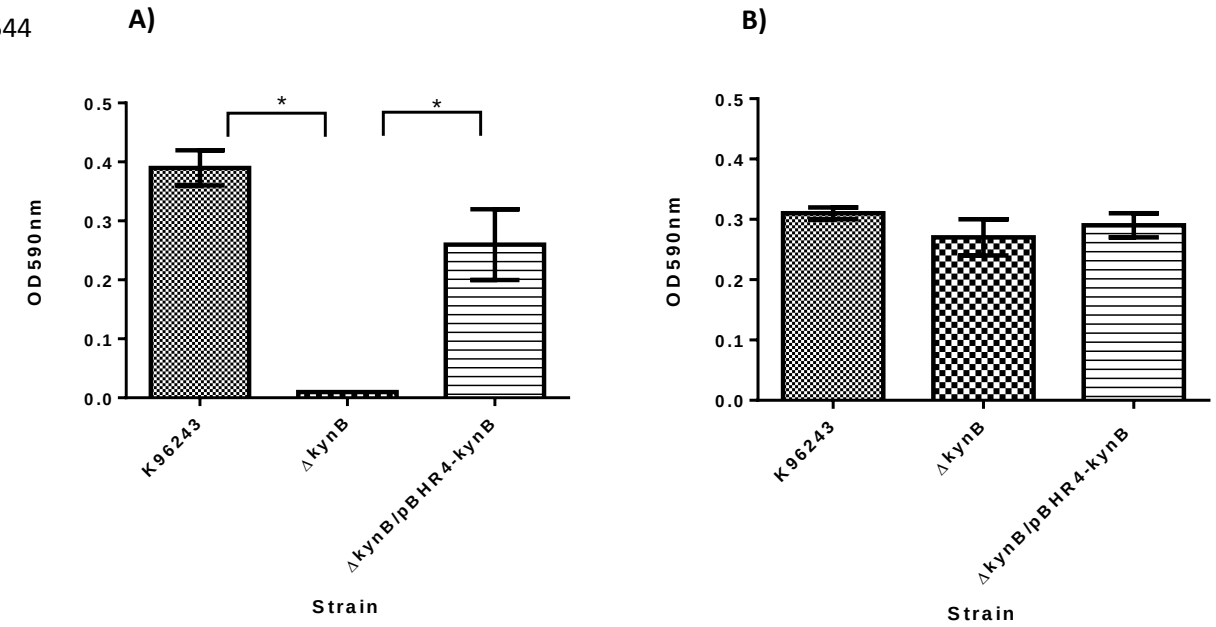


Fig 1. Growth of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB* Δ*kynB*/pBHR-*kynB* strain in M9 media with A) 5mM Tryptophan as the only carbon source B) 5mM L-kynurenine as the only carbon source. Cultures were grown for 48 hours at 37°C, 200rpm in 3ml volumes. Data shown are the average of two biological replicates. Error bars show SEM. *=p<0.05 following one way Anova, Tukey post-test.

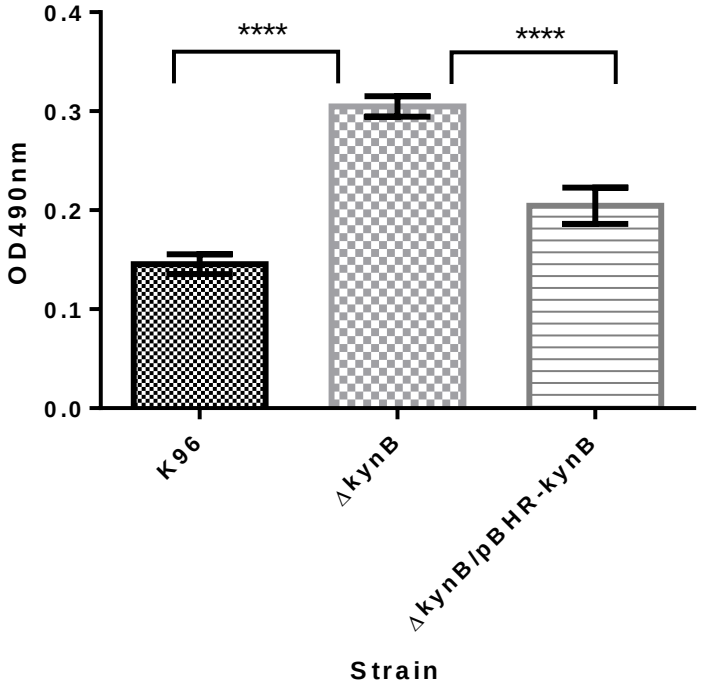


Fig 2. Biofilm production following growth of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB* Δ*kynB*/pBHR-*kynB* on peg lids for 48 hours. Biofilm was quantified by measuring crystal violet released following ethanol treatment. Data shown is the average of two biological repeats. Error bars show SE. ****= p<0.001 following one way Anova, Tukey post-test.

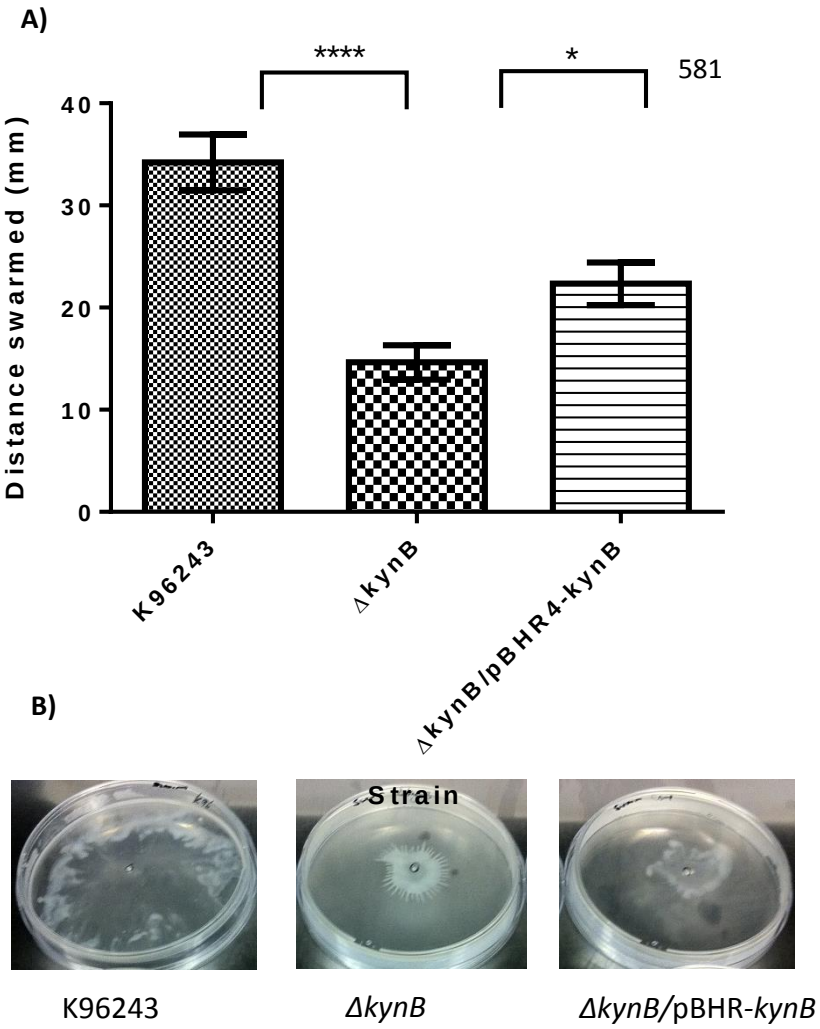
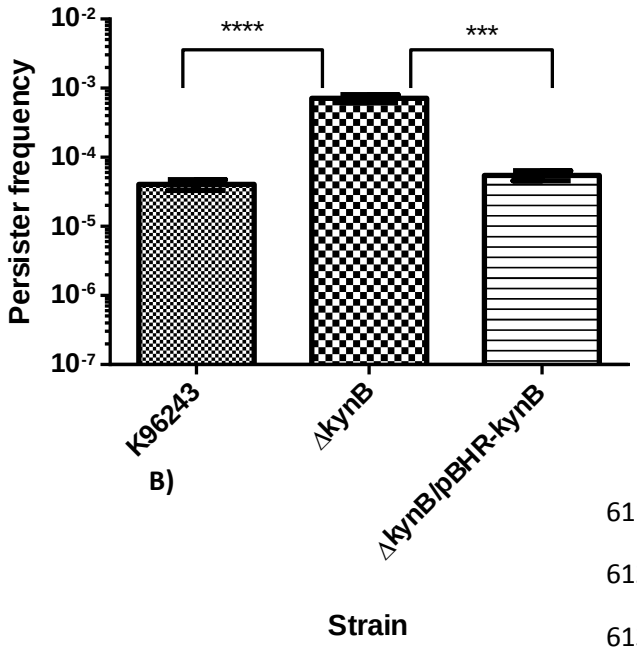


Fig 3. Swarming of motility of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/pBHR-*kynB* on swarming agar for 24 hours at 37 ºC. A) Distance migrated. Data shown are the average of five biological repeats. **** = p<0.0001, *=p<0.05 following One way Anova, Tukey post-test. Error bars represent SEM. B) representative images



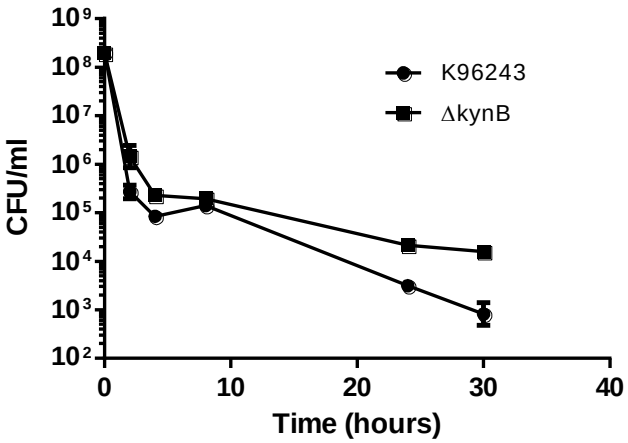
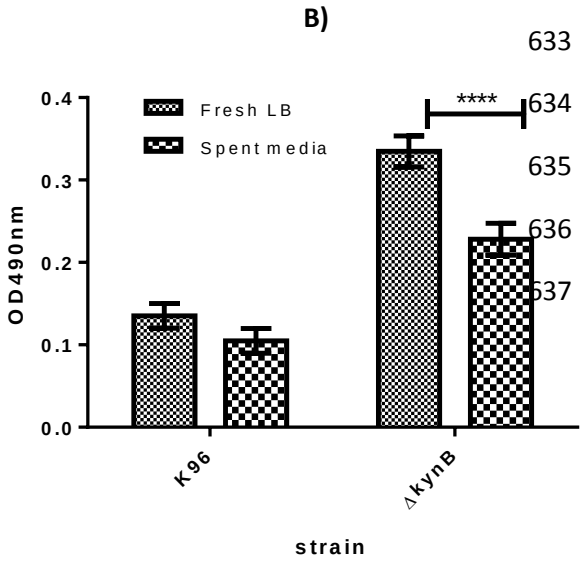
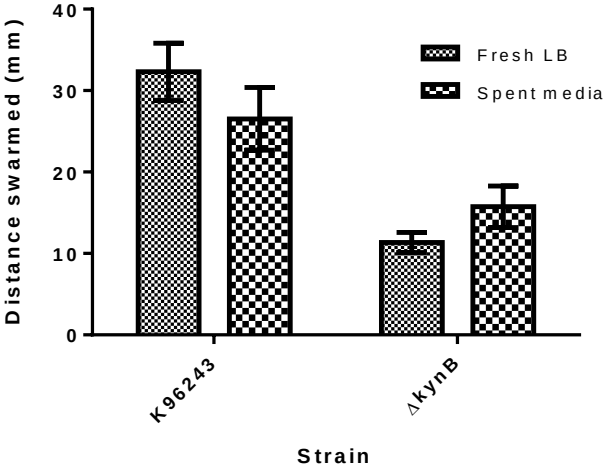


Fig 4.A) Persister frequency following incubation of *B. pseudomallei* K96243, *B. pseudomallei* K96243 Δ*kynB* or *B. pseudomallei* K96243 Δ*kynB*/ pBHR-*kynB* with 100 X MIC ciprofloxacin (200 µg/ml) for 24 hours at 37°C. Persister frequency was calculated as CFU post antibiotic treatment divided by CFU pre-treatment. Data shows the average of three biological replicates. ****= p<0.0001, ***=p<0.001.Error bars show SEM. B) Number of culturable cells of *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*kynB* over a 30 hour treatment with 100 x Mic ciprofloxacin.





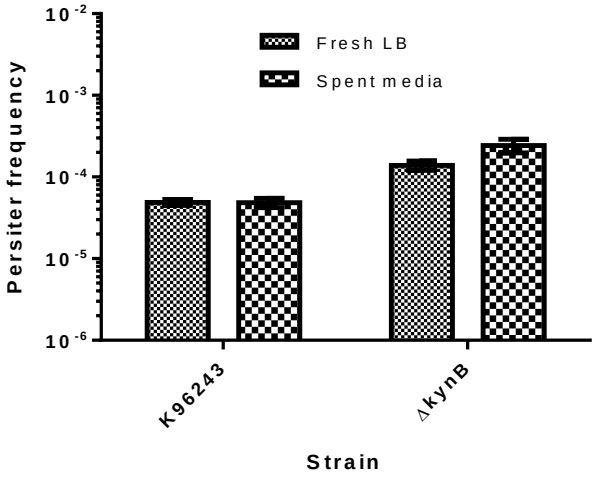


Fig 5. Complementation of *B. pseudomallei* K96243 and *B. pseudomallei* K96243 Δ*kynB* with the addition of spent media isolated from cultures of *B. pseudomallei* K96243. A) Biofilm formation following crystal violet staining. B) swarming distance C) Persister frequency following 24 hour ciprofloxacin treatment. Data are representative from at least three biological replicates. ****= p<0.0001, ***=p<0.001, **=p<0.01. Error bars show SEM.

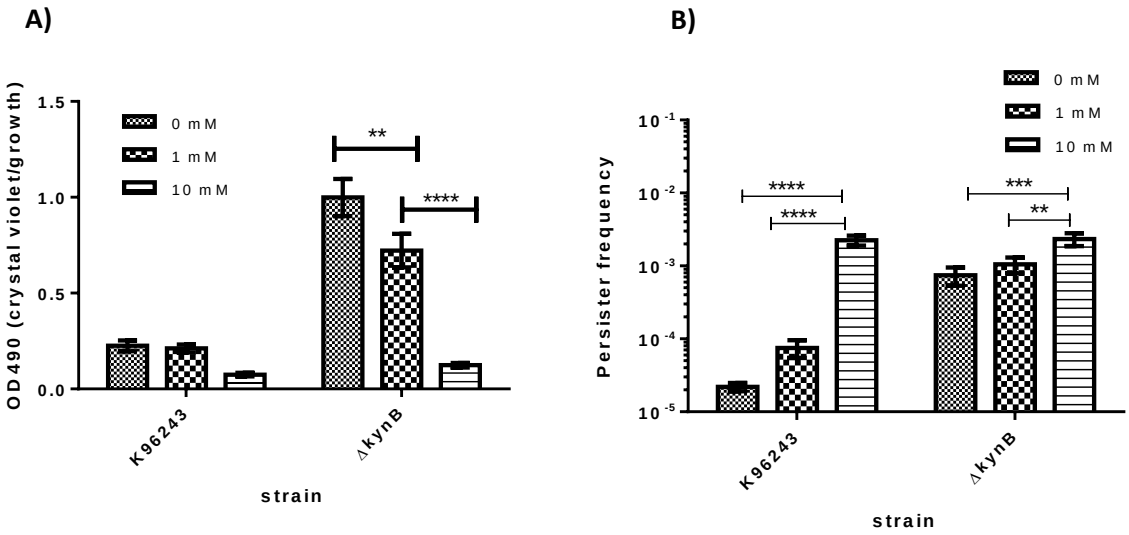


Fig 6. Complementation of *B. pseudomallei* K96243 and *B. pseudomallei* K96243 Δ*kynB* with exogenous anthranilic acid. A) Biofilm formation quantified by measuring the optical density following crystal violet staining. B) Persister frequency following 24 hours treatment with 100 X MIC ciprofloxacin. ****= p<0.0001, ***=p<0.001, **=p<0.01. Error bars show SEM following two-way Anova, Tukey post-test. Data is the average of at least 2 biological repeats.

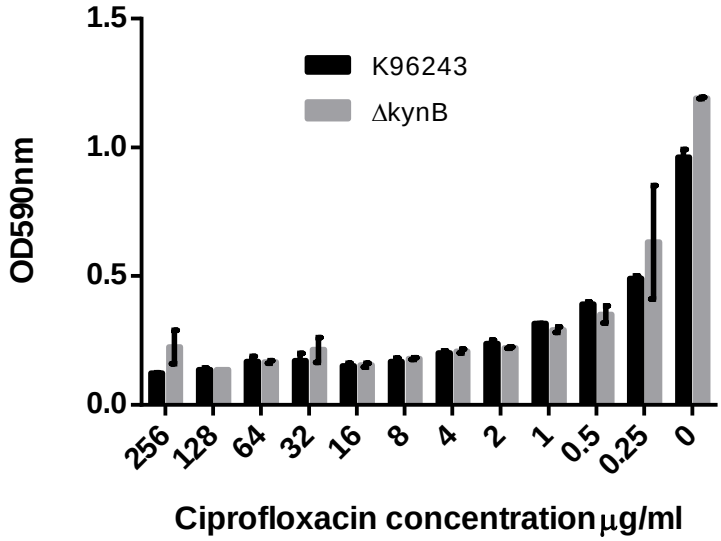


Figure S1. MIC determination of *B. pseudomallei* K96243 or *B. pseudomallei* K96243 *ΔkynB* treated with a range of ciprofloxacin concentrations for 24 hours. MIC was determined as the concentration of ciprofloxacin before an increase in optical density was observed