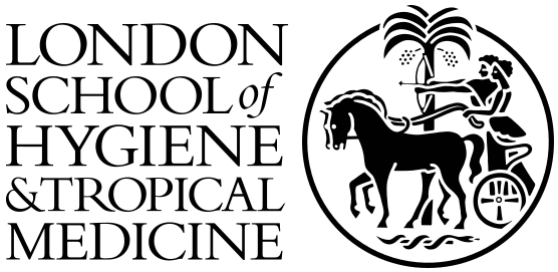

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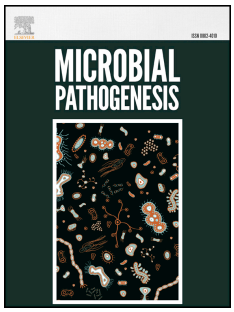

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A proteasome inhibitor produced by *Burkholderia pseudomallei* modulates intracellular growth

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Abstract

The NRPS/PKS cluster encodes the enzymes necessary for glidobactin synthesis it is partially conserved in various members of the *Burkholderia* genus including *B. pseudomallei*. In this study we have shown that the insertional inactivation or deletion of *glbC* in this cluster in *B. pseudomallei* could reduce the ability of the bacterium to survive or grow in murine macrophages or in human neutrophils. Exogenously added proteasome inhibitors were able to chemically complement the mutation. The insertional inactivation or deletion of *glbC* increased virulence in an acute model of infection in Balb/c or C57BL/6 mice but virulence in a chronic model of infection was similar to that of the wild type. Our findings contrast with the previous finding that inactivation of the *glb* gene cluster in *B. pseudomallei* strain 1026b resulted in marked attenuation, and provides evidence of differential roles for some genes in virulence of different strains of *B. pseudomallei*.

keywords; *Burkholderia*, melioidosis, proteasome

1. Introduction

Non-ribosomal peptides and polyketides are natural products with complex chemical structures which are synthesized on modular non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzyme complexes. Although NRPS/PKS clusters are found in all three domains of life, they are most abundant in bacteria (Wang *et al.*, 2014). Many naturally occurring NRPS/PKS products are either exploited as drugs or are the basis for drug development. These drugs include numerous antibiotics, immunosuppressive compounds and anticancer agents (Felnagle *et al.*, 2008).

The role of NRPS/PKS clusters in virulence of bacterial pathogens is much less clear. There are some examples of their role in the virulence of plant and insect pathogens. For example, Groll *et al.* have shown that syringolin, a low molecular weight proteasome inhibitor, plays a role in virulence of *Pseudomonas syringae* in bean plants (Groll *et al.*, 2008). However, in mammalian pathogens the only well documented roles are in the production of low molecular weight iron chelators such as malleilactone, enterobactin, yersiniabactin and mycobactin (Miethke & Marahiel, 2007, Biggins *et al.*, 2012). Against this background, there has been recent interest in establishing whether NRPS/PKS clusters might contribute to virulence of mammalian pathogens, beyond iron acquisition. One starting point for these studies is to investigate gene clusters in mammalian pathogens which are homologues of the clusters in plant pathogens and already shown to play roles in plant disease.

The synthesis of syringolin is directed by a non-ribosomal peptide/polyketide synthase (NRPS/PKS) cluster (Amrein *et al.*, 2004) which encodes proteins with multifunctional activities. The individual activities of these proteins are each encoded in discrete domains (Amrein *et al.*, 2004). The NRPS/PKS cluster encoding the enzymes necessary for syringolin synthesis is reported to be partially conserved in various members of the *Burkholderia* genus, including *B. pseudomallei* a pathogen of humans and other mammals (Schellenberg *et al.*, 2007). A study by Biggins *et al* (2014) has confirmed that in *B. pseudomallei* this cluster encodes the enzymes necessary for glidobactin, which has a structure of a 12-membered ring consisting of two non-proteinogenic amino acids (erythro-4-hydroxy-L-lysine and 4(S)-amino-2(E)-pentenoic acid). The ring is linked to an L-threonine residue which in turn is acylated by unsaturated fatty acids. Two forms of the molecule were identified in *B. pseudomallei* culture supernatant, which have been termed glidobactin C and deoxyglidobactin C (Biggins *et al.*, 2014). Glidobactin C is identical to glidobactin A (Schellenberg *et al.*, 2007), previously identified from a soil-borne member of the *Burkholderia* genus (strain K481-B101; species unidentified). These molecules are similar, but not identical to syringolin. Different naming systems have also been used to identify the similar gene clusters in *B. pseudomallei* (*syrEFGHI*) and in *Burkholderia* strain K481-B101 (*glbABCDEFGH*). In strain K481-B101 the GlbC and GlbF proteins are proposed to be involved in the synthesis of the tripeptide part of glidobactin A and disruption of *glbC* has been shown to abolish the production of glidobactin A (Schellenberg *et al.*, 2007). There is experimental evidence that syringolin and glidobactin bind to and preferentially target the chymotrypsin- and trypsin-like activities of the proteasome (de Bettignies &

Coux, 2010). A recent study indicates that *B. pseudomallei* glidobactin plays a role in virulence in mice (Biggins *et al.*, 2014).

In this study we have determined the function of the glidobactin-encoding enzyme cluster in *B. pseudomallei*. *B. pseudomallei* is the etiological agent of melioidosis, a disease endemic to parts of Southeast Asia and Northern Australia. We have inactivated a key gene (*glbC*; BPSS1269) in the NRPS/PKS *glb* cluster in *B. pseudomallei* and determined the effects of the mutation on growth in phagocytes, intracellular trafficking and virulence in mice.

2. Methods

2.1 Bacterial strains, plasmids and cell lines

All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown with aeration in Luria broth (LB) at 37°C unless otherwise stated. The antibiotics chloramphenicol (Sigma-Aldrich, UK) and gentamicin (Sigma Aldrich, UK) were used at concentrations of 50µg/ml and 100µg/ml respectively. The cell line J774.1 murine macrophage were maintained at 37ºC under 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (Gibco, Life Technologies). Growth curves for wildtype K96243, and K96243-*ΔglbC* were carried out in M9 minimal media with aeration at 37ºC for 24 hr.

123

124 *2.2 Mutant construction*

125 An in frame *glbC* (BPSS1269) deletion mutant (12.711 Kbp) was constructed using the
126 suicide plasmid pDM4 containing regions homologous to up and downstream regions of
127 *glbC*. Briefly, a DNA fragment containing 500bp regions upstream and downstream of
128 the *glbC* (BPSS1269) coding region and flanked by *SpeI* and *XbaI* restriction enzymes
129 was commercially synthesised (GENEART, Invitrogen). The DNA fragment was cloned
130 into the suicide plasmid pDM4 via its *Spe*I and *Xba*I sites. The presence of the DNA
131 fragments in the resulting plasmid pDM4-Δ*glbc* was confirmed by PCR using primers F1
132 – 5'- GCGAGCAGATCGCGAAACAC-3' and R2 – 5'-CTGATCCGCAAGCTGATCTG-
133 3'.The plasmid pDM4-Δ*glbC* was maintained in *E. coli* DH5α cells and then further
134 electroporated into *E. coli* S17 λ*pir* by electroporation. The plasmid pDM4-Δ*glbc* was
135 selected on LB agar containing 50 µg/ml chloramphenicol. Plasmid pDM4-Δ*glbC* was
136 conjugated into *B. pseudomallei* K96243 and gentamicin and chloramphenicol resistant
137 transconjugants (K96243-pDM4-*ΔglbC*) single crossover mutants selected. Double
138 crossover (chloramphenicol sensitive) mutants were obtained after growth on salt free
139 LB agar containing 10% (wt/vol) sucrose. The genotype of the mutants was confirmed
140 by genome sequencing using an Illumina HiSeq 2500 platform. Sequence data was
141 aligned against the K96243 reference genomes using the Illumina GA software. The
142 aligned reads were then visualised using the software program from Galaxy-Zeus
143 (Giardine *et al.*, 2005, Blankenberg *et al.*, 2010, Goecks *et al.*, 2010). Genomic regions
144 with no reads were interpreted as missing from the sequenced genome.

145

2.4 Reverse Transcriptase (RT) PCR

RT-PCRs were performed to investigate transcription of the genes in the glb cluster in K96243-*ΔglbC*. Total bacterial RNA was isolated from stationary phase cultures of wild type K96243 and the two mutants using Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. The quality of the RNA was analysed by carrying out a PCR to determine if there was any residual DNA remaining. Any residual DNA was treated with DNAse (Promega, Southampton, UK) at 37°C for 1 hr. Following this, stop buffer (Promega) was added and incubated at 65°C for 10 min. The RNA was then quantified using NanoDrop™ 1000, (Wilmington, USA) and 200ng/µl was used to prepare cDNA transcripts using Invitrogen ThermoScript™ Reverse Transcriptase according to manufacturer's instructions with random hexamers (Invitrogen, Paisley, UK). The resulting cDNA was then used as a template for PCR using Hot Start Taq (Qiagen) with primers for BPSS 1265 – 1271 (the primers for each of these genes can be found in in Table S2). The PCR amplification cycle consisted of 15 min at 96°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 54°C and 1.5 min at 72°C, and finally with a single extension time of 7 min at 72°C. For each PCR, a water control in the presence and absence of RT (negatives), and K96243 DNA (positive) were carried out to ensure results obtained, were due to cDNA synthesis and not contaminating genomic DNA or RNA preparation and reagents.

2.5 Macrophage uptake and intracellular survival assays

B. pseudomallei uptake and survival were quantified using a kanamycin protection assay. J774.1 murine macrophages were seeded into a 24 well tissue culture plate at a

concentration of 1 x 10^5 cells/ml in DMEM and incubated at 37ºC with 5% CO_2 for approximately 16 hr. Overnight cultures of *B. pseudomallei* were diluted in L-15 medium and 1 ml added to the cells at a multiplicity of infection (MOI) of 10. After incubation for 2hr at 37ºC, to allow bacterial invasion, the cells were washed 3 times with warm phosphate buffered saline (PBS) and incubated with fresh L15 medium containing 1mg/ml kanamycin. After 2hr the macrophage cells were held in fresh media containing 250µg/ml kanamycin to supress the growth of extracellular bacteria. At the indicated times the cells were washed 3 times in warm PBS and lysed with 0.1% (vol/vol) Triton X-100. Serial dilutions of the cell lysate were plated onto LB agar to determine the intracellular bacterial cell counts.

2.6 Neutrophil isolation

Human neutrophils were isolated from heparinised venous blood by 3.0% (w/v) dextran T-500 sedimentation (Pharmacosmos, 551005004007) and Ficoll-Paque PLUS centrifugation (Sigma Aldrich, 10771), as previously described by Chanchamroen *et al* (Chanchamroen *et al.*, 2009). The purity of isolated cells was generally greater than 95%, as determined by FACS Calibur flow cytometry (Becton Dickinson).

2.7 Assay of bacterial intracellular survival

Isolation of neutrophils from human blood was carried out as described previously (Vanaporn *et al.*, 2011). Purified neutrophils from healthy subjects (n=3) were infected with *B. pseudomallei* strain K96243 or K96243-Δ*glbC* at an MOI of 10 and incubated for 30 min at 37°C to allow internalisation. Extracellular bacteria were killed by the addition

of 250 µg/ml kanamycin and further incubation at 37°C for 30 min. At 1, 3 and 6 hours post infection (hpi) intracellular survival of *B. pseudomallei* in neutrophils was determined after host cell lysis and bacterial colony counting. Bacterial numbers were expressed as percentages of the initial inoculums for individuals. This was calculated by dividing the number of recovered bacteria by the total number of *B. pseudomallei* cells added.

2.8 Complementation with proteasome inhibitors

J774.1 murine macrophages cells were prepared as described above. L-15 media (Gibco) were treated with or without N-[N-(N-acetyl-L-leucyl)-L-leucyl]-L-norleucine (alln) or clasto-Lactacystin β-Lactone (cLβ-L) (Calbiochem, Merck-Millipore) at final concentrations of 10 and 5µM/ml respectively before wildtype K96243 or K96243::*glbC* was added at a MOI of 10. The experiments were carried out in the same way as described above and the cell lysate at 2, 8 and 10 hr was analysed for intracellular bacterial cell counts.

2.9 Animal studies

Female Balb/c or C57BL/6 mice (6-8 week-old; Harlan Laboratories, Bicester, Oxon, UK) were used throughout the studies. Groups of 8 mice were given free access to food and water and subjected to a 12 hr light/dark cycle. Mice were challenged under bio-safety level III containment conditions. 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 anesthetised 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 by administering a total volume of 50µl i.n. containing *B. pseudomallei* K96243 wild type or K96243-Δ*glbC* mutant. Control uninfected mice received 50µl of PFS. The animals were observed twice daily for up to 14 days. Humane endpoints were strictly observed and animals deemed incapable of survival were humanely killed by cervical dislocation.

2.10 Statistical Analyses

Differences between average values were tested for significance by performing an unpaired, two-sided Student's t-test. The levels of significance of the resulting *p* values are reported by the following symbols: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and n.s. = non-significant. Log-Rank tests of survival data were performed using the GraphPad Prism software version 5.01 (GraphPad Software, San Diego California USA).

3. Results

3.1 B. pseudomallei glbC plays a role in growth or survival in macrophages

Previous published data shows that the NRPS/PKS cluster encoding glidobactin is partially conserved in various members of the *Burkholderia* genus including *B.*

pseudomallei (Schellenberg *et al.*, 2007). Figure 1 shows the genetic organisation of the *glb* cluster in *B. pseudomallei* K96243. To establish whether this cluster encodes enzymes for a proteasome inhibitor we first made a *glbC* insertional mutant. The mutant was confirmed by whole genome sequencing which showed the presence of a plasmid inserted into the *glbC* only. We compared the behaviour of the wild type and *glbC* mutant in J774.1 macrophages. Compared to the wild type the mutant showed reduced replication in macrophages, which was most pronounced at the latest sampling point (10 hpi.). The pre-treatment of macrophages with the proteasome inhibitors ALLN or cLβ-L restored the ability of the mutant to grow in macrophages (Fig. 2). However, during repeat studies we found that the differences in the abilities of wild type and mutant to grow in macrophages were more pronounced in some J774.1 macrophage sub-cultures than in others;two of the repeats showed reduced intracellular survival of the mutant compared to wildtype at 8 h but not 10 h.

3.2 Construction of a glbC deletion mutant

For our subsequent studies we constructed an in frame deletion mutant of the *glbC* (BPSS1269) gene (K96243-*ΔglbC).* The deletion of BPSS1269 was confirmed by whole genome sequencing of the mutant (K96243-*ΔglbC*) which showed the only the *glbC* had been deleted. RT-PCR revealed expression of all of the genes in the *glb* cluster in *B. pseudomallei* K96243 (Fig S1). In K96243-*ΔglbC* we could not demonstrate expression of BPSS1269 (*glbC*) or BPSS1268 (*glbD*), which is located downstream of *glbC* (Figure 1), but we detected similar expression of the genes upstream of *glbC* (BPSS1270 and BPSS1270) and the genes downstream of *glbD* (BPSS1267 and BPSS1266 and

BPSS1265). Wild type K96243 and K96243-*ΔglbC* grew at similar rates in M9 minimal media or in LB (data not shown).

3.3 A ΔglbC mutant shows a growth defect in human neutrophils

Our recent study demonstrated that macroautophagy is essential for killing of intracellular *B. pseudomallei* in human neutrophils (Rinchai *et al.*, 2015) and we next investigated whether deletion of *glbC* would affect the intracellular survival ability of the bacteria in human neutrophils. PMNs were isolated and infected with an MOI of 10 for 1, 3 and 6 hr and intracellular bacteria enumerated. K96243-*ΔglbC* was more susceptible to bacterial killing by human neutrophils, compared to the wildtype (Fig. 3).

3.4 B. pseudomallei ΔglbC is more virulent in an acute mouse model of infection

To further investigate the role of *glbC* in virulence we infected Balb/c or C57BL/6 mice with *B. pseudomallei* K96243 or K96243-*ΔglbC* at two different doses. A high dose of 2500 CFU of wildtype *B. pseudomallei* has previously been shown to cause acute disease, whereas a lower dose <1000 CFU can lead to chronic infection in mice (Conejero *et al.*, 2011). In this study we found that Balb/c or C57BL/6 mice challenged with high doses of *B. pseudomallei* K96243 survived longer than those infected with K96243-*ΔglbC* (Fig. 4A and B). At low doses, the survival of wild type and mutant was similar. At day 45 all surviving mice were culled and *B. pseudomallei* was readily isolated from the spleens, lungs or livers of these mice (data not shown).

Since our experiments indicated that in acute infection models *B. pseudomallei* K96243-

glbC is more virulent than the wild type, we measured bacterial clearance kinetics (Fig 5). The bacterial burden was significantly higher in the lung, spleen and blood of mice infected with *B. pseudomallei* Δ*glbC* compared to mice infected with wild type *B. pseudomallei* K96243.

Discussion

Gene clusters which have the potential to encode small molecules are frequently identified in the genome sequences of bacteria (Challis, 2008). A previous study has shown that in *B. pseudomallei* the *glb* enzyme cluster, alternatively termed the *syr* cluster, encodes the enzymes for synethsis of glidobactin and deoxyglidobactin (Biggins *et al.*, 2014), two related compounds which differ in the substitution of lysine or hydroxylysine in the warhead of the molecule. These compounds are also related to syringolin A, which has been shown to be a 20S proteasome inhibitor produced by *Pseudomonas syringae* (Groll *et al.*, 2008) and glidobactin A which has antifungal activity and is produced by the soil-borne bacterium *Burkholderia* K481-B101 (Schellenberg *et al.*, 2007). Within the *Burkholderia glb* cluster, *the glbC* gene is believed to encode the NRPS modules responsible for synthesis of the tripeptide component of glidobactin (Schellenberg *et al.*, 2007). The disruption of *glbC* in *Burkholderia* K481-B101 abolished glidobactin A production (Schellenberg *et al.*, 2007). In this study we have shown that the insertional inactivation or deletion of *glbC* in *B. pseudomallei* markedly reduced the ability of the bacterium to survive or grow in un-activated murine macrophages or in human neutrophils. Exogenously added proteasome inhibitors were able to chemically complement the mutation. Our results

confirm that *B. pseudomallei glbC* plays a key role in the synthesis of a proteasome inhibitor which is active towards eukaryotic cells. During repeat studies we did see differences in the replication of bacteria in different sub-cultures of J774 macrophage cells. Two further repeats showed a reduced intracellular survival of the mutant compared to wild type at 8 hours but not at 10 hours. This may reflect differences in the activation state of the cells. This may reflect differences in the activation state of the cells.

A previous study has shown that a gene cluster encoding a proteasome inhibitor plays a role in the virulence of *P. syringae* in plants (Groll *et al.*, 2008). More recently, a study conducted by Biggins *et al.* 2014 found that a mutant of *B. pseudomallei* strain 1026b, in which *glbB* and the 5' region of *glbC* were deleted, was completely attenuated in mice after intranasal challenge (Biggins *et al.*, 2014). In contrast, we found that a *glbC* deletion mutant in strain K96243 showed an increase in virulence in an acute model of disease in two strains of mice compared to the wild type. We found similar results when we tested a *glbC* insertional mutant of K96243 (results not shown). The decreased intracellular survival and the increased virulence in animal model for the *glbC* mutant observed highlights the limitations of using a cell culture system. These results indicate that the *glbC* mutant must exhibit different phenotypes in different cell types. It is not clear why the phenotype of the K96243 *glbC* mutant we have constructed is different from the phenotype of the strain 1026b mutant. In both studies Balb/c mice were used and were challenged by the intranasal route. It is possible that the deletion of *glbB* in strain 1026b mutant is responsible for the attenuation seen, and we have shown that

330 *glbB* was expressed in our *glbC* deletion mutant. The organisation of the glb cluster is
331 the same in strains K96243 and 1026b. However, we found there to be 52 single length
332 polymorphisms (SNPs) between the two *glbC*. A further 5 SNPs between the *glbF*, 1
333 SNP in *glbB* and 0 SNPs between the *glbE* and *glbD* regions of the loci. These SNPs
334 may contribute to the differences in phenotypes seen between strains K96243 and
335 1026b including the reduced transcript levels of downstream *glbD*. Alternatively, the
336 difference may reflect differences in the biochemistry of strains K96243 and 1026b. It is
337 interesting to note that a *tat* mutant of strain K96243 was reported to be essential for
338 growth under aerobic conditions (Moule *et al.*, 2014, Wagley *et al.*, 2014) but a *tat*
339 mutant of strain 1026b grew normally under these conditions (Rholl *et al.*, 2011).

340

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345

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Fig 1. Organisation of the glidobactin (*glb*) cluster in *B. pseudomallei* K96243. The *glbC* (BPSS1269) gene was deleted in K96243-*ΔglbC*. Expression of the genes shown in wild type and mutant was assessed using RT-PCR (see Fig S1 for details).

Fig 2. Survival of *B. pseudomallei* K96243 or K96243::*glbC* in J774.1 macrophages. Macrophages we infected with wild type or mutant at an MOI of 10 and at 2, 8 and 10hr the cells were lysed and intracellular bacteria enumerated. In some cases the proteasome inhibitors ALLN or cLβ-L (10 and 5μM/ml respectively) were added to the cells before infection. Results shown are the mean of 3 replicates, the error bars represent the SEM values.

Fig 3. Survival of *B. pseudomallei* K96243 (white bars) or K96243-*ΔglbC* (black bars) in human neutrophils. Neutrophils from healthy individual (n=3) were infected with *B. pseudomallei* strain K96243 or K96243-Δ*glbC* at an MOI of 10. At 1, 3 and 6 hpi intracellular bacteria were enumerated. * = $p<0.05$, ** = $p< 0.01$, using a unpaired *t*-test.

Fig 4. Virulence of *B. pseudomallei* wild type or Δ*glbC* mutant in mice. Balb/c (A) or C57BL/6 mice (B) (n=6-8 per group) were infected i.n.with either *B. pseudomallei* K96243 or *B. pseudomallei* K96243-Δ*glbC* at the doses stated and survival determined. Stated doses refer to the actual CFU given to each group by CFU counts on the inoculum used on the day of the experiment. * = $p<0.001$.

Fig. 5. Bacterial clearance kinetics following acute i.n. infection. C57BL/6 mice (n=5/group) were challenged i.n. with approximately 2000 CFU *B. pseudomallei* K96243 (actual counts 2150 CFU) or *B. pseudomallei* K96243-Δ*glbC* (actual counts 3495 CFU). Organs (A; lung, B; spleen, C; blood) were harvested at day 1 (d1), 2 (d2) or 3 (d3) p.i., homogenized and plated out on TSA plates. † = (deaths/total). * = $p<0.05$, ** = $p< 0.01$.

443 **Table 1: Bacterial strains and plasmids used in this study**

Bacterial Strain	Description	Source or Reference
B. pseudomallei strains		
K96243	Clinical isolate from Thailand	Holden *et al* 2004
K96243-RFP	Reporter plasmid	Wand *et al* 2011
K96243-*ΔglbC*	Inactivation of BPSS1269 (*glbC*) by complete deletion	This study
K96243:: *glbC*	Inactivation of *glb* cluster by insertional inactivation of BPSS1269. cm^r	This study
***E. coli* Strains**		
DH5α Δpir	*recA1 gyrA* (Nal) Δ(*lacIZYA-argF*) (ϕ80d*lac*Δ[*lacZ*]M15) *pirRK6*	Simon *et al* 1983
S17-1 Δpir	RPA-2 *tra* regulon; *pirRK6*Sm^rTp^r	Simon *et al* 1983
Plasmids		
pDM4	Suicide vector with R6K origin: Cm^r	Milton *et al* 1996
pDM4 - *ΔglbC*	500bp up and down stream of *glbC* cloned into pDM4	This study
pBHR4-groS-RFP	Reporter plasmid -red	Wand *et al* 2011

florescent protein

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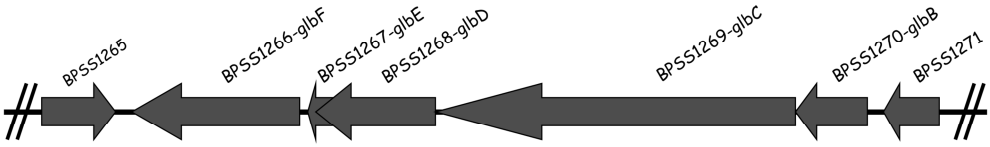

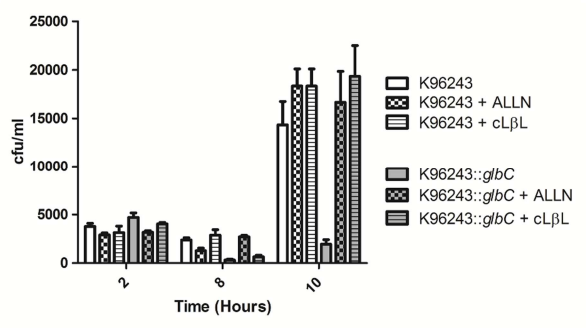

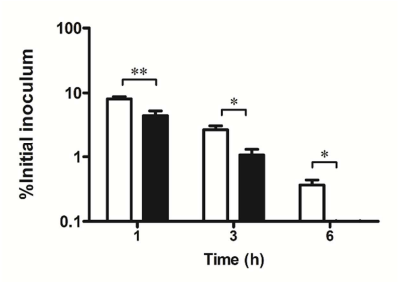

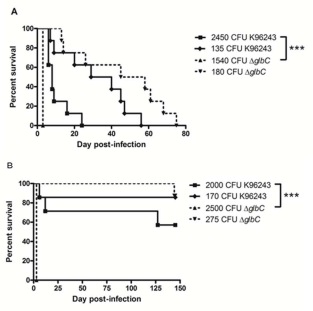

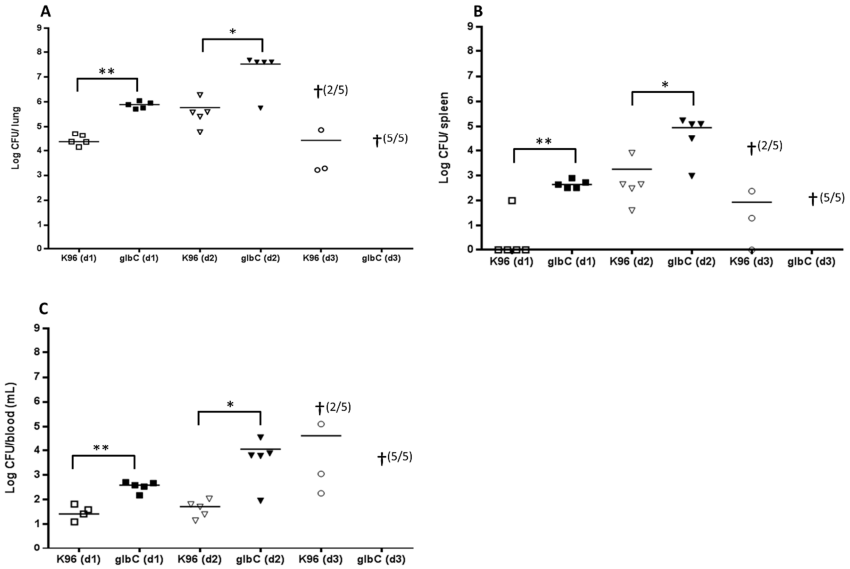

Highlights

1. A NRPS/PKS cluster encoding the enzymes necessary for glidobactin synthesis is partially conserved in *Burkholderia pseudomallei*.
2. We show that the insertional inactivation or deletion of *glbC* in this cluster in *B. pseudomallei* could reduce the ability of the bacterium to survive or grow in murine macrophages or in human neutrophils.
3. The addition of proteasome inhibitors to the glbC inactivated mutant chemically complemented the mutation.
4. The insertional inactivation or deletion of *glbC* increased virulence in an acute model of infection in Balb/c or C57BL/6 mice but virulence in a chronic model of infection was similar to that of the wild type.