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Relationships Between Resistance and Virulence in *Burkholderia pseudomallei*

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Abstract
Purpose of Review Aroused by the capacity of bacteria to develop antimicrobial resistance which allows them to persist in patient under antibiotic treatment, and their adaptation to host defenses by modifying their virulence, we review the relationship between antibiotic resistance and virulence in *Burkholderia pseudomallei*.
Recent Finding Few studies focused on both antibiotic resistance and virulence. The relationship between these two mechanisms is very complex. Main resistance mechanisms such as efflux, biofilm, morphological changes or persistence are linked to virulence but results are still controversial. Recent clinical reports seem to indicate that reductive evolution is involved for balancing antibiotic resistance and virulence in chronic melioidosis cases.
Summary The relation of virulence and resistance should be more considered to better understand the resistance, persistence, and pathogenicity of *B. pseudomallei*. Focusing on these two mechanisms, it will be possible to improve therapeutic options against this important emerging disease and avoid therapy failures or relapses for *B. pseudomallei*.

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Introduction

Burkholderia pseudomallei is the causal agent of melioidosis, an endemic human disease mostly present in Southern Asia and Northern Australia. The endemic area has been recently expanded to other countries and risk zones are also observed in Central America, Southern Asia, and the Middle East [1]. Melioidosis is a polymorphic disease with a wide spectrum of clinical symptoms ranging from asymptomatic form to pneumonia with multiple abscesses and septicemia [2, 3••, 4]. Infection can occur following exposure to contaminated water or soil, by inhalation, ingestion, or percutaneous inoculation [3••]. Melioidosis is associated to a high mortality rate, and resistant strains of *B. pseudomallei* can emerge during the treatment. *B. pseudomallei* relationships between resistance and virulence are misunderstood [5]. The understanding of the interplay between regulation and transmission of antibiotic resistance with virulence must be further expanded. Another important aspect is the micro-evolution of bacteria within the host during chronic melioidosis which could lead to resistance development and may have an impact on strain virulence. Thus, we present here a review describing the main mechanisms implicated in *B. pseudomallei* antibiotic resistance and virulence. Then, we conclude by summarizing the relationships between antibiotic resistance and virulence, highlighting common mechanisms that are involved.

Resistance

Melioidosis therapy is biphasic and lasts several months [6]. An initial acute phase involving intravenous injection of antibiotics during 15 or 20 days is followed by an eradication phase with oral prescription for up to 6 months [7••, 8]. The acute phase of treatment is to prevent septicemia, while eradication phase is to minimize the risk of relapse. Despite the implementation of this treatment, recurrent cases are observed in 13% of patients, certainly due to the observance of the therapy during the eradication phase. The majority of relapse cases (75%) is caused by the strain identified in acute phase, suggesting that most of these were due to failure to eradicate this initial isolate [6, 9]. *B. pseudomallei* ability to persist in intracellular environments may offer havens to the bacteria to survive to the therapy. It could also favor the appearance of multi-drug resistant (MDR) phenotypes which might be associated with persistence. In several cases, it has been shown that the *B. pseudomallei* isolated from relapse cases and persistent infections were resistant to antibiotics treatment [5]. Moreover, most of the *B. pseudomallei* strains are naturally resistant to many antibiotic families like aminoglycosides and some β-lactams and macrolides [10–12].

Several mechanisms are employed by *B. pseudomallei* to develop those resistances such as membrane permeability, efflux, enzymatic inactivation, target alteration, or target overexpression [13••].

Membrane Permeability

Outer membrane permeability plays an important role in *B. pseudomallei* resistance. *Burkholderia* is known to be resistant to cationic peptides such as polymyxin B and colistin. The atypical composition of *Burkholderia* LPS structure is in part responsible for this intrinsic resistance [14]. Indeed, the lipid A component of *B. pseudomallei* consists of a biphosphorylated disaccharide backbone modified by a 4-amino-4-deoxy-arabinose (ARA4N) [15]. This modification of LPS leads to a reduction of the negative charge of the membrane, decreasing the entry of cationic antibiotics and enhancing the resistance to these types of molecules. Porins also play a role in the membrane permeability. The porin Omp38 from *B. pseudomallei* has been characterized in vitro and might be contributing to meropenem, imipenem, or ceftazidime resistance [16, 17].

Efflux

Active efflux plays an important role in antibiotic resistance, and this mechanism is well described for many bacteria. Resistance nodulation cell division (RND) efflux pumps are one of the main mechanisms implicated in intrinsic and acquired resistances. The efflux system that crosses the entire envelop of Gram-negative bacteria is composed of three components, a cytoplasmic membrane, the so called RND transporter protein, an outer membrane channel protein and a membrane fusion protein.

B. pseudomallei genome encodes 10 putative RND pumps. Three of those have been well described [18•]. AmrAB-OprA pump is responsible for the intrinsic resistance to macrolides and aminoglycosides [19]. A mutation of this pumps leads to atypical gentamicin-susceptible strains (0.1% of isolated strains) [20•]. BpeAB-OprB pump is responsible of chloramphenicol, fluoroquinolones, macrolides, and tetracyclines resistance [21, 22]. At last, the BpeEF-OprC pump is expressed in resistant mutants and extrudes fluoroquinolones, tetracyclines, chloramphenicol, trimethoprim, and sulfamethoxazole [23, 24].

β-Lactamases

B. pseudomallei genome encodes also β-lactamase enzymes. Ceftazidime resistance phenotype is rare but mainly due to point mutations in PenA, an A amber class β-lactamases [25–29]. Other classes D β-lactamases, Oxa-57, Oxa-42, and Oxa-43, have been described, but their clinical relevance must be demonstrated [30, 31].

However, those mechanisms are not the only ones employed by the bacteria. Antibiotic resistance can also be adaptive, and environmental conditions can modulate gene expression or induce biofilm formation, morphological variants, or formation of persistence strains.

Biofilm, Morphology, and Persistence

Biofilms are microorganisms' heterogeneous communities, attached to a surface by an extracellular matrix. Bacteria in biofilm are less susceptible to antibiotics than in their planktonic form. As expected, *B. pseudomallei* biofilm reduces susceptibility to several antibiotics including ceftazidime and imipenem [32, 33]. Moreover, *B. pseudomallei* can present morphological changes due to environmental and antibiotic pressure [34]. Seven different morphotypes have been described with the *B. pseudomallei* type I morphotype representing most of the strains, but a switch to a type II morphotype can appear with ciprofloxacin and ceftazidime at sub-inhibitory concentrations [35]. Antibiotic exposure can provoke another morphological change: filamentation [36]. This morphological change is reversible without increase in antibiotic resistance, except for an initial ofloxacin induction. A filamentation induction by ceftazidime leads to small colony variant formation which are known to have high minimum inhibitory concentration level [37].

Persisters are different than resistant strains, because the persistence to antibiotics is not inherited by the next generation. Bacteria persisters are in a dormant state, with a slow or a non-existent metabolism, which enables them to tolerate high

concentrations of antibiotics. Several actors can induce persisters formation: environmental condition, host response, risk factors, strain genotype, antibiotic exposure, growth phase [38, 39]. Toxin-antitoxin systems or metabolic pathways are persistence strategies used by the bacteria that we take as example below.

It is interesting to underline that biofilm is an association of heterogeneous bacterial subpopulations that differ not only by their antibiotic susceptibilities but also by their mechanisms used to resist the environmental stresses [40].

Virulence

B. pseudomallei is a facultative intracellular pathogen able to invade, survive, and replicate in both macrophages and epithelial cells [41]. Several virulence factors have been identified and described [42, 43••]. Considering the complexity and the multiplicity of virulence factors present in *B. pseudomallei*, we describe in this review only the most prominent virulence mechanisms associated with resistance mechanisms.

Pilatz et al. have described the essential genes during intracellular growth [44]. Secretion systems and secreted effectors play an important role in *B. pseudomallei* virulence and especially during the intracellular lifecycle. The genome contains three type III secretion systems (T3SS), six putative type VI secretion systems (T6SS), which are encoded by approximately 15 core genes and a variable number of non-conserved accessory elements, [45–48]. Mutants of T3SS and T6SS present reduced intracellular survival and attenuated virulence. The structure of T6SS is similar to a bacteriophage-like apparatus that allow the injection of effector proteins into host cells [49–51].

A capsular polysaccharide is important in intra-cellular survival and is required for persistence. Capsule mutants displayed a decreased virulence in hamster, and lipopolysaccharide confers resistance to human serum [52, 53].

The cell wall is implicated into *B. pseudomallei* virulence. Recently, several studies have shown the importance of the link between the cell wall structures (colonies morphology) and virulence. *B. pseudomallei* express various morphotypes which are divided into two groups designated “smooth” and “rough.” Seven colony variants (types I–VII) have been described by Chantratita et al. Both smooth and rough colonies could switch to the other type, but the rough morphology is the predominant form isolated from clinical cases [35, 54•, 55]. Variant morphotype strains present different capacities to survive and to persist in mice and to resist to antimicrobial peptides [35, 54•]. It has been hypothesized that type II corresponds to an adaptive persistent phenotype associated with a lower virulent state. Shea et al. described for two *B. pseudomallei* strains the relationship between phenotype and difference of virulence [56]. Virulence assays were performed in macrophage J774 model and mice BALB/C model, and type III appeared more adapted to survive in macrophages.

Quorum sensing is a form of communication between bacteria which is dependent on cell density. It is a two-component system with the first gene encoding homoserine lactone and the second encoding a transcription regulator. *B. pseudomallei* genome contains three *luxI* homologs (homoserine lactones) and five *luxR* homologs (transcriptional regulator). Deletion of *luxI* results in a reduced colonization in a Swiss mouse model of infection [57].

Other factors are implicated in *B. pseudomallei* virulence-like secreted proteins, including three phospholipase C proteins [58]; MprA, a serine metalloprotease [59]; and lipases [44]. Additional proposed virulence factors include adhesins [43••, 60•], flagella, hemolysin, and lethal factor 1 [61].

One of the most important issues in studying *B. pseudomallei* virulence is the variations observed between different strains possibly due to multiple genomic islands (genetic elements transferred from another organism directly into the genome) which are variably present and result in different virulence phenotypes [42, 62, 63].

Virulence/Resistance Relationships

We describe the above various mechanisms implicated in antibiotic resistance or virulence. This part of the review highlights related mechanisms and the relationship between resistance and virulence in *B. pseudomallei* (Table 1).

Efflux Pumps

Efflux pumps are well known in Gram-negative bacteria for their contribution to antibiotic resistance with a wide range of potential substrates. These pumps are ancient genomic elements and are found in microorganisms and mammalian and plant cells. This suggests that efflux pumps are important

Table 1 Mechanisms involved in resistance or virulence

Mechanisms	Resistance	Virulence
Biofilm	xx	xx
Efflux pumps	xx	x
Enzymatic inactivation	xx	
Alteration of target site	xx	
Chronic or latent infection	x	xx
Membrane morphology	x	x
Capsule		xx
LPS	x	xx
Flagella		xx
Quorum sensing system	x	xx
Protein secretion systems		xx

components of cell physiology and are not only antibiotic resistance determinants but could also play a key role in virulence of most of bacteria [64, 65]. In *B. pseudomallei*, several studies have investigated the potential implication of RND efflux pumps in virulence.

In 1999, Moore et al. identified two mutants obtained by a transposon insertion in AmrAB-OprA, which were susceptible to aminoglycosides and macrolides compared to the parental strain [19]. Other study showed the implication of AmrAB-OprA in virulence. *B. pseudomallei* 708a strain is one of the rare strains naturally susceptible to gentamicin and possess a 131-kb deletion in a region containing the *amrAB-oprA* operon. The authors compared this strain to Bp50 AmrAB-OprA-deleted mutants which showed lowered virulence in murine model with a 100% survival rate [20•]. It is noteworthy that this susceptible strain 708a remains highly virulent in patients, with high morbidity but no mortality. Another study with the 708a strain using *Galleria mellonella* model revealed that this strain was susceptible to gentamicin and was less virulent than the other strains analyzed [66]. Finally, Viktorov et al. obtained, by successive antibiotic pressure selection, cross-resistant variants from a clinical isolate of *B. pseudomallei*. In two of the variants, an *amrB* overexpression was observed and their virulence was dramatically decreased in a hamster model [67]. These data seem to indicate that AmrAB-OprA pump is directly or indirectly related to virulence, but the mechanisms remain unclear.

In two studies, Chan et al. showed the relation between another efflux pump, BpeAB-OprB, and quorum sensing in the KHW strain [22, 68]. They first observed that *bpeAB* expression is growth-phase dependent and induced by two homoserine lactones, C8HSL and C10HSL. Mutants deleted of this gene or overexpressing *bpeR* pump repressor are impaired on auto-inducer efflux and have siderophore production, as well as phospholipase C and biofilm decreased [22]. Furthermore, they analyzed invasion and cytotoxic activity of their strains on epithelial cells and macrophages. The invasion and cytotoxicity were severely decreased in deleted mutant as well as in overexpressing *bpeR* strains. Adding the exogenous auto-inducer C8HSL restored the virulence. In a second study, they assessed by HPLC the acyl-HSL profile excreted by the bacteria [68]. In the *bpeAB*-deleted mutant, they observed an acyl-HSL drop down compared to the wild-type strain. These data seem to indicate that the attenuation of bacterial virulence in the pump-deleted mutant is correlated with auto-inducer impairment and with an altered quorum sensing. However, Mima et al. also studied the relationship between BpeAB and quorum sensing and their results were in discrepancy [21]. In their study, they did not observe any acyl-HSL impairment in BpeAB-OprB. They also did not identify significant differences in siderophore production, biofilm formation, and motility in this mutant. Once again, the direct implication of efflux pumps in virulence appears to be controversial.

Biofilm—Morphotype

As previously described, biofilms and morphotypes are related to antibiotic resistance, persistence, and recurrence of diseases in *B. pseudomallei* [32, 33, 69]. Quorum sensing regulates biofilm formation and as a counterpart, biofilm promotes QS communication [32]. Environmental changes induce the biofilm formation and lead to several global regulation modulations, and biofilm is suspected to be associated with bacterial virulence.

Bacteria in biofilm are much more resistant than their planktonic form, increasing 1000-fold their resistance level. In 2005, Taweechaisupapong et al. tried to directly link the biofilm formation with virulence [70]. No significant difference was observed in a murine model between high and low biofilm producers. They conclude that the amount of biofilm is not correlated with virulence. In 2013, Lazar Adler et al. revealed that mutants in a trimeric auto-transporter adhesin (TAA) were affected in biofilm formation [60•]. This gene *bbfA* for "*Burkholderia* biofilm factor A" seemed to be required for initial surface adhesion, a mutation leading to a deficient non-mature biofilm. In a murine BALB/C model, *bbfA* mutant strain showed an attenuated virulence compared to the parental strain and a trans-complementation experiment was able to restore wild-type virulence phenotype. These results indicate that a potential relationship between biofilm formation and virulence existed for this strain.

In a recent study, Chin et al. analyzed virulence and transcriptomic differences between four high and low biofilm-producing strains [71]. In the *Caenorhabditis elegans* nematode model and in the murine BALB/c model, the high biofilm producers killed worms and mice faster than low producers did. Cytokine response was reduced in mice challenged with a high biofilm-producing strain. The authors concluded that biofilm seemed to be directly related with strain virulence by limiting the cytokine response.

There is also a relationship between biofilm formation and colony morphotype (mucoid and non-mucoid phenotype, small colony variant). As previously described, morphology variation could be induced by environmental changes or stresses, and *B. pseudomallei* displays 7 morphotypes [35]. Biofilm is important for *B. pseudomallei* to resist antibiotic pressure in infected patients. Chantratita et al. showed that an antibiotic pressure induced a higher prevalence and switch to type II colonies. Moreover, in the same study, authors observed that this type II morphotype produced more biofilm than parental strain type I.

Another morphological change, filamentation, has been described in case of antibiotic exposure. In 2005, Chen et al. showed that ceftazidime, ofloxacin, and trimethoprim could induce filamentation in *B. pseudomallei* [36]. The authors analyzed the impact of filamentation in virulence by measuring strain cytotoxicity in THP-1 cell line. They observed that

although filamentous bacteria showed reduced cytotoxicity, they were still able to enter and persist into THP-1 cells. Only filaments induced by ceftazidime developed cross-resistance to ofloxacin and gentamicin. Filamentation was reversible if the antibiotic was removed from the medium. However, if revertants were re-exposed to antibiotic, it led to small colony variant (SCV) formation. These SCVs are known to be more resistant to various antibiotics. Filamentation could be a threat in patients because the reversion can contribute to the emergence of multidrug resistance. It is also an issue because filamentous bacteria could be temporarily less virulent, leading to the interruption/cessation of antibiotic administration.

It is complicated to identify direct links and to highlight relations between biofilm production, morphotype, antibiotic resistance, and virulence due to the complex network connecting these different mechanisms.

Lipopolysaccharide

Several types of LPS have been described in *B. pseudomallei*: smooth types A and B and rough LPS [72]. It has been shown that smooth LPS type A strains were low biofilm producers, in contrary to rough LPS strains, which are high biofilm producers. The authors report that the less common LPS patterns (smooth type B and rough) were more often isolated from patients with relapse infections. Bacteria with rough LPS seem to be more able to survive in host than smooth-type LPS. The susceptibility to various antibiotics or cationic antimicrobial peptides of *B. pseudomallei* increased when the LPS core biosynthesis was disrupted [73].

Persisters

The toxin-antitoxin (TA) systems are important in bacterial physiology and have been connected to virulence in several species [74]. In 2014, Butt et al. [75] have linked the HicAB TA system to the formation of antibiotic persisters. *B. pseudomallei* could form persisters under ceftazidime and ciprofloxacin pressures. The authors observed that overexpressing the HicA toxin in K96243 strain led to a growth inhibition. Overexpressing *hicA* caused increased persister formation, from 10^{-5} to 10^{-3} in ciprofloxacin exposure condition and from 10^{-6} to 10^{-3} in ceftazidime exposure. A low level of HicA was necessary to increase ciprofloxacin persisters, and a high HicA level was necessary to increase ceftazidime persisters suggesting a differential role of HicA toxin.

Global Regulation

We have seen that some main antibiotic resistance mechanisms were directly linked with bacterial virulence. However, many results are in discordance and reflect the complex mechanisms behind this phenomenon. Two studies linked virulence and antibiotic resistance by global regulation and metabolic pathway.

Two-component systems are crucial for the bacteria to sense its environment and overcome changes and stresses. These systems act as sensors and modulate gene expression depending on environmental stimuli. Many of those systems are related with bacterial virulence. Lazar Alder et al. identified a new *B. pseudomallei* two-component BprSR system implicated in virulence. They analyzed the whole transcriptome of mutants deleted in BprSR and showed an overexpression for several genes associated with antibiotic resistance such as *bpeAB* and *penA* β-lactamase [76••].

2-alkyl-4(1H)-quinolones (AQs) are implicated in quorum sensing. Their production is dependent of anthranilate, and kynurenine pathway is responsible of the tryptophan metabolized in anthranilate. Recently, Butt et al. associated the AQ synthesis and *B. pseudomallei* virulence. By deleting the *kynB* gene (coding the kynurenine formamidase) which is part of the kynurenine pathway, they observed that the mutant strain was impaired in AQ production. Interestingly, this phenomenon is associated with increased biofilm production and ciprofloxacin-induced persister formation [77]. So once again, virulence and antibiotic resistance mechanisms were associated through a metabolic pathway. However, the reverted phenotypic strain still produces more ciprofloxacin persisters than the parental strain. This may suggest that *kynB* is not only implicated in kynurenine pathway but also in resistance mechanisms.

These results showed that virulence and antibiotic resistance are related with complex mechanisms of regulation.

Antibiotic Resistance and Virulence Relationship in Chronic Melioidosis

All along this review, we have tried to establish the potential relationship between antibiotic resistance mechanism and virulence. However, all the studies are performed in vitro or in vivo in animal models. But, what happens in the patient? Recent studies have investigated how the bacteria could adapt and evolve in patient with chronic infection disease as cystic fibrosis (CF).

In 2013, a study showed the micro-evolution of a strain in a non-CF patient [78••]. Two clinical isolates were obtained from the same patient at two different stages: one at the initial sampling and the other one 139 months later (e.g., more than 11 years). Comparative whole-genome analysis showed for the second isolate, 4 deleted loci in chromosome 2, including virulence factors such as T3SS, efflux pumps BpeEF-OprC and BpeGH-OprD, and secondary metabolism pathways. Several mutations (single nucleotide polymorphism, insertion or deletion) have been also discovered in *penA*, conferring ceftazidime resistance, and in the LPS synthesis pathway with a loss

of O-antigen, inducing rough LPS type. These two isolates differed also by their morphology and growth rate, the second isolate presenting smaller colonies and a lower growth rate. It is important to underline that the initial isolate possessed one deletion in virulence factor WcbR and was avirulent in mice.

Many case reports describe CF patients having chronic melioidosis [79]. In CF patients, *B. pseudomallei* strains can become more resistant to ceftazidime, co-trimoxazole, doxycycline, and carbapenems by mutating known mechanisms such as *penA*, efflux, and antibiotic target mutation [80]. This was the first report of a *penA* duplication implicated in clinical *B. pseudomallei* resistance. Isolates were also mutated in several virulence factors such as T3SS, *virA* (a two-component system activating T6SS), and LPS. It is interesting to note the loss of genome segments in *B. pseudomallei* strain isolated from patient with chronic melioidosis [78••].

Relationship between antibiotic resistance and virulence is highlighted here in those two articles analyzing the micro-evolution of *B. pseudomallei* strains in cases of chronic melioidosis. In patients, it seems that multiple mutations in both virulence and resistance genes and deletion of some genome segments are the main mechanisms implicated in the adaptation to the host. In these cases of chronic carriage of *B. pseudomallei*, there is a balance between attenuated virulence and an enhanced resistance leading to the bacterial persistence in the host. Their reductive evolution seems to be the main link between antibiotic resistance and virulence.

Conclusions

It is important to better understand the antibiotic resistance and virulence of *B. pseudomallei*. Indeed, antibiotic resistance is an important problem and could be associated with the persistence phenomenon in the host. *B. pseudomallei* is considered as an emerging pathogen, with a high rate of fatality rate ranging from 10 to 20% in Australia to over 40% in Thailand, and the virulence should be considered. For some bacteria, relations between antibiotic resistance and virulence are well described, but for *B. pseudomallei* are not so clear. Various detailed studies deal with antibiotic resistance or virulence, but the interaction of these two important mechanisms seems to be left behind for *B. pseudomallei.* As we report in this review, different studies still provide controversial results. Some of them highlighted the theory of "fitness preference." This idea considers that a bacterium could not use its fitness to be resistant and virulent at the same time. Antibiotic-resistant strains cause chronic diseases while virulent ones quickly kill their host and do not need to be resistant. In these cases, the bacteria are not able to use their energy resources to regulate and express both virulence and resistance genes. However, we have here described some cases where overexpressing a resistance mechanism leads to an increased virulence. In other studies, the relationship between resistance and virulence is not proven, certainly due to the complexity and the diversity of the mechanisms employed by *B. pseudomallei* to be resistant and/or virulent.

A new approach considering the inter-relation of both virulence and resistance should be considered to make significant progresses in our understanding of lifecycle of this bacterium and further improve therapeutic options against this important emerging disease.

Compliance with Ethical Standards

Conflict of Interest Marine Schnetterle is supported by DGA/MRIS France.

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- •• Of major importance

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