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Genomic Insights Into the Melioidosis Pathogen, *Burkholderia pseudomallei*

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Abstract
Purpose of Review Genomics has fundamentally transformed our understanding of infectious diseases. Here, we provide an overview of the insights gained from genomic investigations of the melioidosis-causing pathogen, *Burkholderia pseudomallei*.
Recent Findings B. pseudomallei has a large and complex genome that encodes an impressive array of virulence factors, some of which are variably present. Despite frequent lateral gene transfer, phylogenomics resolves *B. pseudomallei* populations to the continental level, enabling strain source tracing in non-endemic regions and shedding light on the origin and anthropogenic spread of *B. pseudomallei* populations across tropical and subtropical regions worldwide. Within-host evolution, outbreak point-sources and antibiotic resistance can now be rapidly elucidated using comparative genomic approaches.
Summary Genomics is an essential tool for understanding the phylogeography, transmission, evolution, virulence, epidemiology, and antibiotic resistance of *B. pseudomallei*. A shift towards more detailed characterization of genetic variation using RNA-Seq, Methyl-Seq, and genome-wide association studies will provide additional insights into this fascinating and deadly bacterium.

Keywords *Burkholderia pseudomallei* · Genomics · Evolution · Phylogeography · Melioidosis · Antibiotic resistance

Introduction

The environmental saprophytic bacterium *Burkholderia pseudomallei* is the causative agent of the potentially life-threatening infectious disease melioidosis. Arguably one of the most important and underrecognized neglected tropical diseases of our time [1], melioidosis has conventionally been considered a disease confined to only a handful of endemic tropical regions. Increasing awareness and detection of *B. pseudomallei* in regions previously not considered endemic for this organism has led to melioidosis cases being unmasked in most tropical and subtropical locales worldwide [2]. Using predictive modeling methods, the contribution of melioidosis to global mortality has recently been estimated at 89,000 deaths annually, a rate similar to the much higher-profile disease measles [3•].

Although melioidosis cases continue to be underreported in some endemic regions, the importance of *B. pseudomallei* as a formidable pathogen has been well recognized for some time. In 1997, *B. pseudomallei* was added to the US select agent list [4], and in 2012, this bacterium was included in the list of Tier 1 select agents, an elevated category reserved for infectious agents posing the greatest biothreat risk to human and animal health in the event of their deliberate misuse [5]. This increased profile provided a much-needed boost for research

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of this pathogen, including genomics-based approaches. To date, over 400 *B. pseudomallei* genomes from at least 33 countries have been made available on public databases. This rich resource has enabled many previously unanswerable questions about *B. pseudomallei* to be addressed at an unparalleled level of resolution and accuracy. Salient examples include the development of DNA-based diagnostics for accurate speciation of *B. pseudomallei* [6, 7] and its nearest genetically related neighbors [6, 8–10], identification of diagnostic loci to differentiate among *B. pseudomallei* strains [11, 12, 13••], determination of the geographical origin of *B. pseudomallei* [14••] and its subsequent dissemination across the globe [13••, 15•, 16•], detection of recombination patterns and restrictions on gene flow [17], characterization of genome structure, pangenome diversity and virulence factors [8, 18, 19••, 20, 21], identification of novel antibiotic resistance mechanisms [22•, 23, 24•, 25•], attribution of outbreak point sources [26•, 27–29], and fine-scale examination of within-host evolutionary processes [22•, 30, 31•, 32]. This review will focus on these genomics-based studies and how they have advanced our understanding of *B. pseudomallei* and melioidosis.

Next-Generation Sequencing Technologies The arrival of the first high-throughput, high-output, and rapid next-generation sequencing (NGS) technology in 2005 [33], which was commercialized by 454 Life Sciences, heralded a paradigm shift in microbial genetics research. Since then, NGS has rapidly evolved in terms of its technological sophistication, data quality, and accessibility. At the time of writing (early 2017), the cost of sequencing a *B. pseudomallei* genome with 100 bp paired-end reads to ×70 coverage using the Illumina HiSeq platform was ~USD$76 per sample. This cost will continue to decrease as new and improved NGS technologies emerge and demand increases. In addition to providing a much richer source of genetic data, NGS yields a greater return on investment and now supersedes many genotypic methods. For example, the relatively low expense of whole-genome sequencing (WGS), a popular application of NGS, means it now costs less to carry out than many genotyping methods (e.g., multilocus sequence typing (MLST) [34], ~USD$100 per sample). Data analysis is also no longer a bottleneck, with several freely available bioinformatics tools for analyzing small through large microbial genome datasets (e.g., breseq [35], NASP [36], and SPANDx [37•]). These tools have been designed to find mutations relative to a reference sequence using re-sequencing data. SPANDx has been optimized using *B. pseudomallei* as one of the model organisms and is designed to compare hundreds and potentially thousands of genomes in a single analysis by identifying single-nucleotide polymorphisms (SNPs), small insertions-deletions (indels), and larger deletions among strains using Illumina, 454, or Ion Torrent sequence data [37•].

The First *B. pseudomallei* Genome In 2004, Holden and coworkers [19••] published the first closed *B. pseudomallei* genome of strain K96243, which was isolated in 1996 from a diabetic patient in Khon Kaen, Thailand. This formative study revealed that the *B. pseudomallei* K96243 genome is comprised of two replicons at 4.07 and 3.17 Mbp, corresponding to chromosomes 1 and 2, respectively. This archetypal *B. pseudomallei* strain encodes ~6300 genes, making it one of the larger prokaryotic genomes currently known. The strikingly high total %G + C (~70%) content of K96243 is punctuated by 16 regions of lower %G + C content, identified as genomic islands (GIs). These GIs comprise ~6% of the genome [19••] and encode for a variety of functions including production of secondary metabolites and enhanced virulence [20].

Genomics has been instrumental in identifying GIs as a major source of genetic diversity among strains, with at least 71 distinct and variably present GIs identified in *B. pseudomallei* [20]. The location of GIs are relatively conserved, with the majority being inserted adjacent to tRNA genes, a process mediated by a tRNA site-specific recombination mechanism. This site specificity has limited the number of locations where GIs can insert into the genome [20]. Notably, the gene content of GIs, and their location and number, are highly variable among strains. A comparative genomic analysis of 37 strains demonstrated that *B. pseudomallei* has an "open" genome, driven largely by variation within GIs, with 136 new genes identified with each new genome [18]. Remarkably, despite the open and highly variable nature of GI loci, the overall gene order of the *B. pseudomallei* genome outside GI regions is quite stable, even between distantly related strains [18].

Phylogenomic Analysis of *B. pseudomallei* Uncovers a Strong Continental Signal *B. pseudomallei* has a strong propensity for homologous recombination, with a rate at least two times greater than that of *Streptococcus pneumoniae*, itself considered highly recombinogenic [14••]. Homologous recombination drives rapid genetic diversification, which presents a quandary when attempting to trace the origin of *B. pseudomallei* strains. This factor is particularly problematic when using genetic methods that rely on a small number of loci, which greatly increases susceptibility to homoplasy, a phenomenon whereby a shared genotype has arisen by convergence rather than descent. However, certain aspects of *B. pseudomallei* biology make it amenable to robust phylogeographic ascertainment, especially when large and deep genetic datasets such as those generated by WGS are used. First, unlike many other bacterial pathogens, *B. pseudomallei* is not readily communicable, with only a small number of reported human-to-human transmission cases (e.g., [38]); infections are almost always acquired from contact with contaminated soil or water. Second, long-range dispersal of *B. pseudomallei* is

uncommon, with closely related *B. pseudomallei* strains in endemic regions typically located not more than 45 km apart [39, 40]. Finally, distinct restriction-modification systems among different *B. pseudomallei* clades restrict gene flow between more distantly related taxa [17]. These fundamental characteristics mean that, even in the face of very high rates of homologous recombination, the *B. pseudomallei* genome provides a reliable predictor of a given strain's geographic origin.

Pearson and colleagues (2009) provided the first phylogenomic examination of *B. pseudomallei* origin and transmission using strains isolated from Australia and Asia [14••]. This seminal study, which compared 43 *B. pseudomallei* and related *Burkholderia* genomes using a phylogenomic approach, was the first comprehensive comparative genomic analysis carried out in *B. pseudomallei*. Based on their phylogeny, the authors proposed an ancestral Australian origin for *B. pseudomallei*, followed by a single, and likely anthropogenically driven, introduction event into Southeast Asia during a recent glacial period. This observation has since been consolidated by more recent genomic studies that have used larger and more globally diverse strain datasets [13••, 15•]. Figure 1a shows a contemporary analysis of the phylogenic approach first described by Pearson and colleagues. Using publicly available genomic data for 467 *B. pseudomallei* isolates recovered from 33 countries and rooted with a closely related *Burkholderia* species, our updated phylogeny confirms the original hypothesis that *B. pseudomallei* evolved from an Australian ancestor. This important discovery has shaped our understanding of *B. pseudomallei* transmission between continents and has impelled ongoing efforts to unmask melioidosis in regions not previously considered endemic for *B. pseudomallei* (e.g., several countries in Africa [41] and the Americas [42], as well as Indonesia [43]).

Human activity has played a central role in the transmission of *B. pseudomallei* between continents and may even be singlehandedly responsible for its global dissemination. The first WGS analysis of *B. pseudomallei* from Africa and the Americas proposed an anthropogenically driven dissemination of this bacterium into tropical regions worldwide [16•]. African strains are much younger than Australian and Asian strains according to phylogenomic analysis (Fig. 1b) and possess evidence of recent transmission from Asia, probably initially via the eastern island of Madagascar, which was inhabited only 1500–2000 years ago by settlers from both the African mainland and Indonesian Borneo. Subsequent large-scale anthropogenic activity from Africa into the Americas during the transatlantic slave trade in the fifteenth through nineteenth centuries is thought to have driven the dissemination of *B. pseudomallei* into this previously naïve region [15•, 16•]. The anthropogenically driven introduction of contaminated soil or water, or chronically infected animals, livestock, or plants, have been raised as plausible explanations for *B. pseudomallei* transmission between continents [44]. WGS of additional *B. pseudomallei* isolates collected from geographically diverse locations over longer timescales will be needed to address the role that humans may have played in these rare yet significant transmission events.

On a much more recent timescale, re-introduction of a Southeast Asian *B. pseudomallei* strain into northern Australia has now been documented for the first time [13••]. In 2005, the first melioidosis case caused by ST-562, a MLST genotype previously only seen in China and Taiwan, was documented in the hyperendemic "hotspot" of Darwin, Northern Australia [13••]. Clinical cases in this region, which have been closely monitored since 1989 as part of the ongoing Darwin Prospective Melioidosis Study [45], showed that no cases prior to 2005 were caused by this ST genotype. Although the precise method of introduction into Darwin is unclear, it does not appear to have been a widespread event, with environmental surveillance for this clone suggesting that, to date, its presence is limited to a restricted geographic region in the Darwin region, and comparative genomic analysis revealing very low levels of genetic diversity [13••]. Phylogenomic analysis was critical for confirming the Asian origin of this clone (Fig. 1b) due to the possibility for homoplasy using MLST [46] or other typing methods. This example provides a concerning demonstration of the relative ease of transmitting *B. pseudomallei* in modern times where global trade and mass human transit are now commonplace.

***B. pseudomallei* Virulence and Melioidosis Presentations Are Highly Variable** The classical presentation of melioidosis is as an acute pneumonia with or without sepsis, which occurs in approximately half of all cases in melioidosis-endemic regions [45, 47]. Melioidosis can mimic multiple infectious diseases and even some autoimmune disorders and cancers [48]. Both disease presentation and severity can differ based on geographic location; for example, mortality rates in the hyperendemic regions of Northern Australia and Southeast Asia are vastly different, at approximately 10 and 40%, respectively [48]. It has previously been postulated that this difference in mortality rate is due to inequitable access to intensive care facilities [49]; however, there remains the possibility that genetic differences among strains also contribute to these disparate mortality rates. Further exploration using genomic approaches such as microbial genome-wide association studies may help to identify virulence loci that are over-represented in Thai strains compared with Australian strains, or conversely, the loss of virulence loci in Australian strains. The open genome of *B. pseudomallei* differs based on locale, with certain loci, including variable virulence factors, much more prevalent in strains from particular regions compared with others [13••, 15•]. Although the correlation of disease severity and clinical presentations with genetic markers in

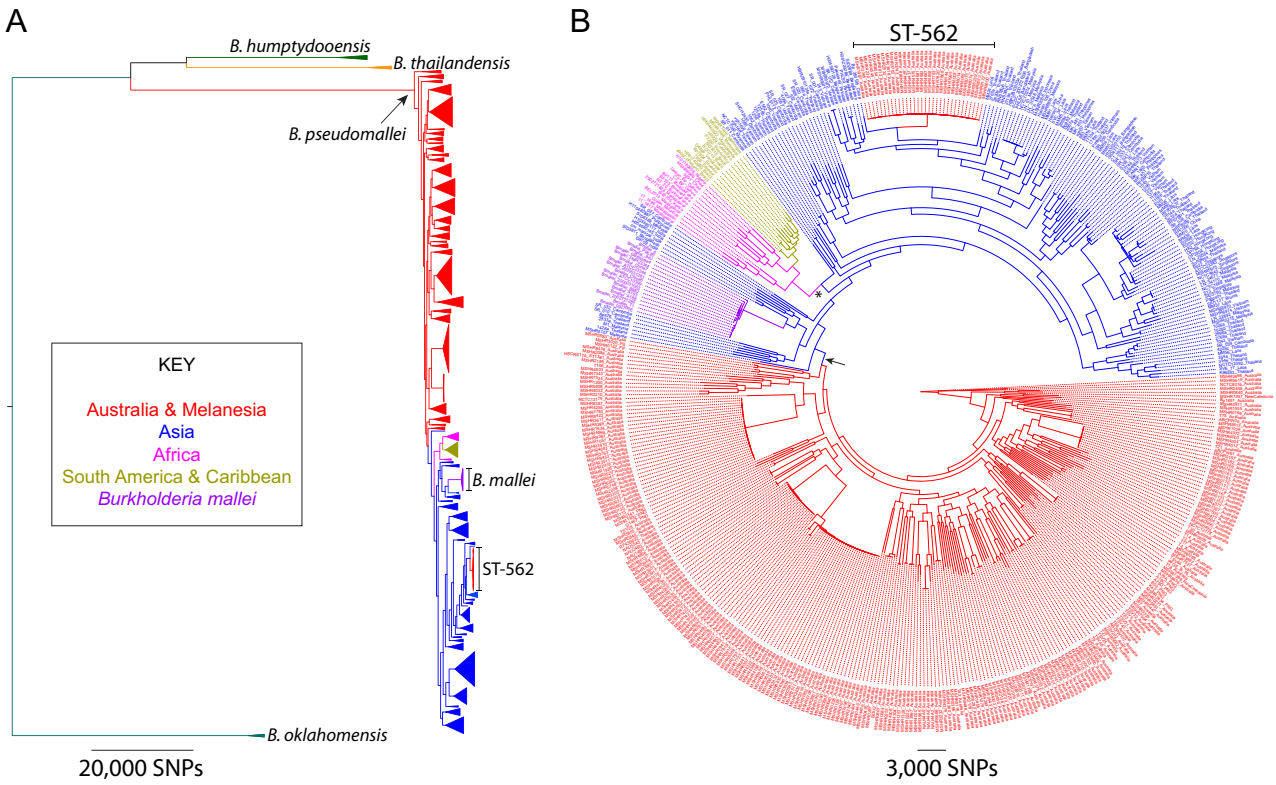

Fig. 1 Phylogenomic reconstruction of the global *Burkholderia pseudomallei* population. **a** Maximum parsimony phylogenetic reconstruction of a diverse *B. pseudomallei* dataset comprising 467 genomes from 33 countries. The tree was rooted with *B. oklahomensis*, a member of the *B. pseudomallei* complex, and includes two other *B. pseudomallei* complex species (*B. thailandensis* and *B. humptydooensis*) for comparison. This phylogeny demonstrates that the Australian *B. pseudomallei* is ancestral to strains isolated elsewhere globally, pointing to *B. pseudomallei*'s origins on this continent. **b** Maximum parsimony phylogenetic reconstruction of *B. pseudomallei* and *B. mallei*. The single transmission event of Australian *B. pseudomallei* into Southeast Asia during a recent glacial period is indicated by a *black arrow*. Subsequent transmission from Asia into Madagascar and Africa, and then Africa into the Americas and Caribbean, is denoted by an *asterisk*. This tree shows that *B. mallei* resides on a node that groups most closely with South Asian strains, suggesting that this equine-adapted subspecies evolved from a *B. pseudomallei* strain from this region. *B. mallei* subsequently disseminated into the other long known glanders-endemic regions in the Middle East, Africa, and the Americas. The recent and unprecedented transmission of an Asian clone, ST-562, into the Northern Territory is labeled on both trees

B. pseudomallei is an ongoing area of investigation, a handful of studies have already identified loci that correlate with specific clinical presentations or severity in humans [11] and in the BALB/c mouse model of infection [21]. Comparative genomics has also identified variation within the lipopolysaccharide biosynthesis pathway [50], which encodes an important virulence determinant in *B. pseudomallei*, although the exact consequence of this variation remains to be ascertained.

Source Attribution of Melioidosis Outbreaks Genomics is a powerful tool for examining, with very high resolution, the probable origin ("point source") of outbreaks. Because of the typically short duration in an outbreak scenario, methods for detecting rapidly evolving loci are essential. Prior to the genomics era, multilocus variable-number tandem repeat analysis (MLVA) was commonly used for detecting genetic variation in bacterial strains sharing a very recent common ancestor, such as a clonal infection, due to its characterization of rapidly evolving repeat loci [51]. MLVA has been used to identify differences in *B. pseudomallei* populations in clonal outbreak clusters and even within an individual patient over a very short duration [52, 53]. However, MLVA suffers from many issues in *B. pseudomallei*, most problematic of which are the interrogation of only a small fraction of the genome and high rates of homoplasy. The roadblock of homoplasy can be somewhat circumvented by the inclusion of another typing method that examines more slowly evolving loci and is thus less susceptible to the effects of homoplasy. For example, multiple studies have used MLST alone to determine whether strains involved in an outbreak are clonally related [54, 55], but due to the lack of resolving power, a solid conclusion is not always able to be reached, especially if the outbreak occurs in an endemic region where the disease is relatively common. An improved fit with the epidemiological data is frequently observed when a highly resolving technique, such as MLVA, is combined with the more stable typing technique, MLST [56]. The shift towards using WGS now provides a much more robust methodology for point-source attribution of

melioidosis outbreaks and bypasses the need to use multiple typing techniques to correctly determine the nature of an outbreak [26•, 27, 28].

Within-Host Evolution of *B. pseudomallei* in the Human Host Comparative genomics has been central in unraveling the molecular mechanisms of *B. pseudomallei* adaption during its transition from an acute to a chronic infection. The longest-known documented melioidosis case, an Australian woman who continues to asymptomatically harbor *B. pseudomallei* in her lungs since first being diagnosed with melioidosis in 2000 [31•], has provided a fascinating model for observing ongoing *B. pseudomallei* evolution within a human host. Notable genetic events in these *B. pseudomallei* strains that have become fixed over time included the loss of several virulence and immunogenic loci, inactivation of the stress response sigma factor RpoS, and deletion of 221 genes on chromosome 2 (285 kb, 4% of the genome); all of this has occurred over a very short (11.5-year) evolutionary period. Striking parallels were observed between this human-adapted infection and the equine-adapted *B. pseudomallei* clone, *B. mallei*. Approximately 50% of genes lost in the latter isolates from the chronic melioidosis patient are also absent in *B. mallei*, demonstrating that these regions are not necessary for prolonged survival in a mammalian host [31•]. In another chronic melioidosis case spanning 2.7 years, comparative genomics revealed that a subpopulation of the *B. pseudomallei* isolates obtained from this patient had undergone large-scale recombination events involving 1.3 Mbp (18%) of the genome, leading to dramatic genetic differences that were not observable using MLST alone [57]. Comparative genomics of *B. pseudomallei* from four relapse melioidosis cases [30], an acute infection [32], and seven chronic cystic fibrosis cases [22•] has also shown that this bacterium can undergo rapid and dramatic adaptive changes within the human host.

Identifying Novel Antibiotic Mechanisms Using Comparative Genomics *B. pseudomallei* is naturally resistant to many antibiotic classes, including aminoglycosides, macrolides, fluoroquinolones, and the majority of β-lactams [58]. The few available antibiotics for treating melioidosis can fail in cases where acquired resistance or decreased susceptibility develops over the course of treatment. This phenomenon has now been documented for all antibiotics used to treat *B. pseudomallei* infections, including carbapenems [25•, 59]. Comparative and functional genomics has greatly accelerated our understanding of the molecular basis for decreased susceptibility or resistance towards antibiotics. This task can be further simplified by genomic comparison of isogenic strains retrieved from the same patient, one of which is antibiotic-sensitive and the other antibiotic-resistant. To date, genomics has been used to identify ceftazidime resistance that has arisen following key SNP mutations in or duplication of the β-lactamase PenA [22•, 25•], or by the entire loss of the penicillin-binding protein 3 gene, *BPSS1219* [24•]. In a similar fashion, doxycycline resistance has recently been shown to be associated with the loss of the efflux pump regulator AmrR in concert with the SAM-dependent methyltransferase mutation [23], and decreased susceptibility towards the carbapenem antibiotic meropenem has been attributed to mutations in AmrR [59]. As *B. pseudomallei* continues to find new ways to evolve resistance towards antibiotics, genomics provides a powerful tool for identifying known and novel mechanisms of resistance. Knowledge of these mechanisms leads to more accurate and faster diagnostics, the ability to provide more effective "personalized" treatments for melioidosis patients, enhanced antimicrobial stewardship, and targets for future innovative therapeutics.

Future Directions

Longer (e.g., PacBio, Oxford NanoPore) NGS reads, and platforms that offer close-to-real-time analysis of sequence read data (e.g. Oxford NanoPore) to enable data collection to be stopped when sufficient coverage has been obtained [60], are already paving the way for new and exciting advancements in bacterial genomics. Longer reads allow genomes to be "closed" by resolving regions that remain refractory to complete resolution using short-read NGS methods due to low-complexity sequences, paralogous loci, or structural rearrangements. The continued evolution of NGS technology speed and capacity, and increased competition among NGS providers, will further drive down the cost of sequencing *B. pseudomallei* genomes to the point that such characterization will be carried out routinely in both research and clinical laboratories. A remaining challenge in genomics is the ability to sequence and accurately parse haplotypes in cases of mixed *B. pseudomallei* infections without the need for passaging and sequencing single colonies [57]. NGS methods that characterize total gene expression (e.g., RNA-seq) and methylation patterns (e.g., Methyl-seq) under different growth conditions, including in vivo, are now being explored to gain further insight into the biology of *B. pseudomallei*. Metagenomic and metatranscriptomic approaches are adding yet another dimension in our understanding of the complex interaction between infectious agents and their hosts.

Conclusion

The review has highlighted the crucial role that genomics has played so far in answering fundamental questions about *B. pseudomallei* origin, transmission, virulence, within-host evolution, and antibiotic resistance mechanisms. These questions, many of which have been unresolved for many years

and even decades, can now be quickly and unambiguously addressed with this powerful and cost-effective technology. The digitization and public availability of *B. pseudomallei* strains via WGS has already begun to provide a rich and invaluable resource for melioidosis researchers worldwide that is facilitating comparison of strains on a global level. New NGS technologies will enable researchers to address even more complex questions concerning this important and deadly pathogen.

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Compliance with Ethical Standards

Conflict of Interest Erin P. Price, Derek S. Sarovich and Bart J. Currie each declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All reported studies with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards.

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