Genetic variation associated with infection and the environment in the accidental pathogen *Burkholderia pseudomallei*

Claire Chewapreecha1,2,3,4*, Alison E. Mather5,6, Simon R. Harris3, Martin Hunt3, Matthew T.G. Holden7, Chutima Chaichana8, Vanaporn Wuthiekanun1, Gordon Dougan4, Nicholas P.J. Day1,9, Direk Limmathurotsakul1,9, Julian Parkhill10,11 & Sharon J. Peacock4,11*

The environmental bacterium *Burkholderia pseudomallei* causes melioidosis, an important endemic human disease in tropical and sub-tropical countries. This bacterium occupies broad ecological niches including soil, contaminated water, single-cell microbes, plants and infection in a range of animal species. Here, we performed genome-wide association studies for genetic determinants of environmental and human adaptation using a combined dataset of 1,010 whole genome sequences of *B. pseudomallei* from Northeast Thailand and Australia, representing two major disease hotspots. With these data, we identified 47 genes from 26 distinct loci associated with clinical or environmental isolates from Thailand and replicated 12 genes in an independent Australian cohort. We next outlined the selective pressures on the genetic loci (dN/dS) and the frequency at which they had been gained or lost throughout their evolutionary history, reflecting the bacterial adaptability to a wide range of ecological niches. Finally, we highlighted loci likely implicated in human disease.

1 Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand. 2 Bioinformatics and Systems Biology Program, School of Bioresource and Technology, King Mongkut’s University of Technology Thonburi, Bangkok 10150, Thailand. 3 Wellcome Sanger Institute, Hinxton CB10 1SA, UK. 4 Department of Medicine, University of Cambridge, Cambridge CB2 0QQ, UK. 5 Quadram Institute Bioscience, Norwich NR4 7UQ, UK. 6 Faculty of Medicine and Health Sciences, University of East Anglia, Norwich NR4 7TJ, UK. 7 School of Medicine, University of St Andrews, St. Andrews KY16 9TJ, UK. 8 Department of Mathematics, Faculty of Science, King Mongkut’s University of Technology Thonburi, Bangkok 10140, Thailand. 9 Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7LF, UK. 10 Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, UK. 11 These authors contributed equally: Julian Parkhill, Sharon J. Peacock. *email: claire@tropmedres.ac; sjp97@medschl.cam.ac.uk
Burkholderia pseudomallei is an environmental Gram-negative bacterium and the cause of melioidosis, a serious infectious disease. A recent modelling study predicted that an estimated 165,000 people were affected globally per year, 89,000 of which died. The bacterium has a broad range of ecological niches, and can be isolated from soil, surface water, amoebae, plants and infected humans and other animals in many tropical and sub-tropical regions. Human infection results from environmental exposure associated with inoculation, ingestion or inhalation of the bacterium, with increasing risk of acquisition for people with predisposing health conditions or activities that increase exposure to soil or water, such as rice farming or drinking untreated water. Infection can be acute, chronic, latent or cleared, with rare cases of human-to-human transmission being reported. Antibody responses to B. pseudomallei can be found in healthy individuals living in endemic areas in the absence of clinical symptoms, suggesting that the majority of the exposure is harmless or results in sub-clinical infection.

B. pseudomallei can be found in the stool of some infected humans and experimental murine models. This provides a potential mechanism for human-to-environmental transmission and the possibility of repeated passage through the human host. Serial passage of Burkholderia cenocepacia in a long-term chronic airway infection model in mice has been shown to increase bacterial fitness. Based on this observation, the natural passage of B. pseudomallei through humans, other animals or its natural predators such as soil amoebae might have enhanced and maintained selection pressure for pathogenicity in a subset of the population. This potentially results in heterogeneity of bacterial virulence, as evidenced by marked variations in severity and pathogenicity in mice challenged by different B. pseudomallei strains. B. pseudomallei has a large and highly variable accessory genome across the species. While the core genome may be sufficient for strain survival, it is possible that specific bacterial genes, gene variants or their combinations may confer additional advantages for survival and replication in specific niches including human infection, or particular environmental conditions. Here, we sought evidence for bacterial genetic factors associated with human disease and environmental adaptation using two independent datasets from major melioidosis hotspots in Thailand, and Australia. These were used as a discovery and validation dataset, respectively.

Results Clinical and environmental isolates are inter-mixed. We first outlined the population structure of the dataset from Northeast Thailand where information from household sampling structure was also available. B. pseudomallei used in this collection was originally cultured from patients presenting to Sunpasitthiprasong hospital in Ubon Ratchathani between 2010 and 2011, together with residential water sources from melioidosis patients as well as non-infected individuals (see Methods for details). With the exception of 1 patient where two isolates were cultured, a single isolate was collected from each patient (n patient = 324, n clinical isolates = 325). Up to 10 water isolates were sampled from each household (n households = 48, n environmental isolates = 428, see Fig. 1 for sampling framework). Unlike many pathogens where isolates associated with disease contain substantially fewer genes, a pan-genome analysis revealed a similar number of genes per genome in clinical and environmental isolates (two-sided Mann–Whitney U test, p value = 0.312). Moreover, both phylogenetic and multidimensional scaling approaches (MDS) indicated that clinical and environmental isolates were largely mixed with each phylogenetic group comprising both clinical and environmental isolates (Fig. 2). Previous studies have noted the importance of recombination in driving B. pseudomallei evolution, demonstrating genetic interactions and co-evolution of multiple B. pseudomallei lineages that shared the same habitat. Evidence for genetic interactions between clinical and environmental isolates was sought for 5 monophyletic groups, each of which had >70% bootstrap node support on the core genome phylogeny to ensure robust analysis (Supplementary Fig. 1). Our results showed that both clinical and environmental isolates in each group had undergone recombination. Moreover, similar numbers of recent recombination events (defined by recombination located at the tips of the phylogeny) were identified in both clinical and environmental isolates (Fisher’s exact test p value = 1, Supplementary Fig. 2). A search for the sources and sinks of recent recombination events (see Methods) revealed that clinical isolates could act as DNA donors for recombination detected in environmental isolates. Similarly, environmental isolates could act as DNA donors for recombination detected in clinical isolates. DNA recipients and DNA donors were more likely to be found in isolates from the same origin. There was a higher probability of clinical isolates being the donor for clinical isolate recipients (two-sided Mann–Whitney U test p value < 2.2 × 10^-16), and environmental isolates being the donor for environmental isolate recipients (two-sided Mann–Whitney U test p value = 9.41 × 10^-9). Together, this suggests a structure to the genetic flux within the clinical and environmental isolates despite the potential for ecological mixing of the population. Not all environmental exposure leads to infection. We next investigated the potential source of infection by comparing the genetically closest environmental isolates to each clinical isolate using the Northeast Thailand data. Given that consumption of untreated household water supply was common in this endemic area, we first considered the link between household water supply and infection. Of 48 households with water samples cultured positive for B. pseudomallei, 27 households belonged to melioidosis patients. Notably, only 6 households of melioidosis patients had environmental and clinical isolates clustered within the same monophyletic group (Fig. 2b, Supplementary Fig. 3a). After removing signals from recombination, comparison of pairwise genetic difference showed that clinical and environmental isolates from these 6 households (median = 6994 single-nucleotide polymorphism (SNP)) were not significantly more similar to one another than to those from randomly paired clinical and environmental isolates (median = 7,090, Mann–Whitney test p value = 0.3901, Supplementary Fig. 3b). This result indicated that the studied patients did not commonly contract melioidosis from their household water supply. It is possible that the infecting isolate represented a minority population in water that was not detected in the study, or it was acquired elsewhere. The availability of Global Positioning information for 134 clinical and 387 environmental isolates allowed us to locate the potential source of infection for a subset of melioidosis cases. After removing signals from recombination events, we mapped the pairwise genetic differences between each clinical isolate and its closest environmental isolate (range: 24–16,866 SNPs, Fig. 3a) and their geographical distance (range: 5–100 km apart, Fig. 3b). We found a lack of genetic and spatial correlation between clinical isolates and their closest environmental isolate (R² = 0.013, p value = 0.352) with no genetic evidence that patients had acquired B. pseudomallei from their neighbourhood or farmland (defined as 10 km² from patient’s household). It is possible that the Mun river, its extensive canal systems and floodplains may have dispersed genetically close isolates over a
Fig. 1 Sampling framework for B. pseudomallei isolates from the case control study. a The chart shows the number of clinical and environmental isolates from patients and/or household water supplies of cases (patients with melioidosis) and controls (patients with non-infectious conditions admitted during the same period). b Temporal distribution of environmental and disease isolates in the discovery dataset collected from June 2010 to January 2012. With the exception of months with no house visits, the number of monthly clinical and environmental samples collected were positively correlated (linear regression, adjusted R-square = 0.259, p value = 0.026). c Spatial and temporal distribution of environmental and disease isolates in the validation dataset from the public database.
large geographical distances (Fig. 3c–g), thereby disrupting the genetic and spatial correlation. It is also likely that our environmental isolates were not sufficiently intensively sampled to capture the source of infection. Nevertheless, the lack of conclusive cases of household contraction despite evidence of exposure supports the hypothesis that not all *B. pseudomallei* exposure leads to infection.

**Genetic factors associated with disease and the environment.** We next investigated potential genetic signals that were associated with infection or the environment by estimating the correlation between the bacterial phylogeny and distribution of source of isolation on the tree using Pagel’s λ (Supplementary Data 5). Only five monophyletic groups were included in the tests to ensure robust analysis. The distribution of “disease” and “environmental” origins was not random (Supplementary Fig. 4), indicating that there may be separable environmental and clinical clades either at deep or shallow nodes. This could reflect the presence of bacterial determinants that mediate survival in human or environmental niches.

We applied two complementary genome-wide association studies (GWAS) (a kmer-based and a pan-genome based approach) to the 325 clinical and 428 environmental isolates, which were controlled for population stratification (see Methods, Supplementary Datas 1 and 2). We note that there was potential cross categorisation as the environmental isolates could be capable of causing disease. While this caveat reduces the power to detect the association which elevates the true negatives, this would be unlikely to impact on the false-positive rate. Of 24,856,071 kmers used to define the population, 38,797 (0.156%) were associated with “disease” or “environmental” origin. These were mapped onto the pan-genome to identify potential genes, resulting in 365 “disease-associated” or “environmental-associated” genes. The pan-genome based GWAS analysis identified 675 disease-associated or environment-associated genes. Comparison of output from the two methods showed that 47 genes were detected by both (38 disease-associated and 9 environmental-associated genes, Supplementary Datas 3 and 4), which account for 0.3% of the pan-genome. Based on the size of transcriptional operons reported in Ooi et al., we grouped these genes into 26 loci (Fig. 4). These 47 genes were evaluated in an independent dataset from Australia (clinical isolates=184, environmental isolates=73), which showed that 12 genes (25.5%) were either enriched in clinical or environmental isolates (Supplementary Data 5, two-sided Fisher’s exact test, FDR < 0.01). The fact that isolates from Australia and Southeast Asia represent distinct phylogenetic clades is consistent with parallel evolution for a proportion of the disease-associated and environment-associated genes.

Functional enrichment analyses of the 47 gene clusters in the discovery cohort showed an elevated frequency of the term “Pathogenesis” and “Replication, recombination and repair” (Supplementary Data 6, one-sided Fisher’s exact test p value 2.30 × 10⁻⁷, and 2.08 × 10⁻¹², respectively). The former may...
Fig. 3 Genetic relatedness between clinical and environmental isolates from households. 

**a** Boxplot summarises pairwise SNPs distance between each clinical and its closest environmental isolate from each monophyletic group after removing recombination signals. A pairwise SNP distance between two clinical isolates cultured from the same patient were included as a threshold. 

**b** Correlation between pairwise SNP distance and geographical distance of clinical and its closest environmental isolates. 

**c-g** Geographical distance between clinical and its closest environmental isolates by monophyletic group. Red and blue dots represent clinical and environmental isolates, respectively. Colour shade of the links indicates the pairwise SNP distance between the pair. Source data used to plot (a) and (b) is available in Supplementary Datas 12 and 13, respectively.
allow the bacterium to compete in specific environmental niches or survive inside single-cell or multicellular organisms during infection. Genes annotated with the term “Replication, recombination and repair” largely comprised transposons that may act as markers or remnant elements for horizontally transferred genes, or may inactivate gene function. Apart from these, 8 of 26 loci consisted of IS, transposons and integrase, which highlights the significance of transposable elements in rearranging bacterial genomes.

Selection pressures maintaining niche-associated genes. We explored whether or not the 38 disease-associated and 9 environmental-associated genes were under selective pressure by calculating the ratio of the rate of non-synonymous substitutions per non-synonymous site to the rate of synonymous substitutions per synonymous site (dN/dS). The average for both groups was below 1, but the ratio was significantly higher for environmental-associated compared with disease-associated and other accessory genes (Fig. 5a, Supplementary Data 3, Mann–Whitney U test p value = 2.87 x 10^{-2} and 5.11 x 10^{-3}, respectively). Despite the small number of genes being compared, this suggests that the subset of genes in the environment-associated genes may be under reduced purifying selection, or elevated diversifying selection, compared to disease-associated and other accessory genes. We further quantified the number of times each cluster was acquired or lost in monophyletic groups that constitute an entire phylogenetic tree (n group = 5, n of isolate in each group ≥ 57, node bootstrap supports ≥70). Assuming an equal rate of gene gain and loss, stochastic mapping of the presence of each disease- or environment-associated cluster highlighted multiple gene gain-and-loss events, one possible reason for which is a constant change in niches that may include switching between extra- and intracellular lifestyles. Notably, 38/47 genes showed a preference for net gain, 4/47 had a preference for net loss, while 5/47 showed ambiguous directions when compared across multiple monophyletic groups (Fig. 5b, c, Supplementary Data 3). Although we did not observe differences in net gain or loss between disease- and environmental-associated genes (ANOVA test, gene p value = 0.841, loci p value = 0.876), our results highlighted a greater proportion of overall net gain for both disease- and environmental associated genes. Some of these may confer the bacterium longer-term advantages, which warrants further investigation.
Examples of disease-and environmental associated genes. Many of the disease-associated loci contained genes that encoded biologically plausible or known virulence determinants. One example was a large toxin complex (tcdB, tcdA, tccC and hemolysin activator fhaC) encoded by a locus of up to 69.7 kb, which was identified in the discovery dataset (Supplementary Fig. 5). This locus has not been characterised in B. pseudomallei but homologues exist in diverse bacterial species including Pseudomonas, Yersinia and Photorhabdus. The latter is an insect pathogen, experimental characterisation of which has demonstrated that tccC has enzymatic activity and that tcdA and tcdB facilitate the translocation of the toxin into host cells. These toxin genes were flanked in B. pseudomallei by several integrases and transposases families including IS2, IS3/IS911, IS4, IS66, IS166, IS407, IS111A/IS1328/IS1533 and IS1478, indicative of a mobile genetic element origin. An analysis of gene gain-and-loss events for the locus was possible for one monophyletic group (group 5, n = 156 isolates), as this locus was variably present in group 5 but fully present or absent in the other groups. For this group, we observed a slightly greater net gain of the whole locus with the toxin genes being acquired and lost 10 and 9 times, respectively. This may suggest not only a selective advantage but also a fitness cost associated with this locus for B. pseudomallei.

An example of environmental-associated loci is a truncated variant of filamentous haemagglutinin (fha), a known adhesin and immunomodulator across different bacterial species. In B. pseudomallei, the number of fha genes varies between isolates and different combinations of fha genes have been observed with patients infected by B. pseudomallei, with a specific fha variant reported to have increased risk of infection associated with positive blood cultures. While our kmer approach identified disease-associated signals from haemaglutination activity domains on this gene, our pan-genome approach detected environmental-associated signals from a truncated form of this gene (Supplementary Fig. 6). A closer inspection highlighted a truncation caused by a premature stop codon upstream of the haemagglutinin repeat domains, which might disrupt gene function. This environmental-associated and truncated form showed a greater net gain in all tested monophyletic groups, suggesting a selective advantage of this variant in the northeast Thailand setting.

Discussion

Our results suggest that despite evidence of direct contact with householders, not all B. pseudomallei exposure led to infection. A transition from exposure to disease likely requires additional risk factors involving B. pseudomallei, host and environment. Our analyses have identified B. pseudomallei gene clusters that are enriched in clinical or environmental isolates. These genes have
arisen repeatedly in different populations with distinct phylo-
geography, demonstrating robustness of the findings from the
Thai discovery dataset. Many of these genes are under relaxed
purifying selection and have been gained or lost multiple times
throughout the organism’s evolutionary history, implying that
there may be several niches to which this opportunistic bacterium is
adapted. This includes environmental and other eukaryotic hosts5,16,41,
the latter potentially providing genetic pre-adaptation for
invasion and survival in the human host. Based on our current
knowledge of the ecology of B. pseudomallei, there are still a
substantial number of disease-associated and environment-
associated genes with unknown function, unidentified interac-
tion partners or unexplored roles in each ecological niche, thereby
limiting the immediate translational applications of our study.
Further exploration into the ecological role of these genes will be
essential to better manage and prevent the infection from this
accidental pathogen.

Methods

Bacterial isolates. Two bacterial collections were used to create independent
discovery and validation datasets. These originated from distinct regions where
melioidosis is highly endemic—northeast Thailand and northern Australia18,22.
The discovery dataset was drawn from a study of the activities of daily living
associated with melioidosis, which was conducted at Sunpatisitphrapong (formerly
Sappasithiprasong) hospital in Ubon Ratchathani, Northeast Thailand between 2010
and 201111. In brief, 330 cases of culture-proven melioidosis and 513 control patients
with non-infectious conditions were recruited. B. pseudomallei can survive in water56,
and this ability contributes to its environmental survival in the endemic area. Five litres
of residential drinking water were collected per household and cultured for B. pseudomallei
from cases and controls who lived within 100 km of the hospital. B. pseudomallei was isolated from 12% of borehole and tap water samples, and 4%
of well water samples. Multiple colonies were picked and individually saved from each
water sample. Consumption of untreated water was common (85% of cases and
72% of controls) and associated with a higher risk of melioidosis5. We assumed that
isolates from water were a fair representation of environmental isolates.
Simultaneous infection with more than one strain of B. pseudomallei was reported to
be uncommon35. Except for 1 case, a single colony was cultured from each melioidosis patient. We noted a differential rate of B. pseudomallei being cultured from clinical (median for blood culture = 1 CFU/mL)44 and water samples (median = 1 × 10−3 CFU/mL)3.
For the purposes of the study described here, we sequenced 325 B. pseudomallei isolates from 324 cases, and 428 B. pseudomallei colonies (isolates) from 48 water samples (including samples from 27 melioidosis patients) (Fig. 1, Supplementary Data 1).
The validation dataset consisted of whole genome sequence data for 258 B. pseudomallei isolated in Australia, which were downloaded from the NCBI database (Supplemental Data 2). These isolates have been described previously18,23. In brief, isolates were from patients with melioidosis (n = 184) and the environment (n = 73). The temporal and spatial distribution of isolates in this dataset is summarised in Fig. 1.

Whole-genome sequencing. DNA was extracted from the 753 Thai B. pseudo-
mallei isolates as described in Supplemental Data 1. DNA libraries were prepared according to the Illumina protocol and sequenced on an Illumina HiSeq2000 with 100-cycle paired-end runs giving a mean coverage of 84 reads per nucleotide. Sequencing of clinical and environmental isolates was done at the same time on the same platform. Taxonomic identity was assigned using Kraken46 to control for potential con-
tamination in each sample with other closely related species. Consumption of untreated water was common (85% of cases and 72% of controls) and associated with a higher risk of melioidosis5. We assumed that isolates from water were a fair representation of environmental isolates. Simultaneous infection with more than one strain of B. pseudomallei was reported to
be uncommon35. Except for 1 case, a single colony was cultured from each melioidosis patient. We noted a differential rate of B. pseudomallei being cultured from clinical (median for blood culture = 1 CFU/mL)44 and water samples (median = 1 × 10−3 CFU/mL)3. For the purposes of the study described here, we sequenced 325 B. pseudomallei isolates from 324 cases, and 428 B. pseudomallei colonies (isolates) from 48 water samples (including samples from 27 melioidosis patients) (Fig. 1, Supplementary Data 1). The validation dataset consisted of whole genome sequence data for 258 B. pseudomallei isolated in Australia, which were downloaded from the NCBI database (Supplemental Data 2). These isolates have been described previously18,23. In brief, isolates were from patients with melioidosis (n = 184) and the environment (n = 73). The temporal and spatial distribution of isolates in this dataset is summarised in Fig. 1.

Genome assembly and pan-genome analysis. New assemblies were performed as described in ref. 37 to give a median of 97 contigs (min = 61 contigs, max = 259 contigs), and median length of 7,114,540 bp (min = 6,884,381 bp, max = 7,404,549 bp). All study genomes were annotated using Prokka48. A predicted median of
5936 coding sequences were assigned onto each genome (median = 5762, max = 6264), which falls within the range of published reference genomes18,49,50. Roary40 was used to detect pan-genome for the discovery dataset together with the two reference B. pseudomallei genomes (K96243 from Thailand and Bp668 from Australia). The inclusion of the well-characterised Thai reference K96243 served as the quality control for the pan-genome analysis, and the Australian reference Bp668 served as an outgroup to root the phylogeny in a subsequent analysis. An all-against-all BLASTP comparison at 92% sequence identity was used as described in ref. 39. Genes were defined as core if present in ≥99% of isolates. This led to 4322 and 10,718 genes being classified as core and accessory, respectively (Supplementary Data 2). The number of core genes identified fell within the range described previously18.

Population structure estimated by multi-dimensional scaling. The population structure of the 753 Thai isolates was estimated from sequence assemblies using Mash v. 1.1.1.151, which captures information from intergenic regions, core and accessory genes. Assemblies were shredded into their constituent kmers. The pairwise distance between assemblies was estimated and computed into the 753 × 753 matrix. Metric MDS was performed using R cmdscale to project the population structure into n−1 coordinates. The top three coordinates were used to control for GWAS population stratification.

Population structure estimated by phylogenetic trees. Phylogenetic approach was employed to determine the overall population structure as well as more detailed subclade analyses. An overall population structure was estimated using SNPs in the core genome. Single-copy core genes from 753 isolates, K96243 and Bp668 were concatenated and aligned using Maff v.7.2052, followed by manual annotation using SeaView43. This comprised 4322 genes, representing on average 73% of genes in individual genomes. Single-nucleotide substitutions in the align-
ment were called using the methods described by Page et al.34, resulting in SNPs. A maximum-likelihood phylogeny was constructed with RAxML HPC v.8.2.8 using a general time reversible nucleotide substitution model with four gamma categories for rate heterogeneity and 100 bootstrap support. The overall phylogeny had 58.4% of external and internal nodes showing ≥70% bootstrap support.

For measuring phylogenetic signals and ancestral reconstruction analyses, only monophyletic branches with >70% bootstrap support were considered. Branches with poor bootstraps were removed using ITOL36. Subsequent tests were performed on 5 monophyletic groups comprising group 1 (n = 57), group 2 (n = 84), group 3 (n = 86), group 4 (n = 91) and group 5 (n = 156), totalling 474 isolates (63% of the northeast Thailand dataset). Each group was rooted on the Australia isolate Bp668.

Maximum-likelihood phylogenies were also used to examine specific disease-
associated clusters by concatenating and aligning genes using Maff v.7.2052, with truncated genes manually checked. A maximum-likelihood phylogeny was constructed as above with 100 bootstrap support and rooted on an Australian gene homologue.

Detection of recombinant sites. Recombination detection required genome alignment with a higher resolution. A pseudo-alignment from each group was generated by mapping sequence reads against a reference genome K9624349 from northeast Thailand. Methods described in ref. 37 was applied to allow greater sensitivity for detection of variants including small insertions and deletions (indels). To determine the impact of recombination within this dataset, we ran Gubbins49 on individual monophyletic group (Supplementary Fig. 1). The regions identified as recombogenic were largely those reported as genomic islands48. The contribution of recombination to the overall diversity was estimated by ratio of recombination events to the number of mutations (r/m) thus avoiding a bias introduced by using number of SNPs that can be affected by DNA donors of varying genetic distances. Phylogenies with recombination removed were used to determine the connections between isolates from the clinic and household water supply.

Identification of recombination donors. Potential sources of recombination fragments were determined by comparing the sequences identity to the recombinant fragments detected the recipient strains. Recombination regions overlapped with genomic islands mobile genetic elements were excluded. Identification of potential recombination donor were focused on recent recombination events with recipient located on the tip of each subclade phylogeny. Recipient blocks were searched using BLAT v.3559 against the rest of the assemblies for identical match (donor blocks). To minimise non-specific match, the search was restricted to recipient block >10 bp with no unknown nucleotides. "N" is used in both recipient and donor blocks.

We next calculated the probability of each isolate being a donor for individual recipient isolate. For each recipient isolate where "n" potential donor isolates were identified, each potential donor isolate was assigned a probability of "1/n". Isolates showing no hit for a particular search were assigned probability of 0. The total likelihood of each isolate for being a donor was calculated as the sum of the above probabilities from all donation events.

Mapping geographical distance. Information of the Global Positioning System were available for 521 isolates (Supplementary Data 1). The pair-wise distance between isolates were calculated using R package geosphere46 with Haversine function.

Estimation of phylogenetic signals. Pagel’s λ1,161 was used to assess phylogenetic signal in each monophyletic group, where bootstrap supports ≥70%. This quanti-
tifies intergroup variation and whether methods were more similar than those outside of the group, and whether the search for genetic signals that distinguish the two groups was productive. Origin of isolation (clinical
or environmental) was reconstructed onto the tree using fitDiscrete from the R package Geiger\(^2\). We compared the model fit of the tree using log likelihood of the untransformed maximum likelihood tree against the model where the tree was transformed to a polytomy or partially transformed trees (internal branches were multiplied by \(\lambda = 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9\), and 0.9, Supplementary Fig. 4a). We also reconstructed randomised origin of isolation (clinical or environmental, 100 permutations) onto the tree and compared log-likelihood scores obtained from reconstruction with the actual origin versus randomised origins.

**Detecting kmers associated with disease and the environment.** Two separate GWAS were performed to screen kmers for associations with source of isolation (clinical or environmental) using the 753 Thai genomes. Assemblies were shredded into overlapping kmers of 19–100 bases, resulting in 24,856,071 kmers. All kmers occurring in more than one assembly were filtered using fsm-lite (https://github.com/wmarmak/fsm-lite) as described in ref \(^5\), and filtered to retain kmers that appeared in 5–95% of samples (\(\text{fsm-lite} – v – s 5 95\) – \(\text{fstat} . \text{txt} \) – \(\text{index} > \text{kmer}\)). Kmers were used to log frequency (5% minor allele frequency cut-off) were removed and thus reduced the data to 24,555,746 kmers. Kmers were next filtered using the \(\chi^2\)-test (1 d.f.). Kmer association with a \(p\) value \(< 10^{-5}\) were has been shown previously through simulations to be true positive associations\(^6\), and thus was retained for further investigation. This step reduced the kmers to 300,325 kmer. Seer\(^\ast\) was then used to fit a logistic curve to binary data (clinical or environmental) for each kmer (‘seer-pheno clin.env.pheno.tsv’ \(\ast\) fsm_kmer.[i].gz) and a \(\chi^2\)-test to evaluate \(p\) value \(< 10^{-5}\) (Supplementary Fig. 4b). Disease-associated or environment-associated genes were outlined in Supplementary Data 10.

**Validating genes associated with disease and the environment.** Disease-associated or environment-associated genes were identified by first filtering the data to retain kmers that had a \(p\) value \(< 10^{-5}\) and the \(\chi^2\)-test to investigate any departures in the mean of \(dN/dS\) for genes associated with disease, the environment and core.

As of October 2019, 38.2% genes had GO terms assigned. A given gene could be associated with multiple GO terms (mean = 2.85, min = 1, max = 14), and were corrected for multiple comparisons using the Bonferroni correction. Comparison of GO terms in disease versus environmental isolates, and their enrichment among disease-associated clusters versus expectation based on the reference genome K96243 was performed using one-sided Fisher’s exact test with all GO terms, with a Benjamini–Hochberg adjusted \(p\) value (Supplementary Data 6). Disease-associated clusters were also annotated with Orthologous Groups of Proteins (COG\(^6\)) and pathway maps (KEGG\(^7\) and MetaCyc\(^8\)) to determine putative function. As of October 2019, COG, KEGG and MetaCyc could be assigned to 78,04, 9,79 and 7,72% of disease-associated genes, respectively. Information on protein domains was sourced from the Conserved Domain Database (CDD)\(^9\).

**Measuring gene selection pressure.** The ratio of non-synonymous to synonymous substitutions (\(dN/dS\) or Ka/Ks) was calculated using the KaKs calculator\(^10\). To reduce computational load, we randomly selected accessory genes to represent equal number as core genes (\(n = 4,520\)). Alignments of core, accessory, disease-associated and environment-associated genes were extracted from the pan-genome\(^10\). The test rejected neutrality (\(Hd, \text{dN/dS} = 1\), Fisher’s exact test \(p\) value \(< 0.05\) in 3031 core genes, 3027 accessory genes, 28 disease-associated and 9 environment-associated genes. A non-parametric Mann–Whitney \(U\) test was used to investigate any departures in the mean of \(dN/dS\) for genes associated with disease, the environment and core.

**Statistics and reproducibility.** We employed chi-squared tests or Fisher’s exact tests to compare categorical variables, and parametric ANOVA or non-parametric Mann–Whitney \(U\) tests to evaluate continuous variables, respectively. Unless otherwise stated, two-sided tests were performed in all cases. Where appropriate, we used the Benjamini–Hochberg procedure and Monte Carlo permutation test to correct \(p\) values for multiple comparisons, thereby controlling for multiple hypothesis testing. To ensure reproducibility, we also used two independent approaches to perform GWAS (kmer-based and gene-based methods) on the discovery dataset and validated the enrichment of candidate genes in an independent validation cohort. Source data used to plot Figs. 2a, 3a, b and 5a, b are archived in Supplementary Datas 11–15, respectively.

**Data availability** All supporting data are included in this published article and its supplementary material. Short reads and assemblies for isolates are archived in ENA or NCBI database. Accession number for each individual isolate in discovery and validation dataset are given in Supplementary Datas 1 and 2. Source data for the figures are available in Supplementary Datas 11–15. Pan-genome analysis listing all genes in the dataset is available via Figshare\(^12\) (details in Supplementary Data 7) Sequences of disease- and environment-associated genes are available via Figshare\(^12\) (details in Supplementary Data 10).

**Code availability** All tools and R packages used for the analysis are publicly available and fully described in the method sections and noted in refs. 33,34,46–48,50–56,59–63,70–72.

Received: 23 March 2019; Accepted: 4 November 2019; Published online: 22 November 2019

**References**

1. Limmahurstosakul, D. et al. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat. Microbiol.* 1, 15008 (2016).

2. Wiersinga, W. J. et al. Melioidosis. *Nat. Rev. Dis. Prim.* 4, 17107 (2018).
3. Noinarin, P., Chareonsudjai, P., Wangsomnuk, P., Wongratanacheewin, S. & Chareonsudjai, S. Environmental free-living amoeba isolated from soil in Khlong Klong, Thailand, Antagonize Burkholderia pseudomallei. PLoS ONE 11, e0167355 (2016).

4. Kaestli, M. E. et al. What drives the occurrence of the melioidosis bacterium Burkholderia pseudomallei in domestic gardens? PLoS Negl. Trop. Dis. 9, e0003635 (2015).

5. Limmetharat, A. et al. Activities of daily living associated with acquisition of melioidosis in northeast Thailand: a matched case-control study. PLoS Negl. Trop. Dis. 7, e2072 (2013).

6. Currie, B. J., Ward, L. & Cheng, A. C. The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. PLoS Negl. Trop. Dis. 4, e90 (2010).

7. Holland, D. J. A., Drinkovic, D. & Currie, B. J. Cystic fibrosis and Burkholderia pseudomallei Infection: an emerging problem? Clin. Infect. Dis. 35, e138–e140 (2002).

8. Ralph, A., McBride, J. & Currie, B. J. Transmission of Burkholderia pseudomallei via breast milk in northern Australia. Pediatr. Infect. Dis. J. 23, 1517–1517 (2004).

9. Wuthiekanun, V. et al. Development of antibodies to Burkholderia pseudomallei during childhood in melioidosis-endemic northeast Thailand. Am. J. Trop. Med. Hyg. 74, 1074–1075 (2006).

10. Vasu, C., Vadivelu, J. & Puthucheary, S. D. The humoral immune response in melioidosis patients during therapy. Infection 31, 24–30 (2003).

11. Goodyear, A., Bielefeldt-Ohmann, H., Schweizer, H. & Dow, S. Persistent gastrointestinal colonization with Burkholderia pseudomallei and dissemination from the gastrointestinal tract following mucosal inoculation of mice. PLoS ONE 7, e37324 (2012).

12. Bragonzi, A. et al. Environmental Burkholderia cenocepacia strain enhances fitness by serial passages during long-term chronic airways infection in mice. Int. J. Mol. Sci. 18, https://doi.org/10.3390/ijms18112147 (2017).

13. Massey, S. et al. Comparative Burkholderia pseudomallei natural history virulence studies using an aerosol murine model of infection. Sci. Rep. 4, 4305 (2014).

14. Welkos, S. L. et al. Characterization of Burkholderia pseudomallei strains using a murine intraperitoneal infection model and in vitro macrophage assays. PLoS ONE 10, e0124665 (2015).

15. Lewis, E. R. G., Kilgore, P. B., Mott, T. M., Pradenas, G. A. & Torres, A. G. Comparing in vitro and in vivo virulence phenotypes of Burkholderia pseudomallei type G strains. PLoS ONE 12, e0175983 (2017).

16. Sahl, J. W. et al. The effects of signal erosion and core genome reduction on the identification of diagnostic markers. MBio 7, https://doi.org/10.1128/mBio.00846-16 (2016).

17. Spring-Pearson, S. M. et al. Pangene analysis of Burkholderia pseudomallei: genome evolution preserves gene order despite high recombination rates. PLoS ONE 10, e0140274 (2015).

18. Chewpreecha, C. et al. Global and regional dissemination and evolution of Burkholderia pseudomallei. Nat. Microbiol. 2, 1266237 (2017).

19. Johnson, S. L. et al. Complete genome sequences for 59 Burkholderia isolates, both pathogenic and near neighbor. Genome Announc. 3, https://doi.org/10.1128/genomeA.00159-15 (2015).

20. Dalgaut, H. E. et al. Whole-genome assemblies of 56 Burkholderia species. Genome Announc. 4, https://doi.org/10.1128/genomeA.01106-14 (2014).

21. Viberg, L. T. et al. Whole-genome sequences of five Burkholderia pseudomallei isolates from Australian cystic fibrosis patients. Genome Announc. 3, https://doi.org/10.1128/genomeA.00254-15 (2015).

22. Price, E. P. et al. Unprecedented melioidosis cases in Northern Australia caused by an Asian Burkholderia pseudomallei strain identified by using large-scale comparative genomics. Appl Environ. Microbiol. 82, 954–963 (2016).

23. Merhej, V., Georgiadis, K. & Raoult, D. Postgenomic analysis of bacterial pathogens repertoire reveals genome reduction rather than virulence factors. Brief. Funct. Genomics 12, 291–304 (2013).

24. Weinert, L. A. et al. Genomic signatures of human and animal disease in the zoonotic pathogen Streptococcus suis. Nat. Commun. 6, 6740, (2015).

25. Heacko-Kang, Y. et al. The heritable natural competency trait of Burkholderia pseudomallei in other Burkholderia species through comE and crp. Sci. Rep. 8, 12422 (2018).

26. Nandi, T. et al. Burkholderia pseudomallei sequencing identifies genomic clades with distinct recombination, accessory, and epigenetic profiles. Genome Biol. 25, 608 (2015).

27. Pearson, T. et al. Phylogeographic reconstruction of a bacterial species with high levels of lateral gene transfer. BMC Biol. 7, 78 (2009).

28. Croucher, N. J. et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 43, 1781–1796 (2015).

29. Floch, P. & Molle, F. Water Traps: the Elusive Quest for Water Storage in the Chi-mun River Basin, Thailand: Working Paper (2009).
Acknowledgements
The authors thank the Wellcome Trust Sanger Institute library construction, sequence and core informatics teams, the pathogen informatics team, Elizabeth Blane for their technical support, and Jukka Corander and John Lees for discussion. C.L.C. is supported by Wellcome International Intermediate Fellowship (216457/Z/19/Z), Thailand National Science and Technology Development Agency (FDA-CCO-2562-8764-TH) and Thailand Science Research and Innovation fund (MRG6280226). A.E.M. is a Food Standards Agency Fellow and is supported by the BBRC Institute Strategic Programme Microbes in the Food Chain BBS/E/F/000PR10348 (Theme 1, Epidemiology and Evolution of Pathogens in the Food Chain) and BBS/E/F/ 000PR10351 (Theme 3, Microbial Communities in the Food Chain). D.L. and V.W. are supported by the Wellcome Trust grant 089275/Z/09/Z. This publication presents independent research supported by the Health Innovation Challenge Fund (WT098600, HICF-T5-342), a parallel funding partnership between the Department of Health and Wellcome Trust. The views expressed in this publication are those of the author(s) and not necessarily those of the Department of Health or Wellcome Trust. This project was also funded by grants awarded to the Wellcome Trust Sanger Institute (098051), and to the Wellcome Thailand and African Programme (106698).

Author contributions
S.J.P. conceived the study. D.L. and V.W. collected and provided samples for the study. CL.C designed and performed the analyses. S.R.H., M.H., A.E.M., M.T.G.H., D.L., N.P.J., D., G.D. and J.P. designed and contributed materials and analysis tools. CL.C and CH.C. curated the publication database for previously characterised genetic loci. CL.C, J.P. and S.J.P. wrote the paper with input from all authors. All authors approved the paper prior to submission.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s42003-019-0678-x.

Correspondence and requests for materials should be addressed to C.C. or S.J.P.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019