Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes

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Abstract

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Accession Codes: Consensus sequences for all 113 sequenced isolates are available at the National Center for Biotechnology Information’s Sequence Read Archive. Accession numbers are listed in Supplementary Table S1.

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Bacterial pathogens evolve during the infection of their human hosts, but separating adaptive and neutral mutations remains challenging. Here, we identify bacterial genes under adaptive evolution by tracking recurrent patterns of mutations in the same pathogenic strain during the infection of multiple patients. We conducted a retrospective study of a *Burkholderia dolosa* outbreak among people with cystic fibrosis, sequencing the genomes of 112 isolates collected from 14 individuals over 16 years. We find that 17 bacterial genes acquired non-synonymous mutations in multiple individuals, which indicates parallel adaptive evolution. Mutations in these genes illuminate the genetic basis of important pathogenic phenotypes, including antibiotic resistance and bacterial membrane composition, and implicate oxygen-dependent gene regulation as paramount in lung infections. Several genes have not been previously implicated in pathogenesis, suggesting new therapeutic targets. The identification of parallel molecular evolution suggests key selection forces acting on pathogens within humans and can help predict and prepare for their future evolutionary course.

During acute and chronic infections, bacterial pathogens can accumulate mutations that allow them to better adapt to their human host, evade the immune response, and become more resistant to antibiotic therapy. The spectrum of beneficial mutations that arise during the course of a bacterial infection is likely to reflect genetic pathways critical to bacterial pathogenesis *in vivo*, and therefore may inspire new therapeutic directions. Recent advances in high-throughput sequencing make it possible to follow the genome evolution of bacterial pathogens, but it is still difficult to tease apart the adaptive driver mutations from the neutral passenger mutations that have been fixed by chance. In the laboratory, this is addressed by following several populations grown in parallel cultures under identical conditions; the adaptive role of mutations is indicated by their recurrence in replicate experiments. In natural and clinical environments, such studies are more difficult and have not yet been systematically performed on a genome-wide scale. As a result, global patterns of adaptive evolution that underlie bacterial pathogenesis in humans are not well characterized.

Here, we systematically identify recurrent patterns of evolution implicated in pathogenesis by comparing the genetic adaptation of a single bacterial strain in multiple human subjects during the spread of an epidemic. The airways of people with cystic fibrosis (CF, a lethal genetic disorder) are particularly prone to long-term bacterial infections. Most individuals with CF become colonized by a dominant bacterial strain that persists for many years, allowing significant time for genetic adaption. In the 1990s, a small epidemic of *Burkholderia dolosa* – a rare CF pathogen that can be transmitted from person to person – broke out among individuals with CF in Boston. A total of 39 individuals were infected with *B. dolosa* (Fig. 1a); and all were followed in a Boston hospital, where bacteria isolated during normal care were routinely frozen.

We conducted a retrospective study of 112 *B. dolosa* isolates from 14 individuals with Cystic Fibrosis from this epidemic outbreak – including the first infected subject in the Boston area (patient zero) – over the course of 16 years (Fig. 1b and Supplementary Table 1). During this period, five of these individuals received a lung transplant, and eight died. Most of the 112 isolates were recovered from the subjects’ airways; a few were recovered...
from the blood of subjects with bacteremia. This collection covers the epidemic with high
temporal resolution and enables us to study the parallel evolution of the same strain in
multiple individuals (Supplementary Fig. 1).

We sequenced the whole-genome of these 112 \textit{B. dolosa} isolates on an Illumina GAIIx
sequencer (75bp single-end reads, average read depth 37x; Supplementary Fig. 2) and
aligned the reads onto a \textit{B. dolosa} reference genome\textsuperscript{26}. We focused our analysis on SNPs;
although structural variants and mobile elements may also be important, they are beyond the
scope of this study. Our analysis identified 492 polymorphic loci (Supplementary Table 2).
These mutations accumulated at a steady rate of $\sim$2 SNP/year ($r=0.79$) (Fig. 1c), with no
discernible difference between subjects (Supplementary Fig. 3). This rate of mutation
accumulation in the presence of selection within the human body is consistent with bacterial
mutation fixation rates reported in long-term human infections\textsuperscript{2,27}. The steady accumulation
of mutations generated enough genetic diversity to resolve evolutionary relationships
between isolates, which were investigated through the creation of a maximum-likelihood
phylogenetic tree (Fig. 2a).

At the epidemic level, the phylogeny suggests a network of transmission between subjects.
Isolates from the same subject tend to form genetically related clusters in the phylogeny
(Fig. 2a, Supplementary Fig. 3). These clusters define subject-specific genetic fingerprints,
from which transmission history can be inferred. We constructed the last common ancestor
(LCA) for each subject's set of isolates, which bears the subject-specific fingerprints. The
phylogenetic relationships between these inferred strains indicated the likely network of
transmissions among the 14 subjects (Fig. 2b). Because these data account for only 14 of the
39 subjects in this epidemic, we cannot determine whether transmission occurred directly
from one subject to another, or indirectly through a subject not in our study, via a healthcare
worker, or through a medical device. Nevertheless, this analysis shows that this specific
epidemic was transmitted through several people during its spread and demonstrates the
strength of the approach in identifying the infection network of an epidemic.

At the level of individual subjects, the phylogenetic analysis evidenced the transfer of
multiple \textit{B. dolosa} clones to the subject's bloodstream during bacteremia. We examined
isolates from the three subjects for whom we obtained more than one blood isolate (subjects
H, K, and N). In two of these individuals, we found pairs of blood isolates that evolved from
distinct lung isolates (Fig. 2c), which is inconsistent with the transmission of a single clone
from lungs to blood (Supplementary Fig. 4a). This evidence for multiclonal transmission is
consistent either with a punctuated transmission of multiple clones from the genetically
diverse lung\textsuperscript{28-31} (Supplementary Fig. 4b), or with multiple transmissions occurring over
time. These different possibilities would lead to recommendations for distinct therapeutic
actions: whereas a lung transplant might be effective in preventing the continuous leak of
bacteria through lesions of the lung, it would not block the proliferation of bacteria already
within the bloodstream. This analysis thus brings into focus unresolved questions about the
mechanistic basis of bacteremia.

Finally, we investigated evolution at the gene level. We looked for genetic correlates of
known pathogenic phenotypes. We first assayed resistance to ciprofloxacin, a
fluoroquinolone frequently prescribed to CF subjects (Fig. 3a). Resistance among the 112 isolates varied over two orders of magnitudes (Supplementary Fig. 5a). We scored each gene for correlation between the presence of mutations and drug resistance (Fig. 3a, inset). This genome-wide association study implicated a single gene in the phenotype, BDAG_02180, homolog to *Escherichia coli* gyrA. All the genotypes associated with resistance had nonsynonymous mutations in T83 or D87, known for their role in fluoroquinolone resistance.\(^4,32,33\) Mutations in these residues occurred in six subjects. In each case, phylogenetic analysis indicated that mutations were independently acquired within the subject, after initial infection (Supplementary Fig. 5b). In some cases, we even found in the same subject mutations in both residues, each carried by a different isolate. These findings support the presence of a strong selective pressure from fluoroquinolones but suggest that there are only few genetic paths to resistance in vivo.

We then focused on a second pathogenic phenotype, the presentation of O-antigen repeats in the lipopolysaccharide (LPS) of the bacterium’s outer membrane, known for its importance to virulence in related species.\(^34-36\) We found that some of our isolates present the O-antigen while others do not (Fig. 3b). A single nucleotide in the glycosyltransferase gene BDAG_02317 correlated exactly with the presentation of O-antigen repeats (Fig. 3b, inset). The ancestral genotype at this locus, a stop codon, corresponds to the absence of O-antigen repeats; two different mutations at the same amino acid position – each restoring a full-length protein – are associated with presence of the repeats. We confirmed this association experimentally; we found that complementation with the full-length glycosyltransferase gene could restore O-antigen presentation (Supplementary Fig. 6a, Supplementary Note). Harnessing the phylogenetic information, we determined that the last common ancestors of strains from each subject presented the truncated genotype. Thus, the gain-of-function mutations occurred in nine subjects independently (Supplementary Fig. 6b), highlighting the strength of the selective pressure for O-antigen presentation during the infection. These results identify a previously uncharacterized genetic mechanism for O-antigen switching and hint at a tradeoff during person-to-person transmission.

We recognize that the human body challenges bacteria with many selective pressures beyond those discussed above. We therefore developed a systematic approach for identifying genes under positive selection without prior knowledge of the phenotypes being selected. At the genome level, we found no evidence for selection in coding regions (\(dN/dS \sim 1\)) and no significant intragenic bias (Supplementary Note). However, we reasoned that genes under selection would be mutated independently in different subjects.\(^17-19\) We leveraged the phylogeny to calculate the number of mutations each gene received, distinguishing genes mutated multiple times from those mutated once but appearing in several subjects through the expansion of the lineage that carried them. We counted 561 independent mutational events in 304 genes (Supplementary Table 3). Assuming neutral evolution, we would expect that these mutations would distribute randomly among the 5,014 *B. dolosa* genes, and that genes would rarely acquire more than a single mutation. Instead, we observed that many more genes than expected contained multiple mutations (Fig. 4a, inset). Seventeen genes were found to have three or more different mutations (neutral
expectation: \(\sim 1\), Online Methods), and four genes had over ten different mutations (expected: 0 genes).

To determine whether genes that acquired multiple mutations were under positive selection, or were merely sites of mutational bias, we calculated the canonical measure for selection, dN/dS (Fig. 4a). The large subset of 247 genes which contained only one mutation exhibited a weak but significant signal for purifying (i.e., negative) selection (dN/dS=0.63, \(p<10^{-3}\), Online Methods). The set of genes with two mutations did not show evidence of selection (dN/dS=1.4, CI:0.7-3.1); this set may include a combination of genes that are under some selection and genes that fixed two mutations by chance (22 expected under neutral drift; 28 observed). By contrast, the 17 genes that acquired three or more mutations received 18 times as many non-synonymous mutations as expected by neutral drift, and are under strong positive selection (dN/dS=18, CI:4.9-152.7). This suggests that these seventeen genes are not neutral mutational hotspots; they are undergoing adaptive evolution under the pressure of natural selection.

The 17 genes under positive selection (Fig. 4b, Supplementary Table 4), which are mostly conserved across the *Burkholderia* genus (Supplementary Fig. 7), indicate genetic pathways that may be involved in pathogenesis. The presence of the two genes previously identified in connection with antibiotic resistance and O-antigen presentation (*gyrA*: 11 mutations; *glycosyltransferase wbaD*: 10 mutations) provides a further connection of these genes to pathogenic phenotypes under selection. Eleven of the 17 genes belong to functional categories related to pathogenicity: membrane synthesis (4 genes, including 2 in LPS biosynthesis), secretion (2), and antibiotic resistance (5). The presence of a second glycosyltransferase in the O-antigen cluster (6 mutations) stresses the importance of this pathway to the disease. Notably, the remaining 6 genes had not previously been implicated in pathogenesis of lung infections. Three of these – a glucoamylase, a methyltransferase, and a sigma factor – have no well-annotated close homologs and their roles in pathogenicity are thus unclear. Another gene trio (homologs of *fnr*, *fixL*, and *fixJ*), including the gene most mutated gene (BDAG_01161, a homolog of *fixL*, that had 17 nonsynonymous mutations), can be linked through homology to oxygen-dependent gene regulation. The large number of mutations in this pathway resonates with reports of lowered oxygen tension in CF mucus and of ties between oxygen sensing and virulence modulation in the gastrointestinal tract. Homologs of these three genes have been implicated in diverse regulatory processes, but their function and the genes they regulate in *B. dolosa* are currently unknown. The identification of 17 *B. dolosa* genes that underwent selective pressure during infection in subjects with cystic fibrosis highlights key pathways involved in pathogenesis and may suggest new therapeutic targets for this and other lung infections.

Tracking the genomic evolution of bacterial pathogens during the infection of their human host provides a direct method for observing evolutionary mechanisms *in vivo* and identifying genes central to pathogenesis. This study, which harnesses the combination of high-throughput sequencing and parallel evolution in the clinical settings, is a step towards a comprehensive understanding of genetic adaptation during pathogenesis. Systematically identifying selective pressures acting on pathogens within their hosts may help point to new therapeutic directions.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Whole-genome sequencing of 112 *Burkholderia dolosa* isolates recovered from 14 epidemic patients indicates steady accumulation of mutations over years

a, An epidemic of *B. dolosa* spread to 39 people with cystic fibrosis over decades (circles). Time of first attested infection for each patient is indicated in years since the collection of an isolate from patient zero (labeled ‘A’). We studied retrospectively a cohort of 14 patients from this epidemic (gray circles, and labels). b, The genomes of 112 bacterial isolates were sequenced (diamonds; each horizontal line corresponds to a patient). Isolates were recovered over time from the patients’ airways (blue), bloodstream (red) or other body compartments (for instance tissue obtained during surgery; orange). c, The number of SNPs between each isolate and the outgroup is plotted as a function of time (years since first isolate). Linear fit is plotted (slope = 2.1 mutations fixed per year).
Figure 2. Bacterial phylogeny reveals a likely network of transmission between patients, and between organs

**a**, The maximum-likelihood phylogenetic tree is displayed (SNP scale shown). The 112 isolates are indicated by thin dashed lines colored according to patient, and labeled according to patient and time (e.g., ‘C-14-5’ was recovered from patient C, fourteen years and five months after the first isolate). Blood isolates are indicated by a dollar sign, and isolates with the same patient and date are distinguished by letters. The last common ancestor (LCA) of isolates from the same patient is represented as a circle of the appropriate color and label. Colored backgrounds indicate patient-specific genetic fingerprints. Patients
B, C, E, and F share the same LCA (white background). b, Phylogeny between the inferred LCAs suggests a likely network of infection between patients (arrows). Dashed arrows indicate less certainty (fewer than 3 isolates). c, Phylogeny between blood and lung isolates recovered from the same patient evidences the transmission of multiple clones to the bloodstream during bacteremia (multiple arrows, patients K and N).
Figure 3. Pathogenic phenotypes are associated with point mutations in key genes

a, Minimal inhibitory concentration (MIC) of ciprofloxacin for each isolate (vertical bars) is correlated with genotypic changes in BDAG_02180, a homolog to *E. coli* gyrA, which result in coding changes at residues 83 and 87. Phylogeny is indicated below as a dendrogram and genotypes at BDAG_02180 are shown in the legend. Inset: *P* values for correlation between the presence of mutations in each gene and resistance levels (Kendall's tau). b, Silver-stained gels showing the presence of O-antigen repeats (banded pattern) in the LPS of twenty isolates. Genotypes of BDAG_02317, a hololog of glycosyltransferase *wbaD*, are shown in the legend. The presentation of O-antigen repeats corresponds to a recurrent gain-of-function mutation. Inset: *P* values for correlation between the presence of mutations in each gene and O-antigen presentation (Fischer's exact test).
Figure 4.
Parallel evolution identifies a set of genes under strong selection during pathogenesis. 

Inset, The number of genes that acquired at least $m$ mutations across the epidemic is plotted as a function of $m$ (gray bars). This distribution contrasts sharply with the distribution expected for neutral evolution (black line). Under neutral evolution, the expectation is that only one gene would receive three or more mutations ($m>3$); instead, we observed 17 such genes. Main, The canonical signal for selection ($dN/dS$) is calculated for these 17 genes (109 mutations in these 17 genes), the 28 genes with $m=2$, and the 247 genes with $m=1$ (Supplementary Fig. 3 presents the contribution of these mutations to the molecular clock). Values of $dN/dS$ greater than 1 indicate positive selection (blue), values smaller than 1 indicate purifying selection (red). Error bars indicate 95% CIs. Calculated over all genes without regard to $m$, this analysis would not show a signal for selection. 

b, Each one of the 17 genes (rows) under positive selection contained an acquired mutated in several patients, signified by squares (color intensity indicate the number of mutations observed within this patient). The total number of mutations observed within that gene, $m$, is indicated left. Genes

| Function                      | Patient | Gene                  |
|-------------------------------|---------|-----------------------|
| $O_2$-related gene regulation |         | histidine kinase flxL |
|                               |         | response regulator fyuJ |
|                               |         | transcriptional regulator for |
| Unknown                       |         | methyltransferase      |
|                               |         | sigma factor          |
|                               |         | glucosamylase         |
| Antibiotic resistance         |         | ribosomal protein L4/psfL |
|                               |         | DNA gyrase subunit A/girA |
|                               |         | beta-lactamase blat    |
|                               |         | ABC-type transporter mtrA |
|                               |         | DNA gyrase subunit B gyrB |
| Outer-membrane synthesis      |         | glycosyltransferase wbbD |
|                               |         | glycosyltransferase mlaA |
|                               |         | transporter lpfT       |
|                               |         | permease lpfG          |
| Secretion                     |         | exoprotein             |
|                               |         | type II secretion gspG |
are grouped by biological function, and labeled with the annotations of close homologs, and, when available, close homolog names.