It is well established that antibiotic treatment selects for resistance, but the dynamics of this process during infections are poorly understood. Here we map the responses of *Pseudomonas aeruginosa* to treatment in high definition during a lung infection of a single ICU patient. Host immunity and antibiotic therapy with meropenem suppressed *P. aeruginosa*, but a second wave of infection emerged due to the growth of oprD and wbpM meropenem resistant mutants that evolved in situ. Selection then led to a loss of resistance by decreasing the prevalence of low fitness oprD mutants, increasing the frequency of high fitness mutants lacking the MexAB-OprM efflux pump, and decreasing the copy number of a multidrug resistance plasmid. Ultimately, host immunity suppressed wbpM mutants with high meropenem resistance and fitness. Our study highlights how natural selection and host immunity interact to drive both the rapid rise, and fall, of resistance during infection.
Antibiotic resistance has emerged as a serious threat to public health by increasing the health and economic burden associated with bacterial infections. Treating patients with antibiotics selects for resistant bacteria, and the emergence of resistance during treatment is associated with poorer outcomes in terms of patient health. Following treatment, resistance in patients typically returns to baseline levels, although there is considerable heterogeneity in the rate of decline for different microbe/antibiotic combinations. Although this link between antibiotic treatment and resistance is straightforward and intuitive, the drivers of evolutionary responses to antibiotic treatment during infections remain poorly characterized. One key challenge in this area is to understand how host immunity impacts resistance. Although it is widely acknowledged that immune responses work in conjunction with antibiotics to suppress bacterial infections, the impact of immunity on evolutionary responses to antibiotics is largely unexplored.

Progress in understanding the evolution of resistance during infections has largely come from longitudinal sampling of patients suffering from long-term chronic infections associated with diseases such as cystic fibrosis and tuberculosis. However, the drivers of resistance in short-term acute infections that cause much of the burden of AMR, such as hospital-acquired infections by opportunistic and commensal pathogens, remain poorly understood. Here, we investigate responses to antibiotic therapy through intensive sampling of a single mechanically ventilated patient before, during, and after treatment for a hospital-acquired Pseudomonas aeruginosa pneumonia. P. aeruginosa is an opportunistic pathogen that is a relatively common cause of nosocomial infections, particularly in immunocompromised patients, and pneumonia caused by P. aeruginosa is associated with a high mortality rate. P. aeruginosa infections are difficult to treat with antibiotics due to low outer membrane permeability and the presence of a large repertoire of both intrinsic and acquired resistance mechanisms, including chromosomal mutations and mobile resistance genes.

To understand the responses to antibiotic treatment, we combined clinical data from the patient with extensive sequencing and phenotypic characterization of isolates that were collected from the lung and gut at regular intervals over a period of 3 weeks. Our clinical data included antibiotic use, bacterial titer data, and host immunity biomarker expression. We collected 12 isolates from each patient sample, and we used whole-genome sequencing and phenotype assays (resistance profiling, fitness) on over 100 isolates to understand the population-level responses to antibiotic therapy and host immunity. Combining these approaches allowed us to understand the population biology of antibiotic resistance during short-term infection at an unprecedented level of resolution.

**Results**

**Clinical data.** A 60-year-old patient was admitted to the intensive care unit (ICU) of the Virgen Macarena tertiary care hospital in Seville, Spain with a primary diagnosis of hemorrhagic shock. The patient was intubated and started on mechanical ventilation, and the tracheal aspirate (ETA) was high at 10^6 colony-forming units per mL (CFU/mL) and P. aeruginosa were the only culturable bacteria were detected in ETA samples taken on day 2 (Fig. 1A). A clinical diagnosis of pneumonia was established by the treating physician on day 2 and the patient was treated with piperacillin/tazobactam (4 g/0.5 g IV q8h for 2 days), meropenem (1 g IV q8h for 2 days) and colistin (3 million IU IV q8h for 13 days) (Fig. 1A). Antibiotic treatment coincided with a dramatic decline in the titer of P. aeruginosa, which fell from >10^4 CFU/mL at day 2 to <40 CFU/mL (assay limit of detection) at day 4. The decline in Pseudomonas titer was associated with improved patient health: between day 2 and 7 the sequential organ failure assessment score (SOFA) declined from 14 to 9, and the Clinical Pulmonary Infection Score (CPIS) declined from 8 to 4.

A second wave of P. aeruginosa growth was detected between day 8 and day 12, suggesting that the patient suffered either a secondary lung infection or that the extant populations of P. aeruginosa recovered. The resurgence of P. aeruginosa was accompanied by the establishment of a culturable lung microbiome. This was initially dominated by enteric bacteria (Enterococcus faecium and Klebsiella pneumonia), but bacteria that are associated with the oral cavity (Streptococcus oralis) and skin (S. epidermidis) increased in prevalence, eventually replacing E. faecium (Fig. 1A). The titer of Pseudomonas in this second wave (10^3–10^4 CFU/mL) was 10-fold lower than in the initial infection, and no new episodes of clinical pneumonia were reported. Ventilator support was withdrawn on day 23 and no culturable bacteria were detected in ETA samples taken on day 27. The patient was discharged from ICU on day 31 with a SOFA score of 1 and a CPIS of 0.

Intestinal carriage of P. aeruginosa was detected upon enrollment, as measured by growth from peri-anal swabs (Fig. 1B). Unlike in the lung, antibiotic treatment (i.e., day 2–4) was not associated with effective suppression of the intestinal population of P. aeruginosa. However, the abundance of intestinal P. aeruginosa declined rapidly after day 7, and no growth of P. aeruginosa was detected in peri-anal swabs that were taken from day 16 onwards (Fig. 1B).

Phenotypic responses of pulmonary and gut populations to antibiotic treatment. To gain a better understanding of the role of antibiotics in the dynamics of P. aeruginosa, we measured the resistance of lung (n = 59) and gut (n = 48) isolates to meropenem, piperacillin/tazobactam, and colistin (Fig. 1C–E). Isolates from early time points (day 1–2) had high levels of piperacillin/tazobactam resistance (minimum inhibitory concentration (MIC) > 256 mg/L) that were well above the clinical breakpoint (2 mg/L), suggesting that piperacillin/tazobactam treatment is unlikely to have had any effect on P. aeruginosa (Fig. 1B). In contrast, colistin MICs (mean = 0.5 mg/L; s.d = 0; n = 24) were below the clinical breakpoints (2 mg/L), suggesting that colistin treatment may have contributed to the suppression of the first wave of lung infection. However, the pulmonary titer of P. aeruginosa recovered under continued treatment (days 13–21) without any accompanying increase in colistin resistance (Fig. 1D) or tolerance (Fig. 1E), suggesting that suboptimal pharmacokinetics and/or adaptive changes in gene expression, limited the in vivo efficacy of colistin.

Meropenem resistance increased from baseline levels (mean MIC = 9.6 mg/L; s.e.m = 0.677; n = 24) following antibiotic treatment (day 13: mean MIC = 29.33 mg/L; s.e.m = 1.04; n = 12), suggesting that meropenem treatment suppressed P. aeruginosa. However, at the outset of the infection, meropenem resistance was approximately equal to the EUCAST clinical breakpoint concentration (8 mg/L), questioning the efficacy of meropenem. Previous work has shown that synergy exists between colistin and meropenem, suggesting that colistin treatment may have increased the efficacy of meropenem. The median meropenem MIC of isolates from the initial infection (n = 4 isolates) was reduced by a factor of 4 in the presence of a sub-lethal dose.
There is growing evidence that bacterial metabolism results in meropenem concentrations being low in the gut lumen relative to the renal system as opposed to the biliary system, implying that meropenem diffuses well into lung tissues but is primarily excreted by antibiotic toxicity varies between these anatomical sites. Mesenchymal stem cells (MSCs) are multipotent cells derived from bone marrow that differ from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in that their differentiation is dependent on the microenvironment of the niche where they reside, rather than on the genome. MSCs can differentiate into various cell types, including adipocytes, osteoblasts, and chondrocytes. In the context of lung injury, MSCs have been shown to promote tissue repair and regeneration through paracrine factors, including growth factors and cytokines, that can modulate the immune response and inhibit inflammation. MSCs are also capable of differentiating into pulmonary epithelial cells, which suggests that they may be involved in the repair of the alveolar epithelium. The mechanisms by which MSCs promote tissue regeneration are not fully understood, but they may involve the production of extracellular matrix proteins, the induction of angiogenesis, and the inhibition of apoptosis. Overall, MSCs provide a promising therapeutic option for the treatment of lung injury, particularly in the context of severe acute respiratory distress syndrome (ARDS).
to the closed reference genome (Fig. 2B). Using this approach, we found a small number of chromosomal SNPs (n = 16) and indels (n = 9), most of which occurred as singletons (n = 13). To identify the variable genetic content among our isolates (the part of the genome found only in some isolates), we compared the genetic composition of each isolate against the gene content of all isolates and validated the potential variable genome by mapping sequencing reads to the sequences of genes in these regions. The only evidence of changes in genome composition was the deletion of a plasmid-carried aacA4 aminoglycoside resistance cassette in a single intestinal isolate.

The genetic diversity found within this patient could reflect either (i) in situ evolutionary diversification of an ancestral bacterial clone or (ii) secondary infection by closely related strains from the ST17 outbreak in this hospital. To discriminate between these possibilities, we reconstructed the phylogeny of our isolates using a closely related ST17 genome (P. aeruginosa H26027) as an outgroup (Fig. 2B), and we then used root-to-tip regression to estimate the time to the most recent common ancestor (MRCA) of the isolates we sequenced (Fig. 2C). We reasoned that in situ evolutionary diversification would be associated with an MRCA within the time frame of the infection, whereas recurring infections by ST17 clones from the same outbreak would be associated with an MRCA that predated this infection. The number of SNPs per isolate was well correlated (r² = 0.5) with the day of infection (slope = 0.063 SNPs/day; s.e. = 0.0062, t = 10.12, P < 0.0001), as we would expect if all of the variants detected evolved in situ during the infection by diversification of a clonal “ancestral strain”. Strikingly, we estimated that the MRCA of the isolates occurred at approximately day 0, suggesting that the initial infection was caused by the rapid growth of a single clone after the patient was admitted to ICU and placed on mechanical ventilation. For this analysis we excluded two genetically divergent gut isolates from day 7 that lacked 8 SNPs found in the reference genome. We argue that these isolates reflect a secondary gut colonization by a distinct, closely related clone of ST17.

Mutational adaptation in the lung. The recovery of the pulmonary P. aeruginosa population following antibiotic treatment was driven by the growth of oprD, wbpM, and MexAB-OprM mutants descended from the ancestral strain (Fig. 3A). The small number of isolates sequenced at each time point (n = 11 or 12) makes it difficult to detect subtle changes in the prevalence of different mutations over time, but two broad patterns are clear.

First, the initial recovery of the Pseudomonas population at day 12 was driven by the growth of oprD and wbpM mutants. Given that this diversity evolved in situ, we argue that the gap in time between the population crash of the ancestral strain and the appearance of these mutants reflects the time taken for the populations of mutants to expand from a single cell to a detectable sub-lineage of cells (minimal observed density approximately 10^2 CFU/mL). The loss of the OprD outer membrane porin is a key mutational mechanism for meropenem resistance in P. aeruginosa and oprD mutations (W277*,
physical barriers, such as bio
mutations are likely to have arisen in sub-populations of cells of
of MexAB-OprM mutants is intriguing, as it suggests that these
= mg/L; s.e.m
resistance than the ancestral strain (Fig. 3B; mean MIC
wbpM
mutation had twofold higher levels of meropenem
wbpM
transposon mutant.
Second, the frequency of oprD mutants rapidly declined, and the
of oprD mutants was accompanied by the rise of mutations in MexAB-OprM, a broad-spectrum antibiotic efflux pump that was constitutively expressed in the ancestral strain.\(^{23,34}\). Strikingly, we observed three independent losses of MexAB-OprM mutants, and the results of these assays tend to correlate well with in vivo measures of competitive ability from animal model systems.\(^{42}\). oprD mutants did not have reduced growth relative to ancestral strain, suggesting that mutations in this gene were not associated with any fitness costs per se (see also\(^ {43}\)). However, oprD mutants had low growth compared to both wbpM (Dunnett’s test \(P = 0.0259\)) and MexAB-OprM (Dunnett’s test \(P = 0.0013\)) mutants, which is consistent with the idea that the prevalence of oprD mutants declined due to low fitness in vivo.

To test this hypothesis, we measured the growth rate of all the lung isolates in nutrient-rich culture medium lacking antibiotics (Fig. 3B). Although lab culture medium lacks many of the stressors encountered by pathogens during infections, this is a standardized approach for measuring the fitness of resistant mutants, and the results of these assays tend to correlate well with in vivo measures of competitive ability from animal model systems.\(^ {42}\). oprD mutants did not have reduced growth relative to ancestral strain, suggesting that mutations in this gene were not associated with any fitness costs per se (see also\(^ {43}\)). However, oprD mutants had low growth compared to both wbpM (Dunnett’s test \(P = 0.0259\)) and MexAB-OprM (Dunnett’s test \(P = 0.0013\)) mutants, which is consistent with the idea that the prevalence of oprD mutants declined due to low fitness in vivo.

The high fitness of MexAB-OprM mutants suggests that the biosynthetic burden and/or activity of this pump was costly. Bacteria often adapt to the cost of resistance through compensatory mutations that recover fitness without compromising resistance,\(^ {41}\), and the loss of MexAB-OprM provides a clear counter-example of selection for the loss of a costly resistance determinant. Intriguingly, MexAB-OprM mutations are detected in \(P.\ aeruginosa\) from patients with cystic fibrosis,\(^ {44}\), suggesting that selection for efflux pump inactivation is a common feature of \(P.\ aeruginosa\) infections. Given the fitness advantage enjoyed by MexAB-OprM mutants in the absence of antibiotics, it is challenging to understand why these mutants were only detected at day 16. Notably, MexAB-OprM mutants only reached detectable frequency after the end of colistin treatment, suggesting that colistin may have played a role in selection for MexAB-OprM mutations. In support of this idea, MexAB-OprM mutants
had increased susceptibility to colistin relative to the ancestral strain (Fig. 3C; Dunnett’s test \( P < 0.0001 \)).

**Plasmid copy number evolves in response to antibiotic pressure.** All of the isolates carried a plasmid (p110820) that included an OXA-10 β-lactamase, suggesting this plasmid may have played an important role in responding to treatment with β-lactam antibiotics. To better understand the impact of this plasmid on antibiotic resistance, we transformed p110820 into the PA01 reference strain and measured antibiotic susceptibility (Fig. 4A). Although OXA-10 is generally considered to be a narrow-spectrum β-lactamase\(^4\,5\), plasmid carriage increased resistance to both piperacillin-tazobactam and meropenem (see also refs. \(46,47\)), suggesting that this plasmid played a key role in mediating the high levels of resistance to these antibiotics in the ancestral strain.

No mutations or structural variation occurred in p110820, apart from the loss of an aacA4 aminoglycoside resistance cassette in a single intestinal isolate (Fig. 2B). However, we found subtle, but pervasive, variation in the copy number of p110820 (Fig. 4B). Copy number in intestinal isolates was \( \approx 3 \) per cell (mean = 2.89; s.e. = 0.054; \( n = 46 \)), with the exception of the fact that isolates from the secondary colonization event had an elevated copy number (mean = 3.67; s.e. = 0.037; \( n = 2 \)). The initial copy number of p110820 in pulmonary isolates was \( \approx 20\% \) higher than in intestinal isolates (mean = 3.55; s.e. = 0.11; \( n = 24 \); \( t_{68} = 7.15, P < 0.0001 \)) and this was associated with a \( \approx 20\% \) increase in meropenem resistance in the lung isolates (mean MIC = 9.6 mg/L; s.e. = 0.67; \( n = 24 \)) compared to the intestinal isolates (mean MIC = 8.17 mg/L; s.e. = 0.17; \( n = 46 \); \( t_{68} = 2.75, P = 0.0076 \)), highlighting the link between variation in plasmid copy number and antibiotic resistance.

Antibiotic resistance plasmids are often associated with fitness costs\(^42,48\), suggesting that selection should favor reduced plasmid copy number following antibiotic treatment. Consistent with this argument, plasmid carriage reduced the growth rate of the PA01 model stain (Fig. 4C; \( t_{57} = 2.48, P = 0.0215 \)), and the recovery of the lung population was associated with a \( \approx 30\% \) reduction in plasmid copy number (Fig. 4B; mean = 2.44, s.e.m. = 0.08, \( n = 35 \); \( t_{57} = 8.15, P < 0.0001 \)). Reduced copy number was not associated with any plasmid or chromosomal mutations, suggesting that copy number declined due to selection on heterogeneity in plasmid copy generated by variation in plasmid replication and partitioning. Although low plasmid copy number is likely to have influenced the fitness and antibiotic resistance of the evolved mutants, it is difficult to quantitatively estimate this impact given that epistatic interactions between resistance plasmids and mutations are common\(^49\). However, it is interesting to note that plasmid copy number was high in MexAB-OprM mutants compared to oprD and wbpM mutants (Fig. 4D), suggesting that chromosomal mutations were more important determinants of fitness than plasmids copy number. In the context of antibiotic resistance, the low plasmid copy number of the evolved mutants suggests that we may have underestimated the increase in meropenem resistance provided by wbpM and oprD mutations and overestimated the loss of meropenem resistance associated with the loss of MexAB-OprM.

Although trade-offs between resistance and fitness can help to explain dynamic changes in plasmid copy number during infection, they cannot explain why plasmid copy number was
high in the lung at the outset of the infection, prior to antibiotic treatment (i.e., on day 1). One possible explanation for this result is that a population bottleneck occurred during the initiation of the lung infection, resulting in increased copy number driven by a founder effect.

**Immunity.** Although antibiotic treatment clearly had important effects on the population dynamics and evolution of *P. aeruginosa* during lung infection, there are several features of the clinical data that antibiotic treatment alone cannot explain. First, the titer of *Pseudomonas* in the lung decreased rapidly (by >1 log) before the onset of antibiotic treatment. Second, the eventual elimination of the entire lung microbiome, including *P. aeruginosa*, was not driven by antibiotic treatment. To investigate the role of host immunity in shaping the dynamics of infection, we measured the abundance of cytokines in ETA samples taken from day 1 and 2 (initial infection), day 8 and day 23 (second wave), as shown in Fig. 5(A–E). The great advantage of this approach is that it allows us to measure the immune response at the site of infection, instead of using a proxy measure of immunity, such as serum levels of antibodies. Importantly, the cytokines that we assayed have been shown to provide protection against *P. aeruginosa* lung infection. Crucially, the decline of *Pseudomonas* titer during both the first wave (day 1 and 2) and second wave (day 23) coincided with high levels of expression of protective inflammatory cytokines relative to the day 8 time point, when the lung of this patient did not contain any culturable bacteria.

Neutrophils are known to play a key role in providing protection against acute *P. aeruginosa* infection in the lung. To assay the functional consequences of elevated IL-8 (neutrophil chemoattractant) expression, we measure the resistance of bacterial isolates to LL37 and HBD-3, antimicrobial peptides that are produced by neutrophils and lung epithelial cells. All of the isolates were highly resistant to HBD-3 (350 μg/mL), but were rapidly killed by a physiologically relevant concentration of LL37 (50 μg/mL), suggesting that this host antimicrobial peptide may have played an important role in eliminating pulmonary bacteria. However, the magnitude of this difference was very small; for example, the time taken for LL37 killing rate = −1.10 log_{10} CFU/h; s.e. = 0.018; n = 12). Surprisingly, the oprD, wbpM and mexA mutants that appeared during the second wave of infection all showed reduced susceptibility to LL37 compared to the ancestral strain (Fig. 5F; ANOVA F_{3,11} = 8.02, P = 0.0085; all Dunnett’s test P < 0.05). However, the magnitude of this difference was very small; for example, the time taken for LL37 to cause a 10-fold reduction in viable cell density was 54.6 min in the ancestral strain (s.e. = 1.5 min; n = 3) and 61 min in the evolved mutants (s.e. = 0.71 min; n = 9). Given the uncertainties over the effective concentrations of LL37 encountered by bacteria in the lung during this infection, it is unclear if these subtle differences in LL37 sensitivity between mutants had any biological significance during this infection.

**Discussion**

Although it has long been known that antibiotic treatment can drive the rise of resistance during infections, the underlying dynamics of this process remain poorly characterized, especially during acute infections. Combining clinical data, resistance phenotyping, genomics, fitness assays and immune response profiling enabled us to produce a very high-resolution understanding of the evolutionary trajectory and drivers of antibiotic resistance during a hospital-acquired *P. aeruginosa* infection, as summarized in Fig. 6.

It is widely acknowledged that antibiotics and host immunity work in conjunction to suppress bacterial infections, but the
is likely to have (i) increased the lag time between antibiotic biotic resistance, there are good reasons for thinking that host these constraints were not able to prevent the evolution of anti-

is that the population following recovery.

case, relaxed antibiotic pressure drove the decline of high resis-

inactivated a costly ef

good evidence of selection on non-mutational variation in increasing the effective exposure of resistant cells to antibiotics58, suggesting that the gut is likely to be a hot-spot for the evolution of

Fig. 6 Summary of bacterial dynamics during infection. This Muller plot summarizes changes in the density and composition of the lung population of P. aeruginosa during infection and it highlights the key phenotypic effects of observed mutations. This plot does not represent the reduction in the copy number of the p110820 plasmid during infection (Fig. 4C).

dynamic interplay between antibiotics, immunity and pathogens remains poorly understood7–9. In this case, host immunity was able to reduce pathogen density by at least 1 log prior to the onset of antibiotic treatment. The toxicity of antibiotics towards bacteria is greatest at low bacterial cell density53, suggesting that early (i.e., day 1–2) immunity-mediated suppression of bacterial population density may have increased the efficacy of meropenem and colistin treatment (day 3–4). Combination therapy with meropenem and colistin contributed to the successful suppression of initial infection, but the Pseudomonas population recovered due to the successful outgrowth of meropenem resistant mutants, highlighting the incredible ability of P. aeruginosa to evolve mutational resistance to clinically important carbapenem antibiotics54–56.

Theoretical considerations suggest that the immune-mediated suppression of bacterial population density is likely to have constrained the evolutionary response to antibiotic treatment57. First, reducing population density must have decreased the absolute number of antibiotic resistant mutants that were present at the time of meropenem-colistin treatment. Reducing pathogen density prior to antibiotic treatment may have also decreased the likelihood of successful outgrowth of resistant mutants by increasing the effective exposure of resistant cells to antibiotics58, an effect that is likely to be particularly important for mutations such as wbpM that lead to small increases in resistance. While these constraints were not able to prevent the evolution of antibiotic resistance, there are good reasons for thinking that host immunity reduced the number of resistant mutants that were able to successfully grow following antibiotic treatment. This, in turn, is likely to have (i) increased the lag time between antibiotic treatment and the detectable recovery of the Pseudomonas population and (ii) decreased the diversity resistant mutants in the population following recovery.

One of the key principles of evolutionary models of resistance is that fitness costs generate selection against resistance following antibiotic treatment, leading to the loss of resistance41,59. In this case, relaxed antibiotic pressure drove the decline of high resistance/low fitness oprD mutants and the spread of mutations that inactivated a costly efflux pump. The copy number of the costly p110820 plasmid also declined following treatment, providing good evidence of selection on non-mutational variation in plasmid copy number. Variation in plasmid copy number arises due to inherent variability in plasmid replication and partitioning, suggesting that altered plasmid copy number may be a very general, and underappreciated, evolutionary response to antibiotic treatment during infections (see also refs. 60–62). While these examples highlight the ability of selection to drive the loss of resistance, it is important to emphasize that meropenem resistance was maintained during the second wave of infection due to the stability of the high fitness wbpM mutant. Our results suggest that the host immune response ultimately suppressed the second wave of infection, thereby limiting the potential for onwards transmission of wbpM. Crucially, these results show that selection and host immunity interact to drive the loss of resistance following treatment.

One important challenge for future work will be to investigate interactions between immunity and resistance in greater depth. In the first place, chemical interactions between antibiotics and host immunity effectors (i.e., synergy or antagonism) may modulate the efficacy of antibiotic treatment and its associated selective pressures. In this case, colistin has been shown to suppress the inflammatory response63, suggesting that continued colistin treatment may have delayed the suppression of the second wave of infection. Antibiotic resistance mutations also alter resistance to host antimicrobial peptides (AMPs)64, suggesting immunity may play an important role in fitness of resistant mutants. For instance, increased resistance to host AMPs may have contributed to the success of the mutants that we observed. An associated challenge will be to understand the role of the microbial community in the dynamics of resistance. Competition between bacterial species is common65, suggesting that lung microbiome may have limited the success of meropenem resistant P. aerugi-

It is common for pathogenic bacteria to inhabit multiple anatomical sites in the body, and this has the potential to generate within-host variation in antibiotic exposure and the selective pressures that this generates67. Although P. aeruginosa is primarily considered to be an opportunistic respiratory pathogen, this bacterium is also capable of colonizing the gut. Antibiotic treatment has profound impacts on the gut microbiome, suggesting that the gut is likely to be a “hot-spot” for the evolution of resistance68,69. However, we found no evidence of clinical or evolutionary responses to meropenem treatment in gut, and this can be explained by the poor penetrance of meropenem into the gut lumen70 and the low toxicity of meropenem under anaerobic conditions. Given this, our data suggests that the gut provided P. aeruginosa with an effective refuge against antibiotic treatment. However, the importance of gut colonization in the infection biology of P. aeruginosa remains unclear. On the one hand, gut colonization may simply be a dead-end, as appears to have been the case in this patient. Alternatively, gut infections that are protected from antibiotic treatment may act as a reservoir that can establish infections in new anatomical locations (i.e., lung, blood stream) or in new hosts. Hopefully, future studies will resolve this issue by estimating the importance of gut populations to the transmission of P. aeruginosa.

Our study was able to capture the evolutionary responses of a pathogen population to antibiotic treatment by characterizing the genetic and phenotypic diversity present in longitudinal samples taken from a single patient. The key insight is that natural selection and host immunity interact to drive the incredibly rapid rise, and fall, of resistance during short-term infections. Previous work that has characterized the evolutionary dynamics in patients has relied largely on long-term sampling of chronic
infections or taking multiple samples from a single time point. Although our study focused on a single patient, our findings highlight that infrequent sampling of pathogen populations may underestimate the rate of evolution of resistance because of the fast turnover of resistant lineages following treatment. Furthermore, capturing host immune responses allowed us to better understand the drivers of resistance, and there is a clear need to better understand both direct and indirect interactions between immunity and resistance. Hopefully, future studies using high-resolution sampling across multiple patients will help to resolve this.

**Methods**

**Clinical data.** The patient was recruited as part of an observational, prospective, multicentre European epidemiological cohort study, ASPIRE-ICU (The Advanced Understanding of Staphylococcus aureus and Pseudomonas aeruginosa Infections in Europe–Intensive Care Units, NCT02413242 ClinicalTrials.gov)27. The intervention was standard of care, and the research protocol was approved by the Andalusian Biomedical Research Ethics Coordinating Committee (CCEIBA). An agreed legal representative of the participant gave written informed consent, according to CARE guidelines and in compliance with the Declaration of Helsinki principles. ASPIRE-ICU enrolled subjects who were mechanically ventilated at ICU admission and with an expected length of hospital stay ≥ 48 h. An assessment of four clinical criteria to establish a clinical diagnosis of ICU pneumonia (e.g., new blood cultures, respiratory use, new radiologic evidence, reason to suspect pneumonia) was performed daily; in case of at least one positive parameter, a combination of objective major and minor criteria was assessed to categorize subjects as having protocol pneumonia or not27. Data on antibiotic use in the two weeks preceding ICU admission and during the ICU stay were reported. During ICU stay, respiratory samples were collected by using all possible combinations of a log2 dilution series of meropenem (0–64 mg/L) and colistin (0–2 mg/L). This assay was carried out using a randomized block experimental design, and we analyzed five replicates of 11 randomly selected isolates and five replicates of a PA01 control in each block. The order and position of each isolate on the experimental plates was selected through randomization, using “sample” command without replacement in R76. We used linear regression of mean log viable cell titer against time to calculate a death rate for each isolate (typically this involved data from 0 to 2 h of incubation). In no case did we observe bi-phasic killing kinetics. We used a one-way ANOVA that included main effects of experimental block and genotype (ancestral, oprD, wbpM or MexAB-OprM) to test for variation in colistin tolerance scores between mutants. We then used a Dunnett’s test to compare the evolved mutants against the ancestral strain. Statistical analysis was performed using JMP v.12.

**Anaerobic meropenem resistance assay.** Six isolates of the ancestral strain were grown from glycerol stocks on LB-Luria-Bertani (LB) Milleragar plates overnight at 37°C. Single colonies were then inoculated into LB Miller broth supplemented with meropenem at increasing twofold concentrations (1–64 mg/mL) and grown anaerobically (Oxoid AnaeroSel® with anaerobic gas generating sachet) for 72 h at 37°C. MIC was calculated as MIC<sub>50</sub> (a 50% reduction) in OD<sub>595</sub> and compared to the same calculation under standard aerobic conditions. We calculated an MIC<sub>50</sub> rather than a conventional MIC, due to the low growth of the ancestral strain under anaerobic conditions.

**Characterization of the wbpM mutant.** In order to determine the effect of the wbpM in resistance, meropenem MICs were determined by EUCAST broth microdilution in triplicate experiments for wild-type reference strain PA14 and its wbpM isogenic knock out derivative obtained from an available transposon mutant library77.

**Gene expression.** The levels of expression of ampC, mexB and rpsL were determined by real-time reverse transcription (RT)-PCR78,79. Briefly, isolates were grown in 10 mL of LB broth at 37°C and 180 rpm to the late log-phase (optical density at 600 nm [OD600] of 1) and collected by centrifugation. Total RNA was isolated by using the RNeasy mini kit (Qiagen), dissolved in water, and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C to remove residual contaminating DNA. A 50 ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the QuantiTect SYBR green RT-PCR kit (Qiagen) with a Bio-Rad instrument (CFX Connect Real-Time System). Primers (Supplementary Table 1) were used for the amplification of ampC, mexB, and rpsL (used as a reference to normalize the relative amount of messenger RNA (mRNA)). To calculate ampC and mexB mRNA expression levels compared to PA01 the following formula was applied:

\[
\frac{2^{\Delta Ct}}{\Delta Ct_{PA01}} = \frac{\Delta Ct_{iso} + \Delta Ct_{iso}}{(\Delta Ct_{PA01} + \Delta Ct_{iso})}
\]

Isolates were considered positive for ampC overexpression when the corresponding mRNA level was at least 10-fold higher than that of PA0180. Likewise, isolates were considered positive for mexB overexpression when the corresponding mRNA level was at least threefold higher than that of PA0180. Moreover, levels of mRNA levels obtained in independent duplicate experiments were considered for each isolate (Source Data file).

**Long-read sequence analysis.** Four isolates were sequenced with the Pacific Biosciences platform using single molecule chemistry on a SMRT DNA sequencing system. Coverage ranged from 122X to 171X. Resulting sequencing reads were assembled using canu v.1.8 and a genome scaffold was generated using error rate of 0.300, corrected error rate of 0.045, minimum read length of 1000 bases, and minimum overlap length of 500 bp. Canu assemblies were circularized using circulator v.1.5.3 using kmer sizes 77, 87, 97, 107, 117, and 127, minimum merge length of 4000, minimum merge identity of 0.95, and minimum contig length of 2000.

**Illimina sequence analysis.** All isolates were sequenced in the MiSeq or NextSeq illumina platforms yielding a sequencing coverage of 69X–134X. Raw reads were quality controlled with the ILLUMINACLIP (2:3:10) and SLIDINGWINDOW
and InDels located in a set of genes known to be involved in microbial population size upon exposure to a lethal concentration of LL-37. Bacterial population size upon exposure to a lethal concentration of LL-37. Tolerance was measured by determining the change in log10 viable cell titer against time. We did not observe any bi-phasic killing curves. We used a one-way ANOVA that included a main effect of genotype (ancestral, oprD, WbpM or MexAB-OprM) to test for variance in LL37 resistance. We then used a Dunnett’s test to compare the evolved mutants against the ancestral strain. Statistical analysis was carried out in JMP v.12.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data is available from figshare [https://doi.org/10.6084/m9.figshare.14219129.v1]. All clinical data analyzed for this patient as part of the study are included in this article. Isolates were obtained from the corresponding author for research use via an MTA subject to permission from the ASPRE research committee. All sequencing data has been deposited on the NCBI short-read archive (PRJNA667268) and all data on isolates can be found at SRR12776324, SRR12776265, SRR12776267, SRR12776268, SRR12776269, SRR12776326, SRR12776327, SRR12776333, SRR12776334, SRR12776363, SRR12776364, SRR12776365, SRR12776375, SRR12776388, SRR12776398, SRR12776400, SRR12776401, SRR12776422, SRR12776434, SRR12776445, SRR12776464, SRR12776465, SRR12776473, SRR12776478, SRR12776489, SRR12776500, SRR12776513, SRR12776523, SRR12776525, SRR12776526, SRR12776528, SRR12776539, SRR12776560, SRR12776611, SRR12776620, SRR12776635, SRR12776636, SRR12776637, SRR12776638, SRR12776648, SRR12776649, SRR12776650, SRR12776651, SRR12776652, SRR12776653, SRR12776654, SRR12776655, SRR12776656, SRR12776657, SRR12776658, SRR12776659, SRR12776660, SRR12776661, SRR12776662, SRR12776663, SRR12776664, SRR12776665, SRR12776666, SRR12776667, SRR12776668, SRR12776669, SRR12776670, SRR12776671, SRR12776672, SRR12776673, SRR12776674, SRR12776675, SRR12776676, SRR12776677, SRR12776678, SRR12776679, SRR12776680, SRR12776681, SRR12776682, SRR12776683, SRR12776684, SRR12776685, SRR12776686, SRR12776687, SRR12776688, SRR12776689, SRR12776690, SRR12776691, SRR12776692, SRR12776693, SRR12776694, SRR12776695, SRR12776696, SRR12776697, SRR12776698, SRR12776699, SRR12777000, SRR12777001, SRR12777002, SRR12777003, SRR12777004, SRR12777005, SRR12777006, SRR12777007, SRR12777008, SRR12777009, SRR12777010, SRR12777011, SRR12777012, SRR12777013, SRR12777014, SRR12777015, SRR12777016, SRR12777017, SRR12777018, SRR12777019, SRR12777020, SRR12777021, SRR12777022, SRR12777023, SRR12777024, SRR12777025, SRR12777026, SRR12777027, SRR12777028, SRR12777029, SRR12777030. Source data are provided with this paper.

Received: 22 September 2020; Accepted: 31 March 2021; Published online: 28 April 2021

References

1. Friedman, N. D., Temkin, E. & Carmeli, Y. The negative impact of antibiotic resistance. Clin. Microbiol. Infect. 22, 416–422 (2016).
2. Bell, B. G., Schellevis, F., Stobberingh, E., Goossens, H. & Pringle, M. A. Antibiotic use in the Netherlands: a systematic review and meta-analysis. BMC Infect. Dis. 14, 13 (2014).
3. Hug, D., N. Piscitelli, S. C. & Daminger, I. H. Development of resistance during antimicrobial therapy: a review of antibiotic classes and patient characteristics in 173 studies. Pharmacotherapy. J. Hum. Pharmacol. Drug Ther. 15, 279–291 (1995).
4. Shorr, A. F., Combes, A., Kollef, M. H. & Chastre, J. Methicillin-resistant Staphylococcus aureus prolongs intensive care unit stay in ventilator-cultivated necrotizing pneumonia patients. Crit. Care Med. 34, 700–706 (2006).
5. Costelloe, C., Maftei, C., Lovering, A., Mant, D. & Hay, A. D. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. BMJ 340, c2096 (2010).
6. Malikzada-Kumar, S., Lammens, C., Coenen, S., Van Herck, K. & Goossens, H. Effect of anthraxcin and clarithromycin therapy on pharyngeal carriage of
macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. Lancet Infect Dis 19, 482–490 (2017).

7. Wang, C.-J., Webster, J. P., Dominguez, C. & Linn, B. R. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577 (2002).

8. Levin, B. R., Perrot, V. & Walker, N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics 154, 985–997 (2000).

9. Ankomah, P. & Levin, B. R. Exploring the collaboration between antibiotics and the immune response in the treatment of acute, self-limiting infections. Proc. Natl Acad. Sci. USA 111, 8331–8338 (2014).

10. Diaz Caballero, J. et al. Selective sweeps and parallel pathoadaptation drive Pseudomonas aeruginosa evolution in the cystic fibrosis lung. Mbio 6, e01981–e01981 (2015).

11. Diaz Caballero, J. et al. A genome-wide association analysis reveals a potential role for recombination in the evolution of antimicrobial resistance in Burkholderia multivorans. PLoS Pathog. 14, e1007433–e1007433 (2018).

12. Marvig, R. L., Sommer, L. M., Molin, S. & Johansen, H. K. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat. Genet. 47, 57 (2015).

13. Smith, E. E. et al. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc. Natl Acad. Sci. USA 103, 8487–8492 (2006).

14. Lieberman, T. D. et al. Genetic variation of a bacterial pathogen within patients with cystic fibrosis. Nat. Genet. 46, 82 (2014).

15. Xu, Y. et al. In vivo evolution of drug-resistant Mycobacterium tuberculosis in patients during long-term treatment. BMC Genomics 19, 640 (2018).

16. Liu, Q. et al. Within patient microevolution of Mycobacterium tuberculosis correlates with heterogeneous responses to treatment. Sci. Rep. 5, 17507 (2015).

17. Haque, M., Sartelli, M., McKimm, J. & Bakar, M. A. Health care-associated infections—an overview. Infect. Drug Resist. 11, 2321 (2018).

18. Pena, C. et al. Carbapenem-resistant Pseudomonas aeruginosa: factors influencing multidrug-resistant acquisition in non-critically ill patients. Eur. J. Clin. Microbiol. Infect. Dis. 38, 519–522 (2019).

19. Jus, C., Peña, C. & Oliver, A. Host and pathogen biomarkers for severe Pseudomonas aeruginosa infections. J. Infect. Dis. 215, S44–S51 (2017).

20. Kang, C.-I. et al. Pseudomonas aeruginosa bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clin. Infect. Dis. 37, 745–751 (2003).

21. Aloush, V., Navon-Venezia, S., Seigman-Igra, Y., Cabili, S. & Carmeli, Y. Multidrug-resistant Pseudomonas aeruginosa: risk factors and clinical impact. Antimicrob Agents Chemother. 50, 43–48 (2006).

22. Botelho, J., Grosso, F. & Peixe, L. Antibiotic resistance in Pseudomonas aeruginosa—Mechanisms, epidemiology and evolution. Drug Resist. Updates 44, 100640 (2019).

23. Goëlein, E., de la Fuente-Núñez, C. & Hancock, R. E. P. N. aeruginosa: all roads lead to resistance. Trends Microbiol. 19, 419–426 (2011).

24. Gellatly, S. L. & Hancock, R. E. P. aeruginosa: new insights into pathogenesis and host defenses. Pathog. Dis. 67, 157–173 (2013).

25. Morita, Y., Tomida, J. & Kawamura, Y. Responses of Pseudomonas aeruginosa to antimicrobials. Front. Microbiol. 4, 422 (2014).

26. Gad, G. F., El-Domany, R. A. & Asfour, H. M. Antimicrobial susceptibility profile of Pseudomonas aeruginosa isolates in Egypt. J. Urol. 180, 176–181 (2008).

27. Paling, F. P. et al. Rationale and design of ASPIRE-ICU: a prospective cohort study on the incidence and predictors of Staphylococcus aureus and Pseudomonas aeruginosa pneumonia in the ICU. BMC Infect. Dis. 17, 643 (2017).

28. Michalopoulos, A. S. & Falagas, M. E. Colistin: recent data on pharmacodynamics properties and clinical efficacy in critically ill patients. Ann. Intensive Care 1, 1–6 (2011).

29. McPhee, J. B., Lewenza, S. & Hancock, R. E. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. Mol. Microbiol. 50, 205–217 (2003).

30. Montero, M. M. et al. Colistin plus meropenem combination is synergistic in vitro against extensively drug-resistant Pseudomonas aeruginosa, including high-risk clones. J. Glob. Antimicrob. Resist. 18, 37–44 (2019).

31. Sabuda, D. et al. Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) position statements on polymyxin B and colistin clinical breakpoints. Clin. Infect. Dis. 71, e52–e59 (2020).
ARTICLE

San Millan, A., Escudero, J. A., Gifford, D. R., Mazel, D. & MacLean, R. C. Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria. Nat. Ecol. Evolution 1, 1–8 (2016).

Matzneller, P. et al. Colistin reduces LPS-triggered inflammation in a human sepsis model in vivo: a randomized controlled trial. Clin. Pharmacol. Therapeutics 104, 773–781 (2017).

Lázár, V. et al. Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. Nat. Microbiol. 3, 718–731 (2018).

Foster, K. R. & Bell, T. Competition, not cooperation, dominates interactions among culturable microbial species. Curr. Biol. 22, 1845–1850 (2012).

Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H. & Björk, L. Proteins of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Mol. Microbiol. 46, 157–168 (2002).

Didelot, X., Walker, A. S., Peto, T. E., Crook, D. W. & Wilson, D. J. Within-host evolution of bacterial pathogens. Nat. Rev. Microbiol. 14, 150 (2016).

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. & Relman, D. A. The application of ecological theory toward an understanding of the human microbiome. Science 336, 1255–1262 (2012).

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology 156, 3216–3223 (2010).

Raza, A. et al. Oral meropenem for superbugs: challenges and opportunities. Drug Discov.Today 26, 551–560 (2020).

Jorth, P. et al. Regional isolation drives bacterial diversification within cystic fibrosis bacteria. Cell Host Microbe 18, 307–319 (2015).

Chung, H. et al. Global and local selection acting on the pathogen Stenotrophomonas maltophilia in the human lung. Nat. Commun. 8, 1–7 (2017).

The European Committee on Antimicrobial Susceptibility Testing. EUCAST Reading Guide for Broth Microdilution. http://www.eucast.org (2019).

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for interpretation of MICs and Zone Diameters, Version 9.0 http://www.eucast.org (2019).

Stover, C. K. et al. Complete genome sequence of Pseudomonas aeruginosa PA01, an opportunistic pathogen. Nature 406, 959 (2000).

Team, R. C. R. A Language and Environment for Statistical Computing. (Team, R. C. R., 2013).

Liberati, N. T. et al. An ordered, nonredundant library of Illumina sequence data. Bioinformatics 30, 551–559, 1501 (2014).

Lee, D. G. et al. Genomic analysis reveals that an international cystic fibrosis clone. Sci. Rep. 7, 1–15 (2017).

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. & Relman, D. A. An ordered, nonredundant library of Illumina sequence data. Bioinformatics 30, 551–559, 1501 (2014).

Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequencing data. Bioinformatics 30, 2114–2120 (2014).

Bankiewicz, A. et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Computational Biol. 19, 455–477 (2012).

Arnold, M. F. et al. Genome-wide sensitivity analysis of the microsymbiont Sinorhizobium meliloti to symbiotically important, defense-like host peptides. MBio 8, e01060–01017 (2017).

Lee, D. G. et al. Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. Genome Biol. 7, R90 (2006).

Soemann, T. Prekopa: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069 (2014).

Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357 (2012).

Li, H. et al. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).

Broad Institute. Picard Toolkit (Broad Institute, GitHub repository, 2019).

DePristo, M. A. et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet. 43, 491 (2011).

© The Author(s) 2021

The authors declare no competing interests.

Author contributions A.R., H.G., J.K., S.K.-S., A.O., S.M.-K., and C.M. contributed to project conception and coordination. H.G. designed and performed experiments. J.K. and S.K.-S. conducted data analysis. A.O. and S.M.-K. conducted literature searches. C.M. guided the project. All authors discussed the results and commented on the manuscript.

Acknowledgements This research was supported by Wellcome Trust Grant (106918/Z/15/Z) and the Innovative Medicines Initiative Joint Undertaking under COMPACT-MAGNET (Combatting Bacterial Resistance in Europe) and COMPACT-NET (Combatting Bacterial Resistance in Europe–Networks, grant agreement no. 115523), resources of which are composed of financial contribution from the European Union’s Seventh Framework Program (FP7/2007–2013) and EFRA companies’ kind contribution. We thank the Oxford Genomics Center (funded by Wellcome Trust Grant 201314/Z/16/Z) for the generation and initial processing of Illumina sequence data.

Author contributions R.W., J.D.C., N.K., F.H.R.D.W., P.J., A.Q., G.T., T.Y.d.S., F.F.-C., A.A., J.H., E.D.B.-T., C.L.-C., B.B.X., C.R., L.T., C.L., contributed to data acquisition and analysis. F.S., O.A., A.R., H.G., J.K., S.K.-S., A.O., S.M.-K., and C.M. contributed to project conception and study design. R.W., J.D.C., A.O., S.M.-K., and C.M. wrote and revised the manuscript.

Competing interests The authors declare no competing interests.

Additional information Supplementary information Supplementary material available at https://doi.org/10.1038/s41467-021-22814-9.

Correspondence and requests for materials should be addressed to C.M.

Peer review communications Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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) 2021