Diversification and evolution of vancomycin-resistant Enterococcus faecium during intestinal domination.

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Abstract

Vancomycin-resistant *Enterococcus faecium* (VRE) is a leading cause of hospital-acquired infections. This is particularly true in immunocompromised patients where the damage to the microbiota caused by antibiotics can lead to VRE domination of the intestine, increasing a patient’s risk for bloodstream infection. In previous studies we observed that the intestinal domination by VRE of patients hospitalized to receive allogeneic bone marrow transplantation can persist for weeks, but little is known about subspecies diversification and evolution during prolonged domination. Here we combined longitudinal analysis of patient data and *in vivo* experiments to reveal previously unappreciated sub-species dynamics during VRE domination that appeared stable from 16S rRNA microbiota analyses. Whole genome sequencing of isolates obtained from sequential stool samples provided by VRE-dominated patients revealed an unanticipated level of VRE population complexity that evolved over time. In experiments with ampicillin-treated mice colonized with single colony forming units (CFUs) VRE rapidly diversified and expanded into distinct lineages that competed for dominance. Mathematical modeling shows that *in vivo* evolution follows mostly a parabolic fitness landscape, where each new mutation provides diminishing returns and, in the setting of continuous ampicillin treatment, reveals a fitness advantage for mutations in the penicillin binding protein 5 (*pbp5*) that increase resistance to ampicillin. Our results reveal rapid diversification of host-colonizing VRE populations, with implications for epidemiologic tracking of in-hospital VRE transmission and susceptibility to antibiotic treatment.
**Introduction**

A healthy intestinal tract contains a multitude of bacteria from diverse species collectively referred to as the intestinal microbiota (1-3). Immunocompromised patients, such as those undergoing allogeneic hematopoietic cell transplantation (allo-HCT), receive broad-spectrum antibiotics to prevent and treat infections, in the process losing large portions of their intestinal commensal bacteria (4-6). In the setting of antibiotic-mediated microbiota destruction, vancomycin-resistant Enterococci (VRE) can rapidly expand to high densities in the gastrointestinal tract, leading to intestinal domination that persists for days after the cessation of antibiotic administration. In animal models of microbiota injury, mice remain susceptible to VRE domination for over 4 weeks after ampicillin treatment; once VRE dominates the intestine it can persist at high density for months. In allo-HCT patients, VRE domination of the gastrointestinal tract raises the risk of bacteremia (7) and the risk of graft-versus-host disease (8-10).

Intestinal bacteria can evolve rapidly in the intestinal tract of mammalian hosts; in some settings strains with higher antibiotic resistances can emerge (11-13). While the majority of VRE that cause bacteremia are classified as *Enterococcus faecium* (77%), this species consists of many strains that differ in antimicrobial resistance (14). Intestinal colonization by *E. faecium* and associated excretion of large numbers of viable bacteria in the healthcare setting are major contributors to patient-to-patient transmission (15). A patient may be colonized by multiple *E. faecium* strains, and each strain could accumulate new mutations, forming a dynamic multi-strain population, which complicates antibiotic treatment (16). Patients already colonized with a VRE strain can even acquire another strain from the hospital, which adds another source of sub-species diversity and can lead to recurrent bacteremia (17). The *E. faecium* dominations we have described (7), which appear stable over time when characterized by 16S amplicon sequencing, may hide underappreciated sub-species diversity and *in vivo* dynamics.
Such dynamics of sub-species diversity could confound epidemiological studies attempting to track antibiotic-resistant infections and contribute to increasing levels of antibiotic resistance.

Here, we demonstrate that VRE populations dominating the gastrointestinal tract of allo-HCT patients are indeed dynamic and undergo day-to-day diversification. Intestinal colonization of mice by a single bacterium revealed the rapid evolution of VRE and the generation of multiple parallel lineages that competed in the gut lumen. Using a longitudinal approach, we show that fitness differences between VRE clonal subpopulations create competition dynamics that guide evolution of the overall population, and mathematical models of in vivo evolution provide insights into the shape of the evolutionary fitness landscape. Our results have implications for the epidemiology of VRE infections in healthcare settings and suggest that tracking pathogen evolution within patients by deeper and broader sequencing of clinical samples and isolates can provide clinically useful information.

Results

Genomic diversity of VRE in the gut of an individual patient. We first characterized the cross-sectional diversity of vancomycin-resistant E. faecium (VRE) in an allo-HCT patient, herein identified as pt110 (Fig. 1a). Microbiota analysis by 16S amplicon sequencing showed that the patient was initially colonized with Enterococcus at <10% relative abundance six days prior to allo-HCT (day -6), after which Enterococcus expanded and remained at high density in fecal samples. To determine the complexity of the patient’s Enterococcus domination, we initially cultured and whole-genome sequenced 3 VRE isolates from fecal samples, one each from days -6, 1 and 8. We selected the genome of an isolate from day -6, classified as E. faecium, as the reference genome and we aligned sequence reads from the day 1 and 8 VRE isolates using breseq to identify genetic differences, generally called variants, which included single-nucleotide variants (SNVs) as well as small insertions-deletions (indels).
Isolates from day 1 and 8 were highly similar to the genome of the day -6 isolate, but they contained unique variants (Fig. 1b) suggesting that these VRE variants might have arisen independently from an ancestral strain prior to day -6.

We analyzed an additional 22 VRE isolates from this patient from days -6, 1, and 8. Using the short reads we assembled draft genomes for each of the 25 isolates and we analyzed the assembled contigs. The analysis revealed that pt110 harbored two distinct VRE strains: 19 isolates belonged to the multilocus sequence typing (MLST) 412 strain and 6 isolates belonged to a less frequent MLST 736 strain (Fig. 2e). To gain more insight into the sub-strain diversification of VRE, we first focused on the predominant MLST 412. We compared the genomes of the 19 isolates, where 7 isolates came from day -6, 2 came from day 1 and 10 came from day 8. As the Enterococcus population increased in abundance between days -6 and 8, reaching over 90% of the microbiota composition determined by 16S sequencing, the numbers of genetic variants detected increased over time (Fig. 1c). A dendrogram of the 19 isolates from MLST 412 based on their core genome indicated that the genetic differences did not accumulate in a constant proportion across the passage of time (Fig. 1d).

Additional allo-HCT patients with Enterococcus dominations reveal hidden dynamics of sub-species diversity. To broaden our analyses of sub-species diversification of VRE within patients, we selected 4 additional patients in whom intestinal domination by Enterococcus was observed, as determined by 16S amplicon sequencing (Fig. 2a-d). We isolated individual VRE colonies from banked fecal samples and genome sequenced a total of 101 isolates, which confirmed that the colonizing species were E. faecium in all patients (Fig. 2e). Similar to pt110, pt1044 was co-colonized with two distinct VRE strains, with MLST 80 predominating initially followed by MLST 789 at later time points. For pt1044, one strain replaced the other and went on to dominate the patient’s microbiota. Interestingly, each VRE strain in the patients demonstrated sub-strain diversity and dynamics (Fig. 2a-e).
To better characterize the pattern of intra-patient VRE diversity, we applied a similar longitudinal analysis to the five patients, where we identified novel variants (SNVs and indels) from multiple isolates from each patient by comparing to a reference genome generated from one of the earliest isolates obtained from each patient (Supplementary Fig. 1). As with pt110, domination by VRE was associated with a diversity of variants that changed in abundance over time and, as in patient pt110, the pattern of new variants that emerged did not reflect the passage of time. To quantify VRE diversification, we determined the number of new variants among isolates obtained from the same patient and belonging to the same MLST and plotted the percent of unique genotypes (determined as the number of genotypes not previously seen per 100 total genotypes) for each patient. The sub-species population dynamics varied between patients, indicating that the path of VRE evolution in patients is likely not deterministic (Fig. 2f). The isolates of patient pt843 and the MLST 412 isolates of patient pt110 showed an increase in proportion of new genotypes detected post-expansion. The MLST 80 isolates of patient pt1142 had a collapse in their population diversity, reverting to the reference strain, as no isolates from this MLST harbored variants on the final timepoint (day 5). We investigated recurrently mutated genes for signatures of selection based on the ratio of non-synonymous substitutions to synonymous substitutions (dN/dS) (Supplementary Table 1). However, we found no significant signatures, possibly due to high variability and few recurrent mutations in patient isolates. We also analyzed the number of unique clones per day using generalized linear regression models to test if there are common patterns across patients (not shown). The high variability even within a patient day-to-day prevents us from making any conclusions from this analysis. Taken together, these analyses suggest complex dynamics of VRE diversification in the gut similar to those observed with other gut bacteria (11), prompting further investigation.

**In vivo experimental evolution in a mouse model reveals rapid diversification.** The deep analysis of VRE dominations in allo-HCT patients, which had previously appeared stable from 16S rRNA microbiota analysis, revealed unexpected dynamics of sub-species diversity. From these data, however,
we could not determine precisely how long each patient had harbored VRE in the gut and without this knowledge, we could not determine whether diversity developed after initial colonization, whether patients were initially infected with a complex VRE population or whether patients kept acquiring new diverse clones during hospitalization. Therefore, we turned to a mouse model where we could monitor the expansion and diversification of VRE more precisely. We used a limiting-dilution experiment to colonize a mouse with ~1 bacterial cell to study the dynamics of intestinal colonization starting from a monoclonal inoculum. Naive C57BL/6J mice, 8-10 weeks old, were all treated with ampicillin in their drinking water for three days, after which they were orally inoculated with a range of VRE concentrations (Fig. 3a). Among a group of 10 individually-housed mice inoculated with 0.2 CFU of VRE per mouse, only one became colonized (Fig. 3b), strongly suggesting that this mouse was colonized by a single bacterium (from a simple calculation based on the Poisson distribution, the probability that the mouse received more than one bacterium is P=0.0011). This mouse was continuously treated with ampicillin and fecal samples were collected for 133 days to investigate the evolution of the colonizing VRE population (Fig. 3a).

The density of VRE detected in fecal samples stabilized 5 days post inoculation (Fig. 3c). We obtained 4 VRE isolates at each of 11 time-points between 1 and 133 days of intestinal colonization (Fig. 3c) and performed whole-genome sequencing to identify new genetic variants. The first mutation was detected on an isolate obtained at day 15 of colonization (Fig. 3d) and was followed by a rapid increase. By 49 days of colonization, we detected VRE diversity within a mouse colonized with a single bacterium that approximated levels of VRE diversity detected in the patients (Fig. 3d, Supplementary Table 2).
To more completely analyze diversity in the intestinal VRE population, we broadened our investigation by shotgun metagenomics sequencing of DNA extracted from fecal samples. We observed expansion of many genetic variants, only a subset of which coincided with those sequenced from isolates obtained from the same mouse (Fig. 3c). The total number of genetic variants in the population stabilized after about 49 days, at a value of ~80 variants, a surprisingly low number given the sheer number of possible genetic variants that could theoretically occur in the *E. faecium* genome (Fig. 3f). As observed with pt110, some mutations stabilized at high or low frequencies, while others disappeared from the population over time (Fig. 3e). Noticeably, a nonsynonymous mutation in penicillin-binding protein 5 (*pbp5*) became fixed in the population by day 49. This A→C *pbp5* mutation occurred in codon 434, changing the negatively-charged aspartate to a nonpolar alanine within the transpeptidase domain of PBP5. The PBP5 protein has been shown to confer ampicillin resistance, and mutations in its gene have been documented in clinical isolates (18-22).

Individual mutations detected by shotgun sequencing displayed varying temporal patterns: while the frequency of some increased steadily and became fixed in the population, others stabilized at a lower frequency or even rose and fell over time (Fig. 3d). This prompted us to characterize this dynamic pattern of VRE diversification quantitatively using a mathematical model.

Mathematical modeling recaptures evolutionary dynamics. While metagenomic shotgun sequencing provided greater sampling depth, isolate sequencing from our *in vivo* model enabled us to link genetic variants within the same genome. Using this information, we classified “genotypes” as isolates that shared identical genetic variants, yielding a total of 22 VRE genotypes observed over the 133-day experiment. These genotypes were then used to assemble a lineage graph with maximum parsimony.
depicting the relationships between the ancestral clone (sc1) and its evolved lineages (Fig. 4a). The grouping of genetic variants into genotypes also allowed us to depict the clonal evolution of VRE using isolate data alone (Fig. 4b,c) or combined isolate and shotgun data (Supplementary Fig. 2a,b).

To understand the population dynamics governing VRE diversification, we generated an in silico simulation of our in vivo experiment. Our simulations began with a population made up of only the ancestral VRE clone, and each iteration of the simulation allowed random mutation at any of the 25 loci observed experimentally. With even just 25 loci, there are $2^{25}$, or 33554432, different possible clones. Yet, experimentally we only observed 22 clones. The purpose of the model was to test various fitness landscapes that guide this very large number of possible clones to the ones that were experimentally observed. We found that under neutral evolution the pbp5 mutation did not fix in the population, unlike our experimental results (Fig. 4d). This clearly demonstrates that in addition to random mutation, competition among clones due to variations in fitness likely shapes VRE diversification.

We therefore tested three possible fitness landscapes that may explain the data from our experiment: 1) linear, in which each additional mutation observed in our panel of sequenced isolates conferred additional fitness; 2) logarithmic, in which additional mutations conferred increased fitness but with diminishing returns; and 3) parabolic, in which few mutations conferred increased fitness but too many mutations resulted in a fitness cost (Supplementary Fig. 2c). The linear and log fitness landscapes implicitly constrain genotypes observed later to be fitter than earlier genotypes. Additionally, we tested the possibility that the pbp5 mutation that may enhance fitness further still, by adding the condition that clones harboring this mutation have higher fitness in linear, logarithmic and parabolic fitness landscapes, for a total of 6 conditions. These clones would follow the same fitness landscape pattern, but
at a higher level (Supplementary Fig. 2c). Importantly, our simulations operated under the assumption that experimentally observed clones fall on the fitness landscapes described above, but that any of the other possible genotypes that were not isolated have a relative fitness that is much lower. Therefore, while they may arise in the course of random mutation, they are unlikely to persist in the population. Hence, our simulations were largely constrained to the clones depicted in the lineage graph (Fig. 4a).

Testing the above six conditions in our in silico experiments revealed that a parabolic fitness landscape where *pbp5* mutation-containing clones have higher fitness most accurately regenerated the population dynamics observed in vivo. This conclusion was based both on the rate of ancestral clone decline and the rate of expansion of *pbp5* mutant sub-lineages (hereby referred to as branch 2), as well as the time to fixation of this mutation in the population (Fig. 4c,d). A simulated linear fitness landscape with no additional fitness given to *pbp5* mutant clones also closely resembled experimental data in terms of expansion of branch 2 lineages, but did not accurately reflect the experimentally determined time to fixation of the *pbp5* mutation in the population and consequent outcompeting of other clones (Supplementary Fig. 2d and Fig. 4d). Our simulations suggest that relative fitness differences between clones play an important role in shaping the evolution of VRE in the gut and posit that a parabolic fitness landscape — in which additional mutations initially confer increased fitness, but beyond a certain point lead to a fitness cost — may govern the accumulation of genetic variants from the ancestral clone.

Since there is biological plausibility that the *pbp5* mutation would be advantageous, it is possible that the positive selection of clones with this mutation is the major force governing VRE evolution in our in vivo model. We therefore expanded our model to allow any clone with the *pbp5* mutation to have high fitness, even if such a clone was not experimentally observed among our isolates. To our surprise, we
found that under a linear fitness landscape the results of the simulation almost never reflect the clones experimentally observed, due to accumulation of a far greater number of genetic variants than contained in our isolates (Supplementary Fig. 2e). Furthermore, a parabolic fitness landscape only partially recaptured the clonal dynamics of the in vivo model (Supplementary Fig. 2f). We hence surmise that if indeed any clone with the \( pbp5 \) mutation has equal fitness, the result of our experiment was just one of many rare events — and it is unlikely we would have ever seen this rare event if the fitness landscape governing VRE evolution was linear, and much more likely if parabolic. If constrained to the lineage graph, the parabolic fitness landscape consistently recaptures the population dynamics observed experimentally as concluded above.

Replicate in vivo experiments demonstrate the stochastic nature of within-host VRE evolution. The stochasticity of evolutionary processes means that replicate experiments will often follow completely distinct paths(23). In order to ascertain how deterministic the pattern of VRE diversification was, we repeated the above in vivo experiments four additional times. Using the same experimental setup of three-day ampicillin pre-treatment and low-dose inoculum, three mice received the VRE strain derived from pt110 (Fig. 5a-c) and one mouse was colonized with the ATCC-700221 VRE strain (Fig. 5d). In order to more closely approximate the conditions encountered by VRE strains colonizing allo-HCT patients, we sub-lethally irradiated two mice and transplanted MHC-matched (minor-antigen disparate) allogeneic donor bone marrow prior to VRE inoculation (Fig. 5b,c).

Our results confirmed the stochastic nature of within-host VRE evolution because each mouse exhibited a unique pattern of VRE diversification (Fig. 5a-d, first column and Supplementary Fig. 3). The total number of genetic variants and the rate of their accumulation varied among the mice. Surprisingly,
despite the differences in population dynamics, the population diversity reached a maximum for all mice, which agrees with our parabolic fitness model (Fig. 5a-d, second column, Supplementary Fig. 4, columns 2-3, and Fig. 5e).

Moderate to high frequency mutations in the *pbp5* gene were detected in two mice (mouse 3 and mouse 5), highlighting the positive selection of various penicillin-binding protein mutants in the setting of continuous ampicillin treatment (Fig. 5b,d). Importantly, similar to our finding with mouse 1, the evolved strains of these two mice outcompeted the ancestral strain (Fig. 5b,d and Fig. 4c). Interestingly, mouse 2 acquired *pbp5* mutations late in the course of the experiment, at day 62, and only achieved a maximum frequency of 10.5%, possibly because these variants may not have had sufficient time to exploit their fitness advantage.

Expansion of *pbp* mutations, however, did not occur in every host. The *pbp5* mutations did not become fixed in the VRE population in mice 3 and 5 (Supplementary Fig. 4c,e). Interestingly, for both of these mice, we detected 3 unique *pbp5* mutation per mouse in the isolates; clonal interference among *pbp5*-evolved sublineages may have prevented fixation of any one mutation.

The genetic variants that accumulated in our replicate *in vivo* experiments differed between mice, leaving open the possibility that minor differences in host environments cause divergent adaptations. A number of mutations in addition to *pbp5* reached high levels, suggesting alternative paths to improved fitness (Supplementary Fig. 4a-e, first column and Fig. 3e).
To determine which genes might harbor a signature of positive selection, we identified 13 genes that were mutated in 3 or more of the 4 mice infected with the pt110-derived VRE strains. We then analyzed the subset of genes, 4 in total, that had also been mutated in the VRE ATCC-700221 strain inoculated mouse. Here, we applied a lower frequency detection threshold of 1%, as we were also interested in rarer mutations. The *pbp5* gene acquired 19 mutations across the 5 mice, and it was mutated over three times more frequently than the other 3 genes (Fig. 5f). As previously discussed, while mouse 2 acquired 11 unique *pbp5* mutations, none of them reached a high frequency (maximum <11%). Mutations in the ABC transporter, SA14-24 serine kinase and gluconate-specific PTS system component genes suggest that changes in environmental information sensing/processing pathways may contribute to VRE adaptation in the gastrointestinal tract. Notably, the isolate data only captured the *pbp5* mutation across the mice in our cohort. As previously mentioned, 3 mice had *pbp5* mutations that were detected in the isolate data, with an average of 2.3 SNVs per gene (Supplementary Table 3). Mutations in the ABC transporter detected in the shotgun data were only present in the isolates from the ATCC-700221-inoculated mouse (mouse 5). Conversely, the SA14-24 serine kinase, gluconate PTS system component and lactose PTS system repressor mutations were found in pt110-derived isolates. This may underscore that the medium used to isolate single clones from intestinal VRE populations may introduce biases (Supplementary Fig. 4a-e, fourth column).

To explore this further, we examined genetic variants in our shotgun data that occurred in 2 or more of the 4 mice inoculated with the pt110-derived VRE strain, out of the total 2672 variants detected within this dataset that achieved ≥1% frequency. We hypothesized that mutations conferring a fitness advantage could be identified by satisfying two conditions: 1) that they arise independently in *in vivo* experiments, and 2) that they achieve a substantial frequency (>20%) in at least one mouse at one or
more time-points. The frequency threshold of 20% was selected after visualizing the variant frequency data obtained from shotgun sequencing. Furthermore, we focused on mutations that could confer a functional change, either by nonsynonymous amino acid change or, for intergenic mutations, by occurring within 300bp of a translation start site (n=781 functional SNVs in our dataset). Out of 38 mutations shared by multiple mice, we found 4 that satisfied both conditions: the nonsynonymous *php5* SNV (locus 1736237), an intergenic SNV upstream of a lactose phosphotransferase (PTS) repressor (locus 390751, -181bp upstream), and two mutations in an ABC transporter (maltose/g3p/polyamine/iron) (loci 1618548 and 1618571) (Fig. 5g). We sought to confirm if any of these mutations reached moderate frequencies (>20%) in the ATCC-700221-inoculated mouse. We detected one in the *php5* gene (locus 1393420) and two in the ABC transporter gene (loci 1500644, 1393420) (Fig. 5h). Taken together, these data highlight the importance of the *php5* gene for intestinal VRE fitness in the presence of ampicillin and suggest other targets of positive selection.

**Parabolic fitness landscape detected in patient shotgun data.** To validate our findings in a patient we shotgun sequenced the samples with >90% VRE domination banked from a sixth patient, pt1252, to better determine the frequency dynamics underlying the sub-species evolution (Fig. 6a). For pt1252, one VRE isolate was cultured from the first day of known VRE domination (day 19, belonging to MLST 412) and used as a reference. Metagenomic binning of the reads obtained from shotgun sequencing revealed that one VRE strain (from MLST 412) dominated the gastrointestinal tract. Individual chromosomal variants displayed diverse dynamics over the course of VRE domination (Fig. 6b), which looked similar to our *in vivo* experimental data (Fig. 3e and Supplementary Fig. 4a-e). Some variants stabilized at high or low frequencies, while others disappeared from the population over time. The accumulation of unique genetic variants in the VRE metagenome of this patient plateaued, a shape expected under a parabolic fitness landscape (Fig. 6c, black line). However, population diversity
appeared to rise and fall when calculating the total number of variants per day (Fig. 6c, red line). These data suggest a rapid diversification that resulted in 2 competing sub-clades, with the more distant of the two from the reference rising in proportion on day 24 and 38. However, an alternative hypothesis that these two sub-clades were acquired together cannot be excluded from this data. Together, this pattern of VRE diversification fits with our in vivo experimental data and highlights the selective pressures of a complex antibiotic treatment regimen.

Discussion

Our survey of intestinal VRE in patients revealed not only previously underappreciated diversity within single strains, but also that such diversity rapidly changes over time. Using a combination of clinical, experimental and mathematical approaches, we characterized population dynamics and revealed evidence of a general fitness landscape that could shape VRE evolution in vivo. Furthermore, sustained antibiotic pressure likely led to positive selection of pbp5 mutations, indicated by its presence in four out of five mice following low-inoculum colonization with VRE.

Our finding that the Enterococcus dominations apparently stable at the level of 16S-based classification can obscure rich sub-species dynamics raises interesting questions. First, from our initial mouse experiment, it is curious that even after gaining the pbp5 mutation, clones continued to evolve and obtained additional mutations that outcompeted clones with the pbp5 mutation alone. This suggests that the pbp5 mutation was not the sole force guiding VRE evolution. It is possible that the mutations compiled on top of pbp5 have a biological mechanism that increased fitness in the presence of an antibiotic, or it is possible that diversity in and of itself was advantageous. For example, one of the mice that presented pbp5 mutations also had a mutation in a gluconate-specific IIC component, responsible
for carbohydrate transport in bacteria, which persisted at a higher frequency than *pbp5* and fixed in the population (mouse 3). VRE with this mutation could have higher sugar uptake and therefore a metabolic fitness advantage; it will be interesting to study how other modes of fitness gains interact with antibiotic resistance. Second, it is interesting that the total number of genetic variants observed at any given time seemed to stabilize rather than continue to increase linearly. We speculate that deleterious mutations and negative epistasis might keep sub-species diversity in check despite the large population sizes and relatively high mutation rates of these bacterial populations. Third, our results showed many more mutations in this species compared with what has previously been observed in other species evolving in intestine of mice (24, 25). The reason for this discrepancy remains unclear, but as more data becomes available on the evolution of gut bacteria *in vivo* it will be interesting to compare how the rates of adaptation differ across taxa — a finding that is both crucial to our basic understanding of gut microbiome biology and to our understanding of the evolution of antimicrobial resistance in enteric pathogens.

Our findings recall the diminishing returns epistasis or negative epistasis in *E. coli* studies, which contribute to decreasing rates of fitness gains during adaptation (26, 27). An important distinction is that these studies only considered mutations shown to have beneficial effects, while our model does not have this constraint. Therefore, we model not only fitness landscapes that have diminishing but positive gains in fitness (log landscape), but also decreased fitness at high mutational load (parabolic landscape).

We chose simple approaches whenever possible, because the simplest approaches sufficed to make our main point that the VRE evolution *in vivo* is underappreciated. First, we chose a method for calling mutations from metagenomic sequencing that relied on simplifying assumptions. Distinguishing error from true mutations is a notoriously difficult task and detection of low frequency variants such as 1% is
especially prone to false positives. Improved methods could use a two-step mutation-calling protocol that would first identify polymorphisms, then look for support in each sample from patient or mouse. This alternative can potentially improve the calculation of genotypes observed at any given timepoint and the comparison to evolutionary simulations. Still, this is not a key issue for the overall result that there is substantial change that would be inaccessible by typical 16S amplicon analysis. To test for this, we reran our analysis of the mutations detected in the metagenomic sequencing of patient 1252 (Fig. 6c). Lowering the threshold for mutation calling from 10% to 1% increased the total number of mutations called—possibly by increasing the number of false positives—but does not alter our overall result consistent with the parabolic fitness landscape (Supplementary Fig. 5).

Second, our mouse experiments rest on the assumption that a single CFU colonized the mouse. Although this cannot be proved, given the inoculum density the likelihood of colonization by 2 or more CFUs is 0.11%, giving us confidence in single CFU colonization. We also note that at higher inoculum density of 27 CFU/mouse, 9 out of 10 mice were colonized, indicating that even at high concentrations where it is very likely that every mouse received at least 1 CFU (probability ~100%), colonization was not guaranteed in every mouse.

Third, our simulation results assumed specific values for population size and mutation rates. Importantly, however, when we varied those values we found that the main conclusion is robust: the penicillin binding protein (pbp) mutation fixes in the population and does so around the same point in time despite changes in population size and changes in mutation rate (Supplementary Fig. 6a). The rate of diversification within “branch 2” (clones containing the pbp mutation) is sensitive to changes in population size or mutation rate, as demonstrated by the varying number of unique clones at the end of a
simulation (Supplementary Fig. 6b). Therefore, for a given population size there is an optimal mutation rate to yield results that recapitulate experimental results, and for a different population size a different mutation rate will yield the same results. Population size and mutation rate are related to each other but fluid; fixing one will then fix the other to optimize results. Based on these results, if we increase population size from 1000 to 5000, the mutation rate should be slightly less than doubled to obtain the same variation at the end of the simulation (Supplementary Fig. 6b). We tested this by running 50 simulations with population size 5000 and mutation rate 0.007, and got nearly identical results to our choice of population size 1000 and mutation rate 0.004 (Supplementary Fig. 6c). We chose the lower population size (1000) to minimize computational time, but find that larger populations give the same result when mutation rate is appropriately adjusted.

Due to the risk of bloodstream infections from the gut carrying high resistance to antibiotics (7, 28), it is critical to understand the in vivo evolution of VRE. Moreover, intestinal microbiome composition can affect tumor responses to anti-cancer therapy (29-31). Overall, we believe our results should caution that whole-genome sequencing from single isolates, taken at a single point of patient colonization, could lead to incorrect interpretations. The rapid evolution of sub-species diversity in VRE may underlie heterogeneous responses to drug treatment across time and should be taken into account in the rational design of antibiotic therapies.

Methods

VRE isolate collection and culturing. Stool samples were collected from allo-HCT patients in an ongoing IRB-approved study and stored in 2ml freezer vials at -80 °C. Stored content was thawed and streaked with an inoculating loop directly onto selective Difco Enterococcusel agar supplemented with 10ug/ml vancomycin (EAPv). Fecal pellets collected from mice were immediately kept on ice after
harvesting and plated the same day on EAPv. Plates were incubated aerobically for 48hrs at 37°C and 4-
10 colonies were selected and re-streaked. Stocks were made by growing a re-streaked colony in 5ml
Brain Heart Infusion liquid media (BHI) overnight and growing in 1:20ml dilution in 20ml BHI for 6hrs
at 225pm at 37 °C. 800ml of the culture was resuspended in 200ml of 75% glycerol to obtain 4 15%
glycerol stocks per isolate, stored at -80 °C. Bloodstream isolates were stored by the MSKCC
microbiology laboratory and re-streaked on EAPv as described for fecal isolates.

Mouse husbandry and in vivo VRE colonization. in vivo experiments were performed in compliance
with Memorial Sloan Kettering’s institutional guidelines and were approved by its Institutional Animal
Care and Use Committee. 8-10-week-old wild-type female C57BL/6 mice were purchased from the
Jackson Laboratories. Mice were housed in sterile cages with irradiated feed and acidified water. 5ml
BHI was inoculated with frozen stock overnight and the next day a 1:20ml dilution was incubated for
2.5hrs at 225pm at 37 °C to obtain a midlog culture of ~6x10^8 CFU/ml. Serial dilutions were performed
in PBS. Inocula were plated to determine the CFU input per mouse. Mice were pretreated with
ampicillin at 0.5g/L for 3 days in the drinking water to abrogate colonization resistance, and orally
gavaged in 100-200ul per mouse. Mice were individually housed at the time of inoculation. The number
of mice per group that were colonized (out of 5 or 10 mice total) was assessed on day 1 and day 5 post-
challenge by serially diluting fecal pellets in PBS and plating on EAPv. Mice in the group that received
the lowest inocula dose were kept to monitor VRE and maintained on ampicillin-treated water (0.5g/L,
changed every 5-7 days). VRE density was measured as described every other week henceforth.

In our first in vivo experiment, mice that were gavaged with an inoculum of ~2 CFU/10 mice
(200ul/mouse) resulted in 1 out of 10 mice infected. For our second experiment, mice were inoculated
with 2 CFU/ms (100ul/mouse), resulting in 3 out of 10 mice infected (mouse 2, 3 and 4). Here, we used
an allogeneic, minor-MHC-antigen disparate BMT model, transplanting 129S1/SvImJ donor cells into female C57BL/6 recipients (Staffas CHM 2018). Briefly, mice were given a split 1100cGy radiation dose and administered 5x10^6 bone marrow cells via tail vein injection. Mice were then infected with VRE by oral gavage on the same day. A similar final experiment was performed with a low-dose inoculum from a laboratory strain of E. faecium, ATCC (700221). Mice were single-housed once inoculated with VRE. When we inoculated 10 mice with pt110 inoculum at a concentration of 0.2CFU/mouse, 1 mouse was colonized. Assuming a Poisson distribution, the probability that the mouse was colonized with 2 or more CFU is 0.0011, giving us confidence that it was colonized with only 1 CFU. (The probability that a mouse with this inoculation density will be colonized by exactly 1 CFU is 0.16, and by 0 CFU is 0.82.) We also find that even at much higher inoculum density (27CFU/mouse), 9 out of 10 mice were colonized, indicating that even at high concentrations where it is very likely that every mouse received at least 1 CFU (probability ~1), colonization was not guaranteed in every mouse. A similar experiment with the ATCC inoculum confirm these results: 3.2(±2.8)CFU/mouse leads to colonization of 1 mouse. At the lower end of this range, the probability that the mouse was colonized with 2 or more CFU is 0.008. Likewise, at much higher inoculum density (72 ± 10.6 CFU/mouse) not every mouse was colonized, even though it is very likely that every mouse received at least 1 CFU (probability ~1). There is some variation in the CFU required for establishment of VRE depending on the inoculum used, although the range is similar for both. (The above probability calculations were done using the poisspdf and poisscdf functions in Matlab.)

**Whole genome sequencing, reference assembly and annotation.** Isolates grown in BHI until early stationary phase and centrifuged. As previously described, DNA was extracted using phenol-chloroform extraction with bead beating using 0.1-mm zirconia/silica beads (BioSpec Products). After extraction, DNA was precipitated in ethanol, resuspended in TE buffer with 200 μg/ml RNase, and further purified with...
QIAamp mini spin columns (Qiagen). Amplicons were purified with the Qiagen QiaAmp kit (Qiagen). The purified PCR products were quantified. Purified DNA was sheared using a Covaris ultrasonicator and size selection with AMPure XP beads (Beckman Coulter) was performed. Libraries were prepared for Illumina MiSeq sequencing with a Kapa library preparation kit with Illumina TruSeq adaptors to create 2x300bp paired-end reads, according to the manufacturer’s instructions. Fecal pellets sent for shotgun metagenomics were sequenced with Illumina HiSeq by 2x100bp paired-end reads. 3 fecal VRE isolates from pt110 were sequenced at the Broad Institute on the Illumina HiSeq platform by 2x101bp paired-end reads (Lebreton 2013).

Sequencing reads were processed using a custom bash shell script (available in Supplementary Files, trim.sh). Trimmomatic was used to quality filter raw sequence reads (version 0.36). Read quality was assessed by FastQC (version 0.11.5).

For pt110, PacBio reads were assembled with HGAP and iteratively corrected using Pilon with Illumina MiSeq reads from the same isolate extracted on day 8. A consensus sequence was constructed using gatk (version 3.6) and picard (version 2.12.1) for an isolate from day -6 using breseq SNV and INDEL calls to correct the PacBio reference. For all other patients, a hybrid assembly was constructed with Oxford Nanopore MinION and MiSeq reads with Unicycler and iteratively corrected with breseq (version 0.31.1). Contigs less than 500bps were removed post-assembly. Quality assessment of finished assemblies was performed using QUAST (version v4.5). References were annotated using the PATRIC web portal. Chromosomal contigs were determined by blasting the assembly fasta file (https://blast.ncbi.nlm.nih.gov/) against the nucleotide collection (nr/nt) for Enterococcus faecium.

**Variant detection.** Trimmed quality-filtered Illumina reads were aligned to our in-house references and variants were called using breseq (version 0.31.1). For isolates, the consensus mode was used. Shotgun
metagenomes were run in the polymorphism mode. breseq's annotated output files were parsed and analyzed in custom Python scripts (available in Supplementary Files). In downstream analyses, we used breseq's default setting frequency cutoff, which called SNVs at 5% frequency. This threshold was lowered to 1% frequency for Figure 5f. For pt1252 shotgun variant analysis, stool samples with >90% VRE domination were analyzed and SNVs were called along 5 chromosomal contigs (#1-4, #8).

For shotgun samples, genes with >15 SNVs per CDS were removed as outliers. Select regions were visually inspected in IGV (version 2.3.97) and for some genes, SNVs were co-localized on the same mapped reads (while other reads had no SNVs), suggesting that these variants were acquired together and did not represent individual mutation events. Other “hotspot” genes had mapping qualities <=2, indicating that poorly mapped DNA might have come from a different bacterial source in the gastrointestinal tract or from another location in the E. faecium genome. Taking a conservative approach, these genes were removed from downstream analysis (n=133 from mouse 5, n=91 from mouse 1, and n=34 from mouse 2). Removing these genes did not alter the shape of the total SNV accumulation over time in our mouse experiments (Supplementary Fig. 4a-e, column 2 and 3).

To detect mutations that occurred independently in our in vivo experiments, we generated a list of chromosomal SNVs for each mouse that occurred at any timepoint in the shotgun data. We compared each position and report the SNVs that occurred in the same position in ≥2 mice in the pt110-inoculated mice.

**Mathematical modeling.** Genotypes were determined based on co-existing mutation patterns in mouse isolates. These genotypes informed phylogenetic graphs that were created using custom Matlab scripts (Fig 4a). (Similar graphs were created for all mice in Fig. 5 in order to create the fishplots depicted, but these are not shown.) VRE genotypes were plotted by day using isolates alone (Fig. 4b) as well as estimated frequencies from shotgun data (Supplementary Fig. 2a) using custom Matlab scripts.
Fishplots were generated in R as described previously (32) (Fig. 4c, Supplementary Fig. 2b, Fig. 5a-d, column 1).

In silico experiments were conducted using custom Matlab scripts based on principles from the quasispecies model (33): we model a group of related genotypes with offspring that accumulate mutations relative to the parent. Our simulations also maintain the assumptions of the quasispecies model, incorporated into a stochastic model. An arbitrary population size of 1000 was chosen, with all members initially the ancestral clone. Based on data from Mouse 1, there were 25 loci over the course of the experiment that had mutations; therefore, our model also allowed mutation in any of 25 sites. There are \(2^{25}\) possible genotypes, and the fitness of each was set to 0.001 (an arbitrary low number), thus giving a low fitness to clones that are not experimentally observed throughout the duration of the simulation. In the neutral evolution condition, further fitness enhancement was not done.

While under neutral fitness all clones are given the same fitness, in the other fitness landscapes clones that acquire mutations are given altered fitness if the mutations match those that were experimentally observed. In the case of a linear fitness landscape, the fitness \(f\) of genotypes experimentally observed was \(f = (0.1 \times \#\text{mutations} \times 2) + 1\). In the case of a logarithmic fitness landscape, the fitness \(f\) of genotypes experimentally observed was \(f = \log(\#\text{mutations} + 2)\). In the case of a parabolic fitness landscape, the fitness \(f\) of genotypes experimentally observed was
\[
f = -0.0625 \times (\#\text{mutations} + 1)^2 + 0.5 \times (\#\text{mutations} + 1) + 0.1.
\]

These formulae were determined empirically by determining which parameters most closely recaptured experimental data in test simulations: For example, many possible linear formulae with varying steepness of slope were tested, and simulation results were compared to experimental data. After many such trials, the formula for a linear landscape that best represented experimental data was selected as the “best case scenario” for a linear landscape. The same was done for the other fitness landscapes tested, and the “best cases” for each was compared to each other. Conditions were also tested where clones with the penicillin binding protein mutation gained an additional fitness advantage,
mathematically incorporated by adding (+1) to the above formulae. During each round of the simulation,
certain members of the population were randomly selected to divide, with genotypes with higher fitness
more likely to be selected. Each of these selected individuals were then allowed to randomly mutate any
of the 25 loci. These new offspring were then added back to the population. To keep the total population
constant, random individuals were removed. This was repeated 5000-8000 times. The condition was also
tested where if, during the course of random mutation an individual acquired the php mutation, that
offspring now has the fitness conferred by either the linear, log, or parabolic landscape being tested,
regardless of whether that genotype was experimentally observed. The outcome of the above in silico
experiments show simulated VRE evolution over time under various possible fitness landscapes (Fig.
4d-e, Supplementary Fig. 2c-f).

Determining the fitness landscape that best captured experimental data was done by 1) comparing time to fixation of the php mutation in in vivo and in silico experiments, and 2) calculating
how well the scale of time in terms of days for the experiments matches with the scale of time in terms
of number of iterations for simulations. This was calculated as following:

In the experiment, two major events occur: 1) decline of the ancestral clone, and 2) expansion of
branch 2 (php containing clones). For the experimental data, we calculated rates of decline and
expansion which we call \( K_a \) and \( K_b \), respectively. For each fitness landscape, we also calculated \( K_a \) and
\( K_b \) based on simulation results. The ratio between K values were calculated, such that \( \alpha_1 = \frac{K_a(\text{simulated})}{K_a(\text{experimental})} \) and \( \alpha_2 = \frac{K_b(\text{simulated})}{K_b(\text{experimental})} \). Each alpha value has the
units days/iteration, giving a scaling between experimental days and simulation iterations. Closely
matching \( \alpha_1 \) and \( \alpha_2 \) values indicated consistent population dynamics between experimental and
simulated data. Of the 6 fitness landscapes tested, only 2 had \( \alpha_1 \) and \( \alpha_2 \) that were close in value,
indicating that the simulation accurately captured both the rate of ancestral decline and rate of php clone
expansion in the same simulation. These 2 landscapes (linear and parabolic with boost to \textit{pbp} mutation) were therefore the ones considered as “top hits,” with parabolic as the best match given the rate of fixation of the \textit{pbp} mutation. The average between \( \alpha_1 \) and \( \alpha_2 \) for the linear landscape led to the 60 iterations/day scaling for linear, and the average between \( \alpha_1 \) and \( \alpha_2 \) for the parabolic landscape led to the 50 iterations/day scaling for parabolic.

**Construction of phylogenetic trees.** Trimmed reads were assembled into contigs using the short-read genome assembler SPAdes (version 3.11.1) and annotated with PROKKA (version 1.12). Strains were typed by MLST using mlst (version 2.8). Core genomes and accessory genes were identified by Roary (version 3.8.2) and SNVs were called with SNV-sites (version 2.3.2)(34). Maximum-likelihood trees based on SNVs in the core genomes were created with RAxML using the model ASC-GTRGAMMA for trees with fewer than 50 genomes. The phylogenetic tree of patient VRE strains was created using FastTree, which infers the approximately maximum likelihood tree from alignments of sequences(35, 36). The tree was then rooted by the outgroup \textit{E. hirae}.

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Figure Legends

Figure 1 | Within-host diversity of a single allo-HCT patient at MSKCC within and across time. a) The relative abundances of microbiota components measured by 16S rRNA sequencing and classified at the species level are shown for 3 stool samples collected. Days are relative to HCT. b) Lollipops
indicate the presence of a SNV or short indel along the chromosome for 3 VRE isolates taken on day -6, 1 and 8. (Markers without stems denote positions of mutations found in other genomes.) 

c) Bar chart of the number of variants per isolate on days -6, 1 and 8. 

d) A dendogram of the MLST 412 isolates cultured across day -6, 1 and 8 based on whole genome comparisons using the PATRIC web portal. 

Branch lengths are proportional to the number of genetic differences.

Figure 2 | Unique patterns of genetic variation are detected on each day for VRE-colonized allo-HCT patients and are acquired at variable rates. 

(a-d) The relative abundances of microbiota components (ASVs) measured by 16S rRNA sequencing and classified at the species level are shown for 4 patients. Days are relative to HCT. Vertical dotted black line denotes day of cell infusion. 

e) Multiple VRE isolates per sample were cultured from 5 additional allo-HCT patients and sequenced by Illumina MiSeq. De novo assemblies were constructed from Illumina reads using Spades. Core genome alignments were obtained using Roary, and a maximum-likelihood tree was assembled using FastTree. 

f) One isolate per patient was sequenced by both Illumina Miseq and Nanopore MinION platforms. A hybrid assembly was generated with Unicycler and variants were called and corrected with breseq and bcftools. Reads for all patient isolates were aligned to their own reference and breseq was used to detect mutations. The proportion of new unique genotypes observed per day were plotted at each day. The reference strain (which contains no variation by definition) is not counted as a unique genotype.

Figure 3 | VRE expanded rapidly in the gastrointestinal tract after acquiring a nonsynonymous mutation in the gene encoding penicillin binding protein 5 (pbp5). 

(a) Diagram of experimental design: an initial cohort of 30 mice was split into 3 groups, each given a different concentration of CFU and then single-housed. One mouse with the lowest level of inoculation was followed for 133 days. All mice were kept on ampicillin for the duration of the experiment. 

(b) Low inocula of VRE (CFU/mouse) were gavaged into ampicillin-treated mice. Height of bars depict mean CFU/g fecal content for each condition. The number of mice that were successfully colonized per inoculated group by day 5 are plotted; n=10 per experimental group. The dose of 0.2 CFUs/mouse, which is equivalent to 2 CFUs/ml, infected 1 mouse out of 10 (circled in red). 

c) This individual mouse was followed for a total of 133 days. The density of VRE was measured by plating fecal pellets on selective media. 4 isolates were cultured from fecal content obtained at the 11 timepoints indicated. 

d) A heatmap of the 25 mutations acquired in the chromosome across the 44 isolates. The pbp5 mutation is indicated, as are other loci mutated in 3 or more isolates. 

e) Frequency traces are shown for the 219 chromosomal mutation picked up by shotgun sequencing of fecal pellets from a single colonized mouse. 

f) Total number of mutations that achieved ≥5% frequency in the shotgun data per day over the course of the experiment.

Figure 4 | Sub-lineages coexist after rapid diversification in which the pbp5 mutation becomes fixed in the total intestinal VRE population. 

(a) A parsimonious graph of the 22 unique genotypes found amongst the 44 isolates. The branch of isolates that evolved harboring the pbp5 mutation stems from sc2. The starred clone (sc23) was not experimentally observed but must have existed in order for
the observed sc19, 21, and 22 genotypes to occur. b) The frequency of each genotype found in the 4 isolates collected per day is plotted; related isolates are grouped into colored bins. c) Clonal dynamics are captured in a fishplot. d) The number of clones with a penicillin binding protein mutation under neutral, linear, and parabolic fitness landscapes are plotted over time. Each curve is an average of 50 independent in silico experiments. e) Simulated VRE evolution under a parabolic fitness landscape with a fitness boost given to experimentally observed genotypes with a pbp5 mutation. Each genotype corresponds to a color, the same scheme used in Fig 4b, with the addition of white clones corresponding to genotypes that arise during the course of the simulation but that were not experimentally observed. One “day” corresponds to about 50 simulation iterations.

**Figure 5 | Within-host intestinal VRE evolution is a stochastic process.** Population dynamics are represented in fishplots based on combined isolate and shotgun data for mouse 2 (a), mouse 3 (b), mouse 4 (c) and mouse 5 (d) (left column). Mouse 5 was colonized by a laboratory E. faecium ATCC strain (700221), while mice 2-4 were inoculated with pt110-derived VRE isolate used in Figure 3. 129-donor bone-marrow transplants were performed on mouse 4 and 5 prior to VRE infection. Total number of mutations that achieved ≥5% frequency are calculated from the shotgun sequencing data (a-d, right column). e) The number of mutations in various simulation conditions (neutral, linear, and parabolic fitness landscapes) were plotted over time. f) A list of genes mutated in ≥3 pt110-VRE-colonized mice by mutations at ≥1% frequency in the shotgun data was generated, and the subset that were also mutated at least once in ATCC-inoculated mouse are shown (n=4). (Mouse 1 is the mouse from figure 3.) The total number of mutations per gene within each mouse that appeared at any point during the experiment are represented in a heatmap. n reports the total number of mutations for each gene summed across the mice. The number of shotgun samples per mouse are displayed at the bottom of the graph (5-7 samples per mouse). g) 4 mutations were found that were shared in ≥2 pt110-VRE-colonized mice and that had reached >20% frequency in at least one mouse at one timepoint in the shotgun data. Mutation frequencies over time are represented in a heatmap for the 4 mice inoculated with the pt110-derived isolate. Note, for the pbp5 mutation (locus 1736237), the maximum frequency reached in mouse #2 is 4%. h) In the ATCC-colonized mouse (#5), 3 mutations were found in the ABC transporter and pbp5 genes (no mutations occurred in the lactose PTS repressor gene).

**Figure 6 | VRE diversification plateaus early in the intestinal domination of an allo-HCT patient.** a) The relative abundances of microbiota components measured by 16S rRNA sequencing and classified at the species level are plotted over the course of hospitalization for pt1252. Days are relative to transplantation (color key can be found in Figure 1a). b) Stool samples with >90% VRE domination were analyzed by high-throughput shotgun sequencing. The frequencies of all variants found along the 4 contigs that represent the chromosome in the pt1252 reference are shown. c) The number and cumulative number of variants achieving ≥5% frequency per day are reported from the shotgun sequencing data.
Patient 110: Microbiota composition measured by 16S amplicon sequencing

Chromosomal position

Chromosomal mutations found in E. faecium isolates by whole-genome sequencing and breseq analysis

Dendrogram built from whole genome of E. faecium isolated MSLT 412 from patient 110

Number of different loci found in E. faecium isolates

Number of genetic differences/isolate relative to isolate -6.5

7 isolates from day -6
2 isolates from day 1
10 isolates from day 8

Microbiota taxa composition

phylum: Bacteroidetes

E. faecium

D-6_5
D-6_7
D-6_3
D-6_1
D-6_9
D-8_10
D-8_7
D-8_4
D-1_3
D-6_1
D-6_3
D-6_7
D-8_1
D-8_5
D-8_8
D-8_6
D-8_1
D-8_2

Scale: 1 genetic difference

Figure 1
Figure 3

a. 

b. 5 days post VRE inoculation

VRE density in mouse colonized with ~1 CFU

Shotgun sequencing:
219 SNPs in chromosome over 133 days

c. VRE density in mouse colonized with ~1 CFU

Number of isolates (out of 4) found with mutation

Number of new mutations

Inoculation density CFU/mouse

Days post VRE inoculation

Figure 3
Figure 4

Lineage graph

a. % isolates VRE clones found in isolates
Day 0 Day 133
Fishplot of clonal dynamics based on isolates

b. c.

Time to fixation of penicillin binding mutation in various simulated conditions

average # clones with penicillin binding mutation

Time (# iterations)

Day 0 Day 133
Simulation of VRE evolution with parabolic fitness landscape
d. e.

75 50 25 0
100 75 50 25 0
100
Figure 5

Fishplot of clonal dynamics based on isolates

- Mouse 2
- Mouse 3
- Mouse 4
- Mouse 5

Shotgun sequencing mutation count

- Time (# iterations)
- average # mutations/day

Number of genetic variants in various simulated conditions

- Mouse inoculated with pt110-derived VRE isolate
- Mouse inoculated with ATCC strain

Mice inoculated with pt110-derived VRE isolate

- PTS repressor
- ABC transporter

Mouse inoculated with ATCC strain

- pt5
- ABC transporter

Penicillin-binding protein 3
Two-component sensor kinase SA14-24
ABC transporter, substrate-binding protein (cluster 1, maltose/G3P/polyamine/iron)
PTS system, glucone-specific IIC component

Figure 5
Patient 1252: Microbiota composition measured by 16S amplicon sequencing

a.  

b.  

Shotgun sequencing

C.  

Shotgun sequencing mutation count

Days relative to HCT

Mutation frequency (%)