INFECTIOUS DISEASE

Increasing tolerance of hospital *Enterococcus faecium* to handwash alcohols

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Alcohol-based disinfectants and particularly hand rubs are a key way to control hospital infections worldwide. Such disinfectants restrict transmission of pathogens, such as multidrug-resistant *Staphylococcus aureus* and *Enterococcus faecium*. Despite this success, health care infections caused by *E. faecium* are increasing. We tested alcohol tolerance of 139 hospital isolates of *E. faecium* obtained between 1997 and 2015 and found that *E. faecium* isolates after 2010 were 10-fold more tolerant to killing by alcohol than were older isolates. Using a mouse gut colonization model of *E. faecium* transmission, we showed that alcohol-tolerant *E. faecium* resisted standard 70% isopropanol surface disinfection, resulting in greater mouse gut colonization compared to alcohol-sensitive *E. faecium*. We next looked for bacterial genomic signatures of adaptation. Alcohol-tolerant *E. faecium* accumulated mutations in genes involved in carbohydrate uptake and metabolism. Mutagenesis confirmed the roles of these genes in the tolerance of *E. faecium* to isopropanol. These findings suggest that bacterial adaptation is complicating infection control recommendations, necessitating additional procedures to prevent *E. faecium* from spreading in hospital settings.

INTRODUCTION

Enterococci are members of the gut microbiota and usually have low virulence, but they have nevertheless emerged as a major cause of health care–associated bacterial infections (*1*). Enterococci now account for about 10% of hospital-acquired bacteremia cases globally, and they are the fourth and fifth leading cause of sepsis in North America and Europe, respectively (*2*). Hospital-acquired enterococcal infections are difficult to treat because of their intrinsic and acquired resistance to many classes of antibiotics (*3*). The difficulties associated with treatment, coupled with the risk of cross-transmission to other patients, make enterococcal infections an increasingly important hospital infection control risk (*4*).

Among the medically important enterococci, *Enterococcus faecium* in particular has become a leading cause of nosocomial infections (*5*). *E. faecium* population analysis has revealed the emergence of a rapidly evolving lineage referred to as Clade-A1 and includes clonal complex 17 (CC17), comprising strains associated with hospital infections across five continents (*6*, *7*). These hospital strains are resistant to ampicillin, aminoglycosides, and quinolones, and their genomes contain a high number of mobile genetic elements and are enriched for genes encoding altered carbohydrate utilization and transporter proteins that distinguish them from community-acquired and nonpathogenic *E. faecium* strains (*6*).

A recent Australia-wide survey demonstrated that *E. faecium* caused one-third of bacteremic enterococcal infections, and 90% of these were ampicillin-resistant CC17 strains, of which 50% were also vancomycin-resistant (*8*). Costs associated with the management of patients infected with vancomycin-resistant enterococci (VRE) are high because of the need for isolation rooms, specialized cleaning regimens, and the impact on staff, bed availability, and other resources. Treatment of invasive VRE infections requires higher-cost antibiotics, with patients experiencing side effects and treatment failure due to further acquired bacterial drug resistance (*8*).

Alcohol-based disinfectants, such as hand rubs and associated hand hygiene programs, are a mainstay of infection control strategies in health care facilities worldwide, and their introduction is aligned with declines in some hospital-acquired infections, particularly those caused by hospital-adapted multidrug methicillin–resistant *Staphylococcus aureus*. The compositions of hand hygiene solutions typically contain at least 70% (v/v) isopropyl or ethyl alcohol (*9*–*11*). The application of alcohol-based hand rubs for 30 s has better disinfection efficacy than traditional approaches using soap and water, with greater than 3.5 $\log_{10}$ reduction in bacterial counts considered effective (*12*). The presence of alcohol in these hand rubs is responsible for rapid bacterial killing at these concentrations, although some species are capable of surviving alcohol exposure at lower concentrations (*9*, *13*). The ability to withstand the addition of a certain percentage of alcohol is referred to as alcohol tolerance, and this phenomenon has been described across several bacterial genera (*13*, *14*).

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To control VRE, many health care facilities perform active surveillance cultures on all patients and then use contact precautions that involve the use of gowns, gloves, and single room isolation for colonized patients (*15*). However, this approach is expensive and cumbersome, particularly when VRE endemicity is high. Because of the relatively low virulence of VRE, other facilities rely on standard precautions, predominantly alcohol-based hand rub usage, and only selectively perform active surveillance cultures in high-risk areas such as hematology departments and intensive care units (*15*). At Austin Health and Monash Medical Centre, two university teaching hospitals in Melbourne, Australia, patients are screened for VRE rectal colonization on admission and weekly for all inpatients in defined high-risk clinical areas. VRE-colonized patients are colocated, and contact precautions (including strict adherence to alcohol-based hand rub guidelines) are used routinely (*16*). Here, we investigated the tolerance of 139 *E. faecium* recent isolates to the isopropyl alcohol used in alcohol-based hand rubs.

RESULTS

Increasing isopropanol tolerance among hospital *E. faecium* isolates over time

Alcohol-based hand rubs were systematically introduced to Australian health care facilities beginning at Austin Health and Monash Medical Centre in December 2002 (*17*–*19*). One consequence of this changed practice has been the substantial increase in the volume of alcohol-based hand rub products used by institutions. For instance, the volume of alcohol-based hand rubs used at Austin Health and Monash Medical Centre increased from 100 liter/month in 2001 to 1000 liter/month in 2015. We tested the hypothesis that *E. faecium* isolates adapted to this changed environment, becoming more tolerant to alcohol exposure than earlier (pre-2004) isolates. We developed an alcohol killing assay based on exposure to 23% (v/v) isopropanol for 5 min because this concentration and time provided a discriminating dynamic range among the *E. faecium* isolates. Results were expressed as a $\log_{10}$ reduction in colony-forming units (CFU) from a starting inoculum of 10^8 CFU. We assessed the isopropanol tolerance of 139 *E. faecium* isolates collected from two major Australian hospitals over 19 years. There was considerable variation in isopropanol tolerance, with a range of 4.7 $\log_{10}$ CFU reduction between isolates. These differences were independent of *E. faecium* genotype (table S1). We noticed that later isolates were more likely to be tolerant to isopropanol killing than earlier isolates (Fig. 1A), an observation that was supported by significantly different population mean tolerance when comparing pre-2004 with post-2009 isolates (0.97 $\log_{10}$ mean difference, $P < 0.0001$; Fig. 1A). There was genetic diversity among the *E. faecium* population across this time period with two dominant CC17 multilocus sequence types (MLST), ST17 and ST203, that each incrementally displayed increasing isopropanol tolerance (Fig. 1, B and C). Isolates representing the most recently emerged clone (ST796 in 2012) exhibited uniformly high isopropanol tolerance ($n = 16$; median, 1.14 $\log_{10}$ reduction; Fig. 1D and table S1). There was no relationship between acquired vancomycin resistance and isopropanol tolerance with 34 vancomycin-sensitive *E. faecium* (VSE) strains and 28 vancomycin resistant *E. faecium* (VREfm) strains both displaying <1.5 $\log_{10}$ reduction (Fisher's exact test, $P = 0.738$; table S1). Exposure of a selection of *E. faecium* isolates to ethanol showed similar tolerance patterns as isopropanol, with the ST796 strain also being significantly more ethanol-tolerant compared to representatives of the other dominant *E. faecium* sequence types ($P < 0.0002$; fig. S1).

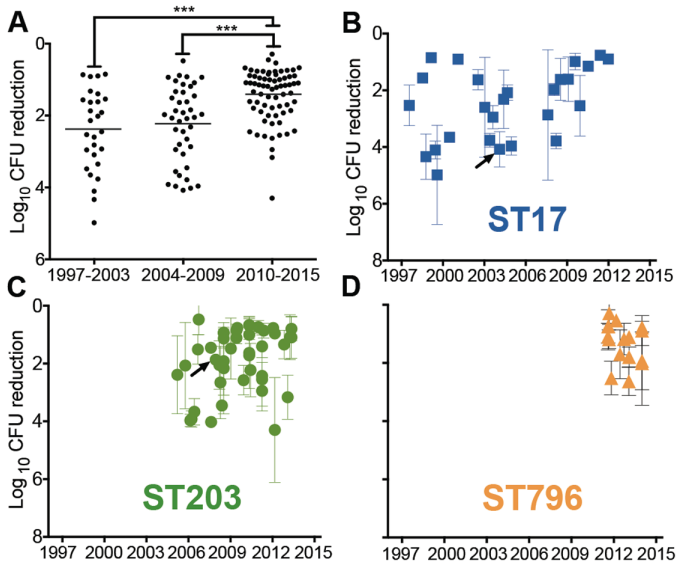

Fig. 1. Isopropanol tolerance variation among *E. faecium* isolates. Tolerance to isopropanol of 139 *E. faecium* isolates collected over 19 years at two hospitals in Melbourne, Australia. (**A**) The changing tolerance to isopropanol of *E. faecium* isolates collected between 1997 and 2015 is shown. The mean $\log_{10}$ CFU reduction values for each *E. faecium* isolate after exposure for 5 min to 23% (v/v) isopropanol are plotted against specimen collection date and clustered in 5- or 6-year windows. Two-tailed, unpaired Mann-Whitney test, ***$P < 0.0001$. (**B** to **D**) Mean $\log_{10}$ CFU reduction for the three dominant *E. faecium* clones ST17, ST203, and ST796 with the range (at least biological duplicates) displayed. The black arrows indicate the two isolates used in a previous handwash volunteer study (*24*).

Clinical relevance of *E. faecium* alcohol tolerance

To assess the clinical relevance of the alcohol tolerance differences uncovered by our 23% (v/v) isopropanol killing assay, we established a contaminated surface transmission model for *E. faecium* in mice. We compared the impact of an intervention, using 70% isopropanol impregnated surface wipes on a contaminated surface, on transmission of two VREfm and two VSE *E. faecium* isolates. We used a mouse gastrointestinal tract colonization model of transmission, first establishing that the colonizing dose-50 (CD_{50}) among the four *E. faecium* isolates was not significantly different (Fig. 2A). We selected a 2012 alcohol-tolerant isolate (Ef_aus0233, 0.45 $\log_{10}$) and a 1998 reduced-tolerance isolate (Ef_aus0004, 4.34 $\log_{10}$). Groups of six BALB/c mice, pretreated for 7 days with vancomycin, were dosed by oral gavage with decreasing doses of each isolate. The CD_{50} for each isolate was low and not significantly different [Ef_aus0004. 14 CFU; 95% confidence interval (CI), 6 to 36 CFU; compared to Ef_aus0233, 3 CFU; 95% CI, 1 to 6 CFU] (Fig. 2A, left). We then coated the floor of individually vented cages with about 3×10^6 CFU of each *E. faecium* isolate and subjected each cage to a defined disinfection regimen, wiping with either water or a 70% (v/v) isopropanol solution (Fig. 2B, left). The density of bacteria on the cage floor after isopropanol cleaning ranged from 0.4 to 30 CFU/cm^2, which is consistent with concentrations of *E. faecium* reported on environmental surfaces in health care settings (Fig. 2B) (*20*). Groups of six BALB/c mice were then released into the treated individually vented cages for 1 hour, before being rehoused in individual cages for 7 days, and then screened for gastrointestinal colonization by *E. faecium*. Across three independent

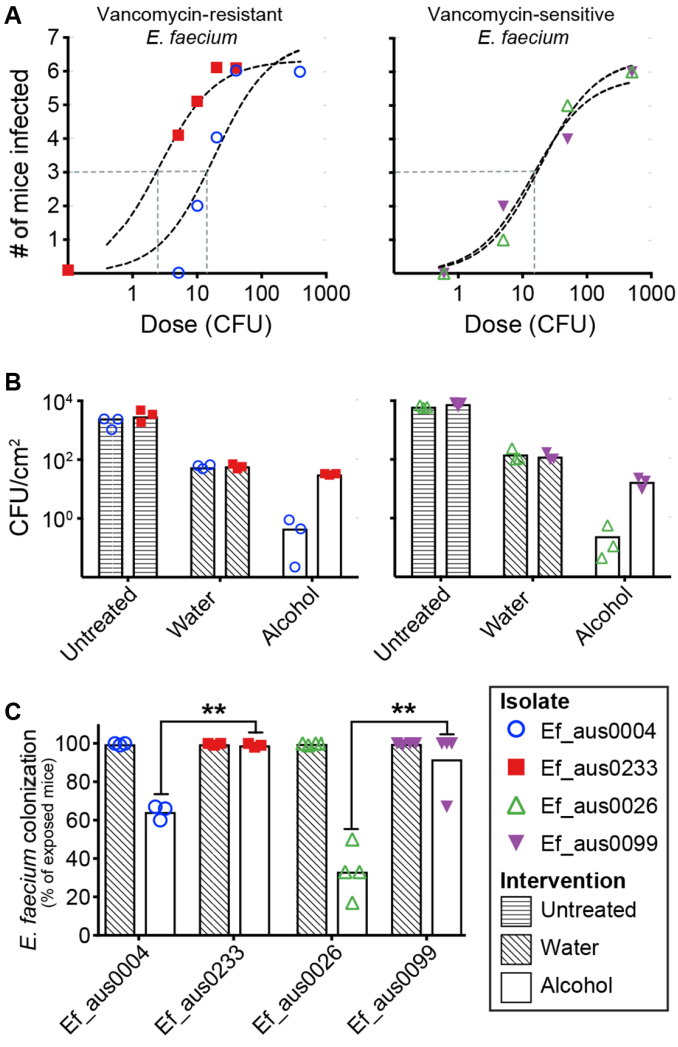

Fig. 2. Isopropanol-tolerant *E. faecium* resists disinfection. A mouse gastrointestinal colonization assay was used to assess transmission of *E. faecium*. (**A**) The CD_{50} (gray dashed lines) for two vancomycin-resistant *E. faecium* isolates (Ef_aus0004 and Ef_aus0233) and two vancomycin-sensitive isolates (Ef_aus0026 and Ef_aus0099) was established (table S6). (**B**) The results of the contaminated cage floor mouse gut colonization experiment to quantify transmission of *E. faecium* are shown. The concentration of *E. faecium* before (the inoculum) and after standardized cage floor cleaning with 70% (v/v) isopropanol versus cleaning with water is plotted (table S7). The symbols show *E. faecium* CFU for the floor of three cages. (**C**) Percentage of mice with gut colonization by vancomycin-resistant or VSE after standardized cage floor cleaning with either 70% (v/v) isopropanol or sterile water. The results of at least three independent experiments based on six mice per experiment are shown (*$P < 0.01$; table S8). The null hypothesis (no difference between *E. faecium* that is sensitive versus tolerant to isopropanol) was rejected for $P < 0.05$, unpaired *t* test with Welch's correction.

experiments, we then assessed the percentage of mice from each experiment colonized by *E. faecium*. The alcohol-tolerant *E. faecium* isolate (Ef_aus0233) was better able to withstand the 70% (v/v) isopropanol disinfection and to colonize the mouse gut than was the more alcohol-susceptible Ef_aus0004 isolate ($P < 0.01$; Fig. 2C, left). We decided to challenge our alcohol tolerance and transmission hypothesis further by selecting a pair of VSE isolates with a different tolerance to alcohol but much closer genetic identity than Ef_aus0004 and Ef_aus0233, thereby reducing potential confounding phenotypes that might arise with divergent genomes. The VSE isolates (Ef_aus0026 and Ef_aus0099) shared only 29 core genome single-nucleotide polymorphism (SNP) differences but had opposing alcohol tolerance phenotypes (4.4-fold difference) (tables S1 and S2). Each isolate had the same low CD_{50} (Ef_aus0026, 19 CFU; 95% CI, 9 to 41 CFU; compared to Ef_aus0099, 12 CFU; 95% CI, 3 to 62 CFU) (Fig. 2, A and B, right). Across four independent experiments, a significantly greater number of mice were colonized by the isopropanol-tolerant *E. faecium* isolate (Ef_aus0099) than the more susceptible *E. faecium* isolate (Ef_aus0026) ($P < 0.01$; Fig. 2C, right).

Population structure of *E. faecium* isolates

To look for signatures of genetic adaptation that were associated with alcohol tolerance, we first compared the genomes of 129 of 139 *E. faecium* isolates (10 isolates were not sequenced). A high-resolution SNP-based phylogeny was inferred from pairwise core genome comparisons and Bayesian analysis of population structure (BAPS) that stratified the population into seven distinct genetic groups, coinciding with previous MLST designations (Fig. 3A and fig. S2A). The population had a substantial pan-genome, comprising 8739 orthologous gene clusters, underscoring the extensive genetic diversity of this *E. faecium* population (fig. S3). There was also a temporal pattern to the appearance of each genetic group. Beginning with the previously described displacement of ST17 with ST203 in 2006 through to the emergence of ST796 in 2012, we observed the introduction to the hospitals at different times of distinct *E. faecium* clones, with each clone exhibiting increasing alcohol tolerance (Fig. 1 and fig. S2B).

Identifying bacterial genetic factors linked to alcohol tolerance

High alcohol tolerance was observed within distinct *E. faecium* lineages, suggesting that multiple genetic events leading to isopropanol tolerance occurred (Fig. 3). We searched for the genetic basis of alcohol tolerance by evolutionary convergence analysis to identify regions of the *E. faecium* genome that might potentially harbor genes or mutations linked to alcohol tolerance. We first identified 19 matched genetic *E. faecium* isolate pairs across 129 isolates that shared less than 1000 core genome SNP differences but exhibited a greater than 1.5-fold difference in alcohol tolerance (Fig. 3A and table S2).

We then searched for core genome mutations that occurred in at least three pairs at the same chromosome nucleotide position and in the same direction of change (that is, homoplasies). We identified 400 nucleotide positions mutated in two or more pairs, which reduced to 75 nucleotide positions mutated in three or more pairs, and only three of these 75 sites had mutations in the same direction (Fig. 3B). One of these loci was the *rpoB* gene, encoding the β subunit of RNA polymerase. The H486N/Y substitution in RpoB seen in three pairs was associated with reduced alcohol tolerance (Fig. 3). Mutations in this region of *rpoB* are known to cause resistance to the antibiotic rifampicin, and it is exposure to this drug rather than an evolutionary response to alcohol that likely selects these mutations. Nevertheless, the *rpoB* mutations served as additional support for the approach and its capacity to detect homoplasic mutations associated with a changed alcohol tolerance phenotype. The two additional loci detected spanned an amino acid substitution in a putative galactoside symporter in three *E. faecium* pairs at chromosome position 519,608 and two mutations in six *E. faecium* pairs in a putative phage region (around position

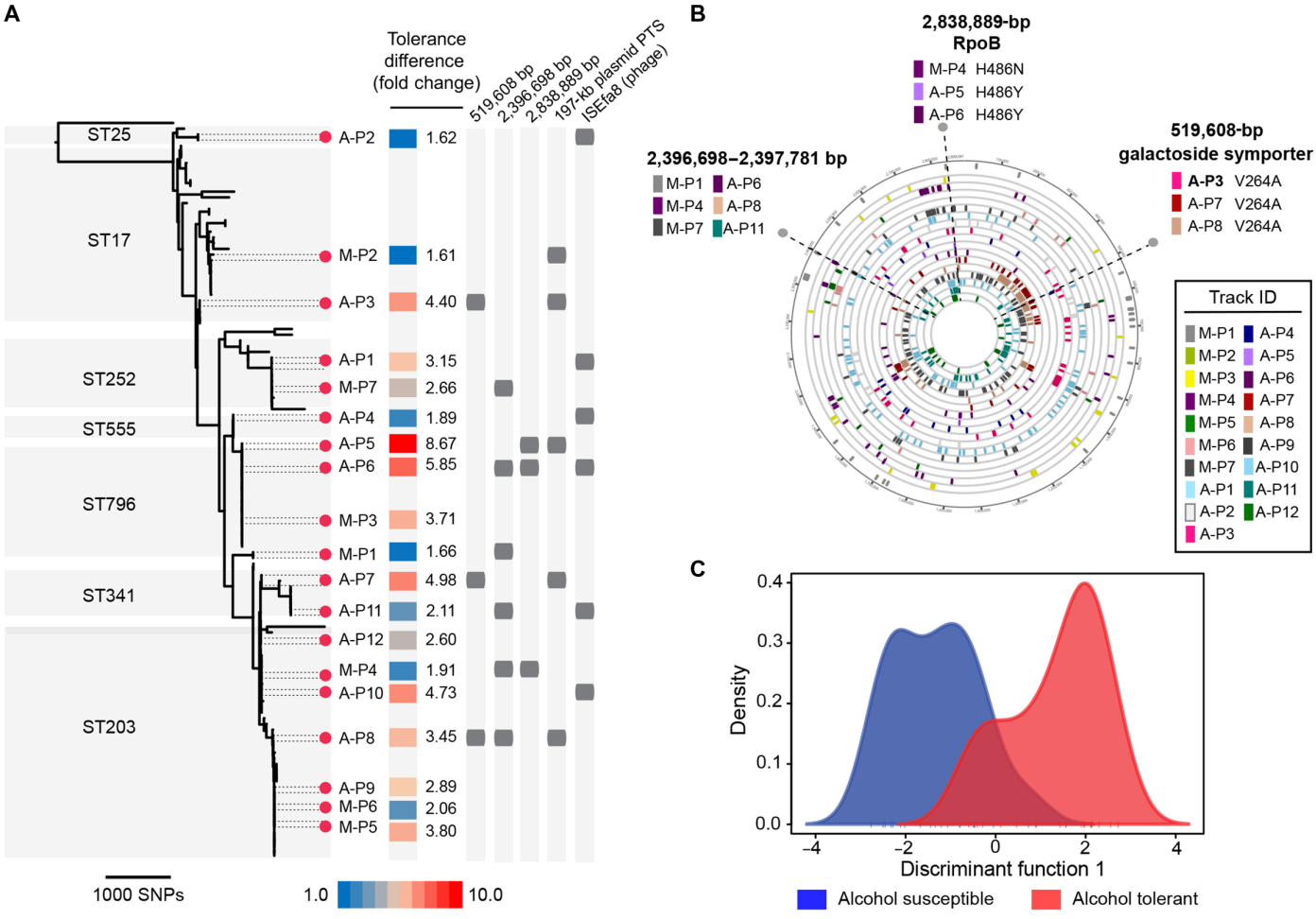

Fig. 3. Population structure of *E. faecium* isolates. (**A**) One hundred twenty-nine *E. faecium* isolates were subjected to whole-genome sequencing and alcohol tolerance testing, and their population structure was determined. The phylogeny was inferred using maximum likelihood with RAxML and was based on pairwise alignments of 18,292 core genome SNPs against the *E. faecium* Ef_aus0233 reference genome (filtered to remove recombination). Previous MLST designations are indicated by sequence type. A heat map summary of the fold difference in $\log_{10}$ kill for each selected pair of taxa is shown, with blue being the smallest fold difference in tolerance and red being the largest fold difference in tolerance. The prefix "A" or "M" before each pair number indicates pairs of strains from the Austin Hospital or Monash Medical Centre, respectively. (**B**) Analysis of convergent SNP differences among phylogenetically matched pairs of *E. faecium* isolates. The circular map represents the 2,888,087–base pair (bp) chromosome of the *E. faecium* Ef_aus0233 reference genome, showing the location of convergent core genome SNPs for each *E. faecium* pair. Track IDs are indicated by the color-coded key. The three homoplasic mutations (at chromosome positions 519,608, 2,396,698, and 2,838,889) that were conserved in the direction of nucleotide sequence change and present among three or more *E. faecium* pairs are highlighted and annotated. (**C**) Probabilistic separation of alcohol-sensitive (blue) and alcohol-tolerant (red) isolates according to a DAPC model built using accessory genome variation (table S3).

2,396,698) (Fig. 3, A and B). One of these three pairs included Ef_aus0026 and Ef_aus0099, the vancomycin-sensitive pair that were used in the mouse gut colonization transmission study (pair A-P3; Figs. 2 and 3B and table S2).

In addition to SNP variations, we also compared the presence or absence of patterns of genes between alcohol-tolerant and alcohol-sensitive *E. faecium* isolates in each of the 19 pairs. We first used a supervised statistical learning approach called discriminant analysis of principal components (DAPC) to build a predictive model and identify genes that contributed to the separation of pairs based on their isopropanol tolerance values. Using only the first 25 principal components (PCs), the model showed good separation of alcohol-tolerant and alcohol-sensitive isolates, with the resulting loading values used to guide the ranking of genes that associated with the alcohol-tolerant phenotype (table S3). This analysis suggested that there is a genetic basis for the alcohol tolerance phenotype, with distinct separation of the alcohol-tolerant and alcohol-sensitive populations (Fig. 3C). We then ranked genes according to (i) their contribution to DAPC separation of the phenotypes, (ii) the frequency of gene presence/absence among the 19 pairs, and (iii) the direction of gene presence/absence (that is, always present in alcohol-tolerant isolates; table S3). This analysis identified two high-scoring loci, a copy of ISEfa8 inserted adjacent to a putative prophage region around chromosome position 953,094 and a 70-kb region of a 197-kb plasmid that spans genes encoding several putative proteins, a predicted LPXTG motif cell wall protein and two carbohydrate phosphotransferase system (PTS) proteins, PTS-1 and PTS-2 (Fig. 4A).

Validation of bacterial genetic factors linked to alcohol tolerance

To test the validity of the predictions based on convergence analysis and DAPC, we used allelic exchange to make targeted mutants in the isopropanol-tolerant ST796 reference isolate Ef_aus0233. Given the reported role of PTS proteins in solvent tolerance (*21*), we focused first on one of the plasmid-associated PTS regions, deleting a 6.5-kb region of PTS-2, a putative glucoside-specific PTS (Fig. 4A). We also made a deletion mutant of the gene locus_00501 encoding a putative galactoside symporter (Fig. 4B), where there was a specific V264A amino acid substitution associated with isopropanol tolerance. A *rpoB* mutant (H486Y) was also made because this locus was also identified in the genome convergence analysis and so should present an altered alcohol tolerance phenotype, although here, the mutation was associated with loss of alcohol tolerance. An absence of unintended second-site mutations was confirmed by whole-genome sequencing, and the PTS and 00501 mutations were also repaired. Screening these three mutants and their repaired versions using our isopropanol exposure killing assay showed no change in alcohol tolerance (Fig. 4C). To further explore the sensitivity of the two mutations in 00501 and PTS to isopropanol, we also conducted growth curve assays in the presence of 3% isopropanol, a concentration we determined provided useful discrimination among our *E. faecium* isolate collection. All mutants showed significant increases in their doubling times compared to wild type, a phenotype that was restored in the repaired mutants ($P < 0.01$; Fig. 4D and fig. S4). The mutants showed no growth defect in the absence of isopropanol (fig. S4). These experiments confirmed predictions from convergence testing and DAPC that these loci are involved in promoting isopropanol tolerance. Loss of individual loci, however, did not affect sensitivity to isopropanol killing, suggesting that isopropanol tolerance is a polygenic phenotype, with multiple genetic changes across different loci likely to have occurred in alcohol-tolerant *E. faecium* strains.

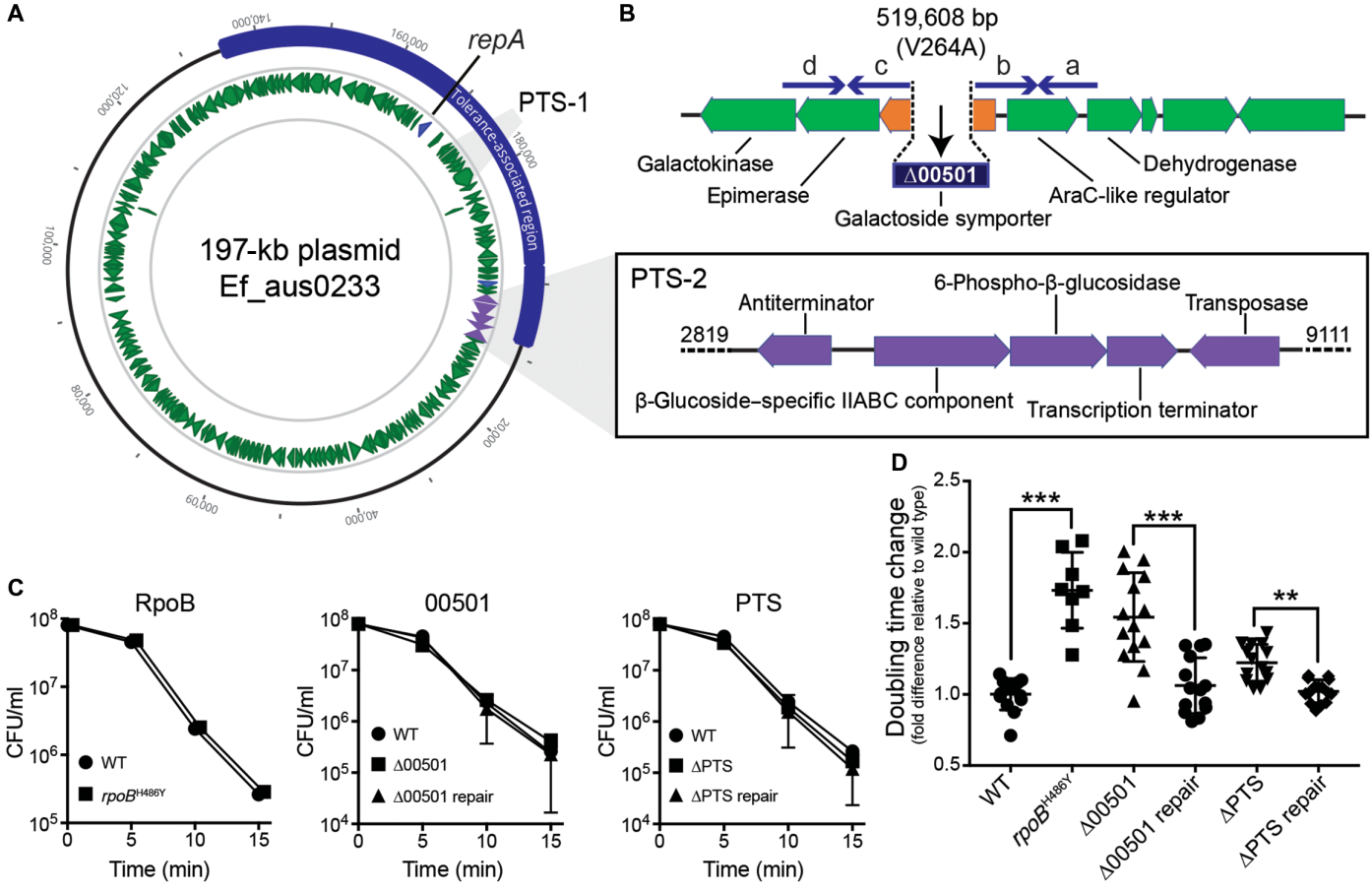

Fig. 4. Functional confirmation of genes associated with isopropanol tolerance in *E. faecium*. (**A**) Map of the 197-kb *E. faecium* plasmid showing the 70-kb region associated with isopropanol tolerance (blue) and the two carbohydrate PTS loci, including the 6.3-kb PTS-2 locus (purple) deleted by allelic exchange in the *E. faecium* reference strain Ef_aus0233. (**B**) Layout of the region around Ef_aus0233 chromosome position 519,608 showing the region deleted by allelic exchange in the gene 00501 encoding a putative galactoside symporter. Dark blue arrows indicate position of primers used to generate the recombination substrate for mutant construction. Primer positions: (a) 521,394 to 521,371; (b) 520,396 to 520,420; (c) 518,946 to 518,918; (d) 517,961 to 517,985. (**C**) Impact of the *rpoB*^{H486Y} mutation, 197-kb plasmid ΔPTS-2, and Δ00501 galactoside symporter mutation on the ability of Ef_aus0233 to survive exposure to isopropanol. The means and SDs for biological triplicate experiments, with no differences between any of the mutants and wild-type (WT) *E. faecium* (table S9), are shown. (**D**) Growth phenotypes of the same *rpoB*^{H486Y} mutation, 197-kb plasmid ΔPTS-2, and Δ00501 galactoside symporter mutation in the presence of 3% (v/v) isopropanol. Fold-change difference in doubling time for each mutant compared to wild-type *E. faecium* is shown. The phenotypes of the repaired mutants relative to wild-type *E. faecium* are also depicted. The null hypothesis (no difference between mean doubling time of mutant and repaired mutant or wild-type *E. faecium*) was rejected for $P < 0.01$, unpaired Mann-Whitney test (**$P < 0.01$, ***$P < 0.0001$; table S10). Error bars depict SDs. All data points are shown for at least three biological replicates and three technical replicates for each condition.

DISCUSSION

In 2005, we published a 3-year study describing a progressive decline in rates of hospital acquired methicillin-resistant *S. aureus* and Gram-negative infections after the introduction and promotion of alcohol-based hand rubs (*22*). Similar programs were progressively rolled out to all major hospitals in Australia, and compliance with the use of alcohol-based hand rubs is now a nationally reportable key performance indicator (*19*). The 2015 Australian National Hand Hygiene program report shows high (>80%) compliance rates in health care facilities across the country (www.hha.org.au), and staphylococcal infection rates have declined nationally (*18*, *23*). However, coincident with the introduction of alcohol-based hand rubs and high compliance, there has been a paradoxical nationwide increase in *E. faecium* infections (*8*). Here, we have shown that more recent *E. faecium* clinical isolates were more alcohol-tolerant than their predecessors and, using our in vitro alcohol tolerance assay, that the date of isolation rather than genotype is a better predictor of *E. faecium* survival. To obtain a practical dynamic range and allow meaningful comparison between isolates, the tolerance assay used concentrations of alcohol lower than the usual 70% (v/v) of most alcohol-based hand rub products. However, with our mouse gut colonization model, we were able to demonstrate that differences detected by this in vitro assay translated to an increased likelihood of transmission for alcohol-tolerant *E. faecium* strains when subjected to a full 70% (v/v) isopropanol surface disinfection intervention (Fig. 2). As alcohol tolerance increases, we hypothesize that there will be skin surfaces in contact with alcohol-based hand rubs or inanimate surfaces in contact with other alcohol-based cleaning agents that do not receive the maximum biocide concentration or contact time required for effective bacterial killing. This idea is supported by our previous clinical research using full concentration alcohol-based hand rubs in 20 human volunteers and two strains of vancomycin-resistant *E. faecium* ST17 and ST203, where we identified a mean 3.6 $\log_{10}$ reduction in vancomycin-resistant *E. faecium* on the hands of test subjects, but with a very large intersubject variance (*24*). For two volunteers, the reduction of vancomycin-resistant *E. faecium* was less than 1.6 $\log_{10}$, suggesting that some host factors not only might result in vancomycin-resistant *E. faecium* containment failure but also might enhance the clinical likelihood for selection of alcohol-tolerant *E. faecium* (*24*).

There has been growing interest in tolerance to other biocides such as chlorhexidine, a second active agent sometimes added to alcohol-based hand rub products (*25*, *26*), including attempts to identify tolerance mechanisms through mutagenesis screens that have pinpointed a specific two-component regulator (*27*). Alcohol tolerance has been reported in other clinically relevant bacteria. For example, studies have reported the enhanced growth of *Acinetobacter baumannii* when exposed to low, nonlethal concentrations of alcohol and alcohol-based hand rubs, and increased pathogenicity after the addition of ethanol (*14*, *28*).

Research on alcohol tolerance mechanisms used by enterococci has been largely derived from studies of Gram-positive bacteria associated with spoilage of sake, particularly the lactic acid bacteria that are known to survive and grow in ethanol concentrations of greater than 18% (v/v) (*29*). The increase in tolerance over time displayed by *E. faecium* isolates in our study is consistent with the accumulation of mutations and genes that have shifted the phenotype. Stepwise alcohol adaptation has been observed in laboratory experiments with a related Gram-positive bacterium, *Clostridium thermocellum*, that eventually tolerated up to 8% (w/v) ethanol (*30*). For bacteria in general, short-chain alcohols such as ethanol and isopropanol are thought to kill by disrupting membrane functions (*31*, *32*). The penetration of ethanol into the hydrocarbon components of bacterial phospholipid bilayers causes the rapid release of intracellular components and disorganization of membranes (*33*). Metabolic engineering of solvent-tolerant bacteria has uncovered major mechanisms of tolerance, showing that membrane transporters are critically important (*31*). For solvents such as ethanol and isopropanol, potassium ions and proton electrochemical membrane gradients are general mechanisms that enhance alcohol tolerance (*34*).

Our phylogenetic convergence and DAPC analyses across distinct *E. faecium* lineages identified changes in several genetic loci likely contributing to alcohol tolerance. Specific mutagenesis for three regions confirmed these predictions, showing that multiple mutations are required and loci involved in carbohydrate transport and metabolism are likely under selection. Not one mutation showed a change in a bacterial killing assay after exposure to 25% (v/v) isopropanol (Fig. 4C), but significant differences were observed on growth rate in the presence of 3% (v/v) isopropanol ($P < 0.01$; Fig. 4D). The gene 00501 encodes a putative major facilitator superfamily galactoside symporter, and the SNP at position 519,608 (V264A) occurs within one of its 12 transmembrane regions (Fig. 4B). We speculate that mutations such as V264A might help alter the membrane proton gradient to favor an alcohol-tolerant state (*34*). In Gram-negative bacteria, transport systems are known to be up-regulated or required in response to exposure to short-chain alcohols (*35*, *36*). Bacterial major facilitator transporters, such as 00501, are frequently identified in screens for proteins linked to increased solvent tolerance. However, the specific mechanisms by which they promote tolerance are not understood (*31*). The enrichment in alcohol-tolerant *E. faecium* strains for PTS loci is also noteworthy (Fig. 4) (*37*). PTS proteins catalyze the phosphorylation and transport of different carbohydrates into the bacterial cell. However, there is a growing understanding that their various regulatory roles are as important as their sugar uptake functions (*37*). They have also been implicated in solvent and stress tolerance. In *Escherichia coli*, up-regulation of a mannose-specific PTS led to increased tolerance to *n*-hexane exposure (*21*). In *Enterococcus faecalis*, PTS loci appear to be important for survival under low pH and oxidative stress conditions (*38*). Notably, PTS loci are enriched in health care–associated *E. faecium* lineages, with specific systems associated with mouse gut colonization, biofilm formation, and survival in serum killing assays (*39*–*42*).

Limitations of our study include the geographic restriction of the *E. faecium* isolates. Although we screened a large number of bacteria from two major Australian hospitals over 19 years, it will be important to test whether alcohol tolerance is emerging in other *E. faecium* populations. We have proposed here that the significant positive relationship between time and increasing alcohol tolerance ($P < 0.0001$; Fig. 1) is a response of the bacteria to increased exposure to alcohols in disinfectant preparations and that the more tolerant strains are able to displace their less alcohol-tolerant predecessors. However, it is also conceivable that *E. faecium* populations are responding to another factor. For instance, modified or acquired transport systems might be conferring acid tolerance, leading to improved survival during passage through the gastrointestinal tract. Secondary phenotypes such as alcohol tolerance are then coselected (passenger phenomenon) and, together with the primary phenotype, multiply the environmental hardiness of the pathogen.

Whatever the drivers, the development of alcohol-tolerant strains of *E. faecium* has the potential to undermine the effectiveness of alcohol-based disinfectant standard precautions and may, in part, explain the increase in VRE infection that is now widely reported in hospitals in Europe, Asia, the Americas, and Australia. Alcohol-based disinfectants remain an important general primary defense against cross-transmission of most microbial and some viral pathogens in health care settings. In hospitals with endemic VRE, it would seem prudent to optimize adherence to alcohol-based disinfectant protocols to ensure adequate exposure times and use of sufficient volumes of product, particularly each time a health care worker cleans their hands. In addition, consideration may need to be given to the use of various formulations of alcohol-based hand rubs (for example, foams and gels) because they are known to have variable (generally reduced) efficacy compared to solutions (*43*). Furthermore, extending active surveillance cultures outside high risk areas of the hospital and return to strict contact precautions during outbreaks with new emergent strains of VRE may be required to prevent widespread cross-contamination.

MATERIALS AND METHODS

Study design

This study aimed to test the hypothesis that the pathogen *E. faecium* has become more tolerant to exposure to short-chain alcohols in alcohol-based disinfectants. One hundred thirty-nine clinical *E. faecium* isolates collected over 19 years from two major hospitals in Melbourne, Australia were screened for their ability to resist killing after exposure to 23% (v/v) isopropanol. The clinical significance of increasing bacterial tolerance to alcohol killing was then tested in a contaminated surface study involving mouse gastrointestinal tract colonization by *E. faecium*. This was achieved by comparing the ability of two different pairs of *E. faecium* isolates that had contrasting alcohol tolerance phenotypes to resist disinfection with 70% (v/v) isopropanol of a cage floor surface and then to colonize the gastrointestinal tract of mice. The *E. faecium* isolates were then subjected to whole-genome sequencing and population genomic analyses. Evolutionary convergence testing was then used to try and identify mutations arising in the population that conferred tolerance to alcohols. The contribution of mutations identified from genome comparisons and potentially linked to this phenotype was then evaluated by site-directed mutagenesis, with testing of the mutants for isopropanol tolerance or altered growth in the presence of isopropanol.

Bacterial isolates

Table S1 lists the 139 *E. faecium* isolates investigated in this study that were randomly selected within each year from predominantly blood culture isolates obtained at the Austin Hospital and Monash Medical Centre between 1998 and 2015. Isolates were stored at −80°C in glycerol. Sixty-six isolates were vancomycin-resistant (60 *vanB*-type and 6 *vanA*-type). Some of these isolates have been described in a previous study on the epidemiology of *E. faecium* at the hospital between 1998 and 2009 (*16*) and included recently emergent epidemic clones ST203 and ST796. Six ST341, one ST414, and four ST555 isolates from an Australian-wide enterococci sepsis screening program conducted by the Australian Group on Antimicrobial Resistance were also included because they were noted emergent clones in other Australian states but were only rarely isolated at our hospitals (*44*). Isolates were grown using brain heart infusion (BHI) medium at 37°C, unless otherwise stated.

Alcohol tolerance assays and analysis

In preliminary experiments, various concentrations of alcohol and *E. faecium* inoculum sizes were assessed. At "full strength" isopropanol [70% (v/v)], killing was complete and resulted in greater than 8 $\log_{10}$ reductions in broth culture and an inability to detect differences between isolates. However, by lowering the alcohol concentration in a stepwise fashion, we were able to identify a dynamic range in which we observed marked differences in the time-kill curves between isolates. Guided by these experiments and published literature (*45*), we then measured *E. faecium* survival after exposure to 23.0% (v/v) isopropanol. Overnight cultures were grown in 10 ml of BHI medium (Difco, BD). After overnight growth, each strain was diluted to an optical density at 600 nm (OD_{600nm}) value of 0.5 using phosphate-buffered saline (PBS). To 1 ml of the diluted culture, either 23% (v/v) isopropanol or 23% PBS was added, and samples were vigorously vortexed, followed by a 5-min incubation at room temperature. Immediately before sampling, each culture was again vortexed for 5 s, and samples were serially diluted between 10- and 1000-fold in 7.5% Tween 80 in PBS (v/v) to inactivate alcohol killing and to give a countable number of colonies on each plate (*46*). An automatic spiral plater (Don Whitley) was used to plate 50 μl of aliquots of an appropriate dilution of each strain in triplicate onto BHI agar plates. Plates were incubated overnight at 37°C, and colonies were counted using an aCOLyte 3 colony counter (Synbiosis). The limit of detection with this technique was 6000 CFU/ml. For later isopropanol tolerance experiments with mutants, the above killing assay was varied slightly such that 1 ml of 32.5% (v/v) (final concentration of 25%, v/v) isopropanol was added to 300 μl of cells, equating to an OD_{600nm} of 1.66 (~8×10^7 CFU/ml). These experiments were conducted as described above, except that spot plates (10 μl of dilutions in triplicate) were conducted instead of spiral plating and additional sampling points were added (10 and 15 min). Biological replicates were performed for each isolate, and average CFU values for cultures exposed to isopropanol and those exposed to PBS (as a control) were obtained. From these data, a mean $\log_{10}$ CFU reduction was calculated for each isolate by subtracting the $\log_{10}$ CFU remaining after exposure to isopropanol from the mean $\log_{10}$ CFU of cultures treated with PBS. Differences in population means for *E. faecium* isopropanol tolerance were explored using a Mann-Whitney test with a two-tailed *P* value. The null hypothesis (no difference between sample means) was rejected for $P < 0.05$.

Growth assays in the presence of isopropanol were performed as follows. Single colonies of *E. faecium* were grown in BHI medium overnight at 37°C with shaking. The bacterial cell culture concentration was then standardized to an OD_{600nm} of 3.5. Cells were diluted 10-fold in BHI, and 10 μl was inoculated into 190 μl of BHI broth with or without 3% (v/v) isopropanol. Cells were dispensed in 96-well plates and incubated at 37°C with agitation, and the OD_{600nm} was measured every 10 min over 24 hours using an EnSight Multimode Plate Reader. The maximum doubling time was determined by fitting local regression over intervals of 1 hour on growth curve data points and by taking the maximum value of the fitted derivative using the R package cell growth (www.bioconductor.org/packages/release/bioc/html/cellGrowth.html). The growth rate for each bacterial strain was determined from a minimum of three technical replicates for at least three biological triplicate experiments.

Mouse models of *E. faecium* gut colonization

Animal experimentation adhered to the Australian National Health and Medical Research Council *Code for the Care and Use of Animals for Scientific Purposes* and was approved by and performed in accordance with the University of Melbourne Animal Ethics Committee (application no. 1413341.3). Female BALB/c mice (6 to 8 weeks old) were used to develop the VREfm and VSE gut colonization models. For VREfm colonization, mice were provided drinking water ad libitum containing vancomycin (250 mg/liter) for 7 days before exposure. For VSE colonization, after dosing with vancomycin as above, mice were then provided drinking water with ampicillin (250 mg/liter) for a further 7 days. Before exposure of the animals to VREfm or VSE, fecal pellets were collected from each mouse to check their *E. faecium* status. Briefly, at least two fecal pellets from each mouse were collected and cultured in 10 ml of tryptone soy broth (TSB) at 37°C with agitation overnight. The cultured broths were then inoculated onto VRE-chrome agar plates for VREfm screening or Enterococcosel Agar plates for enterococci screening. After 1 week of antibiotic pretreatment, the mice were dosed by oral gavage with a 200-μl volume of *E. faecium*. The bacteria were prepared by culturing overnight in TSB at 37°C with shaking. Bacteria were harvested by centrifugation, washed three times with sterile distilled water, and diluted in sterile distilled water as required before use.

The *E. faecium* cage cross-contamination assays were performed in a blinded manner. Bacterial suspensions prepared as described above were diluted in sterile distilled water to ~1×10^6 CFU/ml. This dose was chosen because it provided both sufficient bacteria to survive the cleaning intervention and a density of *E. faecium* consistent with reports from environmental surveys of health care settings (*20*). Each cage floor was then completely flooded with 10 ml of the diluted *E. faecium* suspension. Seven milliliters of the suspension was removed from the inundated cage floor. The contaminated cages were left in the biosafety cabinet for 1.5 hours to dry under laminar air flow. The dried cage floors (150 mm × 300 mm) were wiped with 40 mm × 40 mm sterile filter paper soaked in either 850 μl of freshly prepared 70% (v/v) isopropanol or 850 μl of water in a consistent manner, with 8 vertical wipes and 24 horizontal wipes in one direction using the same surface of the filter. Each wiping movement partially overlapped the previous one. After the isopropanol or water cage floor treatment, six naïve mice were released into the cage for 1 hour. Each animal was then relocated to a fresh cage, singly housed, and provided with appropriate antibiotics in the drinking water. Fecal pellets were collected from each mouse after 7 days to check the *E. faecium* colonization status, as described above. After the 1-hour exposure of the mice to either water- or isopropanol-wiped cages and rehousing, the cage floors were flooded with 10 ml of PBS, and 3 ml was withdrawn to determine the remaining concentration of viable *E. faecium* by dilution plate count. The CD_{50} values were calculated by interpolation using the nonlinear regression and curve-fitting functions in GraphPad Prism (v7.0b).

Whole-genome sequencing and bioinformatics analyses

Twenty-two isolates examined in the current study have been sequenced previously (*47*–*49*). Genomic analysis and comparisons were performed using established bioinformatics methods that involved assessing *E. faecium* population structure and defining core and accessory genomes. Whole-genome DNA sequences were obtained using either the Illumina HiSeq or MiSeq platforms, with library preparation using Nextera XT (Illumina Inc.). Resulting DNA sequence reads and existing sequence reads were analyzed as previously described to define a core genome by aligning reads to the 2,888,087-bp ST796 reference chromosome (GenBank accession no. NZ_LT598663.1) (*50*) using Snippy v3.1 (https://github.com/tseemann/snippy). The resulting nucleotide multiple alignment file was used as an input for Bayesian analysis of population structure using hierBAPS v6.0 (*51*) and phylogenetic inference using RAxML v8.2.11 (*52*). Whole-genome alignments generated by Snippy were used for subsequent assessment of recombination using ClonalFrameML (*53*) with filtering as described (*50*). Pairwise SNP differences were calculated using a custom R script (https://github.com/MDU-PHL/pairwise_snp_differences). Genomes for each isolate were also assembled de novo using Velvet v1.20.10 (*54*), with the resulting contigs annotated with Prokka v1.10 (*55*). A pan-genome was generated by clustering the translated coding sequences predicted by Prokka using Proteinortho (*56*) and visualized with Fripan (http://drpowell.github.io/FriPan/).

To identify potentially causative variants while reducing the impact of lineage specific effects, pairs of *E. faecium* isolates that exhibited greater than 1.5-fold alcohol tolerance difference and less than 1000 core genome SNP differences (before recombination filtering) were examined. With these criteria, 19 pairs were identified across the 129 isolates. Separate core genome comparisons were undertaken for each the pair using Snippy. The resulting GFF files of each within-pair comparison were intersected using bedtools v2.26.0 (*57*) and inspected on the Ef_aus0233 chromosome in Geneious Pro (version 8.1.8, Biomatters Ltd.; www.geneious.com).

The potential role of gene content variation in the alcohol tolerant phenotype was examined by using a supervised probabilistic approach to assess the contributions of gene presence/absence at separating between sensitive and tolerant isolates. Here, an alignment of accessory genome orthologs was used as input for the generation of a DAPC model using the R package adegenet v2.0.1 (*58*). DAPC is a linear discriminant analysis that accommodates discrete genetic-based predictors by transforming the genetic data into continuous PC and building predictive classification models. The PCs are used to build discriminant functions under the constraint that they must minimize within group variance and maximize variance between groups.

Allelic exchange mutagenesis in *E. faecium*

To delete a plasmid-encoded region encoding a PTS system (6.5 kb) and a symporter from the chromosome (1 kb), first deletion constructs were polymerase chain reaction (PCR)–amplified (Phusion polymerase, New England Biolabs) from Ef_aus0233 genomic DNA (tables S4 and S5). The construct included 1 kb of DNA up/downstream of the region to be deleted and was joined by Splice Overlap Extension–PCR. Gel-extracted amplimers were cloned into pIMAY-Z (*59*) by SLiCE (*60*). Electrocompetent cells of Ef_aus0233 were made using the method of Zhang *et al.* (*61*). Purified plasmid (1 μg) was electroporated into cells, and the cells were selected on BHI agar containing chloramphenicol (10 μg/ml) at 30°C for 2 to 3 days. Allelic exchange was conducted as described (*59*), except that cells were single colony–purified twice preintegration (30°C) and postintegration (37°C). *E. faecium* exhibit intrinsic β-galactosidase activity, however cells containing pIMAY-Z could nonetheless be differentiated from pIMAY-Z–cured cells after 24 hours at 37°C. To repair the symporter deletion mutant, the wild-type allele for the symporter (amplified with the A/D primers and cloned into pIMAY-Z) was recombined into the

symporter deletion mutant. All mutants and complemented strains were subjected to whole-genome sequencing to ensure that no secondary mutations cofounded the analysis using Snippy (see above) and ISMapper to identify unintended SNP/indel and insertion sequence changes, respectively (*62*).

Isolation of spontaneous *rpoB* mutants in Ef_aus0233

An overnight BHI culture of Ef_aus0233 was concentrated 10-fold, and 100 µl was spread plated onto BHI agar containing rifampicin (200 µg/ml). A total of three potential *rpoB* mutants were screened by Etest for stable rifampicin resistance. All were resistant to above rifampicin (32 µg/ml). The strains were subjected to whole-genome sequencing, and single mutations were identified in the *rpoB* gene with one mapping to amino acid position 486 (Ef_aus0233 annotation) representing the H486Y substitution.

Statistical methods

Unless otherwise stated, the null hypothesis (no difference between means) was rejected for $P < 0.05$ and was assessed using an unpaired Mann-Whitney test with a two-tailed P value. Curve fitting and interpolation were achieved using nonlinear regression. Analyses were performed using GraphPad Prism (v7.0b).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/10/452/eaar6115/DC1
Fig. S1. Tolerance of *E. faecium* to ethanol exposure.
Fig. S2. Isopropanol tolerance phenotype summary for 129 genome-sequenced *E. faecium* isolates.
Fig. S3. Core and pan-genome analysis of 129 *E. faecium* genomes.
Fig. S4. Growth curves of mutants.
Table S1. Summary of *E. faecium* strains used in this study, with associated isopropanol tolerance data.
Table S2. Pairwise comparisons of high-low alcohol-tolerant *E. faecium*.
Table S3. DAPC analysis based on ortholog comparisons versus alcohol tolerance.
Table S4. Oligonucleotides used in this study.
Table S5. Bacterial strains and plasmids used for *E. faecium* mutagenesis.
Table S6. Data for *E. faecium* CD_{50} experiments.
Table S7. Data for *E. faecium* density (CFU/cm^2) on the cage floor pre- and posttreatments.
Table S8. Data for mice colonized by *E. faecium* after either water or alcohol cage floor disinfection.
Table S9. Data for *E. faecium* (CFU/ml) remaining over time after exposure to isopropanol.
Table S10. Data for change in doubling times of *E. faecium* mutants grown in the presence of isopropanol.

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Increasing tolerance of hospital *Enterococcus faecium* to handwash alcohols

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Alcohol loses its luster

Alcohol-based disinfectants are a key way to control hospital infections worldwide. Pidot *et al.* now show that the multidrug-resistant bacterium *Enterococcus faecium* has become increasingly tolerant to the alcohols in widely used hospital disinfectants such as hand rub solutions. These findings may help explain the recent increase in this pathogen in hospital settings. A global response to *E. faecium* will need to include consideration of its adaptive responses not only to antibiotics but also to alcohols and the other active agents in disinfectant solutions that have become so critical for effective infection control.