

1 **Title: Increasing tolerance of hospital *Enterococcus faecium* to hand-wash**  
2 **alcohols**

3

4 **Authors:** Sacha J. Pidot<sup>1,#</sup>, Wei Gao<sup>1,#</sup>, Andrew H. Buultjens<sup>1,#</sup>, Ian R. Monk<sup>1</sup>, Romain Guerillot<sup>1</sup>,  
5 Glen P. Carter<sup>1</sup>, Jean Y. H. Lee<sup>1</sup>, Margaret M. C. Lam<sup>1</sup>, M. Lindsay Grayson<sup>2</sup>, Susan A. Ballard<sup>3</sup>,  
6 Andrew A. Mahony<sup>2</sup>, Elizabeth A. Grabsch<sup>2</sup>, Despina Kotsanas<sup>4</sup>, Tony M. Korman<sup>4</sup>, Geoffrey W.  
7 Coombs<sup>5,6</sup>, J. Owen Robinson<sup>5,6</sup>, Anders Gonçalves da Silva<sup>5</sup>, Torsten Seemann<sup>7</sup>, Benjamin P.  
8 Howden<sup>1,2,3,8</sup>, Paul D. R. Johnson<sup>1,2,8,\*</sup> and Timothy P. Stinear<sup>1,\*</sup>

9

10 **Affiliations:**

11 <sup>1</sup> Department of Microbiology and Immunology, The Doherty Institute for Infection and Immunity, University of  
12 Melbourne, Victoria, 3010, Australia.

13 <sup>2</sup> Infectious Diseases Department, Austin Health, Heidelberg, Victoria, 3084, Australia.

14 <sup>3</sup> Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, The  
15 Doherty Institute for Infection and Immunity, University of Melbourne, Victoria, 3010, Australia.

16 <sup>4</sup> Monash Infectious Diseases, Monash Health, Clayton, Victoria, Australia, Victoria, 3168, Australia

17 <sup>5</sup> Antimicrobial Resistance and Infectious Diseases (AMRID) Research Laboratory, School of Veterinary and Life  
18 Sciences, Murdoch University, Murdoch, Western Australia, 6150, Australia.

19 <sup>6</sup> Department of Microbiology, PathWest Laboratory Medicine-WA, Fiona Stanley Hospital, Murdoch, Western  
20 Australia, 6150, Australia.

21 <sup>7</sup> Melbourne Bioinformatics, University of Melbourne, Carlton, Victoria, 3053, Australia.

22 <sup>8</sup> Department of Medicine, University of Melbourne, Heidelberg, Victoria, 3084, Australia.

23

24 # Contributed equally

25 \*Correspondence: [tstinear@unimelb.edu.au](mailto:tstinear@unimelb.edu.au); [paul.johnson@austin.org.au](mailto:paul.johnson@austin.org.au)

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27 Short title: Alcohol tolerance of *Enterococcus faecium*

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29

30 **Abstract:**

31 Alcohol-based hand rubs are international pillars of hospital infection control, restricting  
32 transmission of pathogens such as *Staphylococcus aureus*. Despite this success, health care  
33 infections caused by *Enterococcus faecium* (Efm) - another multidrug resistant pathogen - are  
34 increasing. We tested alcohol tolerance of 139 hospital Efm isolates, obtained between 1997 and  
35 2015 and found Efm post-2010 were 10-fold more tolerant to alcohol killing than older isolates.  
36 Using a mouse infection control model, we then showed that alcohol tolerant Efm resisted standard  
37 70% isopropanol surface disinfection and led to gastrointestinal colonization significantly more  
38 often than alcohol sensitive Efm. We next looked for bacterial genomic signatures of adaptation.  
39 Tolerant Efm have independently accumulated mutations modifying genes involved in carbohydrate  
40 uptake and metabolism. Mutagenesis confirmed their roles in isopropanol tolerance. These findings  
41 suggest bacterial adaptation and complicate infection control recommendations. Additional policies  
42 and procedures to prevent Efm spread are required.

43 [146 words]

44

45 **Introduction:**

46 Enterococci are members of the gastrointestinal microbiota with low virulence but they have  
47 nevertheless emerged as a major cause of healthcare associated infections (1). Enterococci now  
48 account for approximately 10% of hospital acquired bacteraemia cases globally, and they are the  
49 fourth and fifth leading cause of sepsis in North America and Europe respectively (2). Hospital  
50 acquired enterococcal infections are difficult to treat because of their intrinsic and acquired  
51 resistance to many classes of antibiotics (3). The difficulties associated with treatment, coupled with  
52 the risk of cross transmission to other patients, make enterococcal infections an increasingly  
53 important hospital infection control risk (4).

54

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

63

64 A recent Australia-wide survey demonstrated that Efm caused one third of bacteraemic  
65 enterococcal infections, and 90% of these were ampicillin-resistant clonal complex 17 strains, of  
66 which 50% were also vancomycin-resistant (8). Costs associated with the management of  
67 vancomycin resistant enterococci (VRE) colonised patients are high because of the need for  
68 isolation rooms, specialised cleaning regimes and the impact on staff, bed flow and other resources.  
69 Treatment of invasive VRE infections requires higher-cost antibiotics, with patients experiencing  
70 side effects and treatment failure due to further acquired resistance (8).

71

72 Alcohol-based hand rubs (ABHR) and associated hand hygiene programs are a mainstay of  
73 infection control strategies in healthcare facilities worldwide and their introduction is aligned with  
74 declines in some hospital-acquired infections, in particular those caused by hospital-adapted  
75 multidrug methicillin-resistant *Staphylococcus aureus* (MRSA). The compositions of hand hygiene  
76 solutions typically contain at least 70% (v/v) isopropyl or ethyl alcohol (9-11). The application of  
77 ABHR for 30 seconds has better disinfection efficacy than traditional approaches with soap and  
78 water, with greater than 3.5 log<sub>10</sub> reduction in bacterial counts considered effective (12). The  
79 presence of alcohol in ABHR is responsible for rapid bacterial killing at these concentrations  
80 although some species are capable of surviving alcohol exposure at lower concentrations (9, 13).  
81 The ability to withstand the addition of a certain percentage of alcohol is referred to as alcohol  
82 tolerance, and this phenomenon has been described across several genera (13, 14).

83

84 To control VRE many healthcare facilities perform active surveillance cultures (ASC) on all  
85 patients and then employ contact precautions that involve the use of gowns, gloves and single room  
86 isolation for patients colonised (15). However, this approach is expensive and cumbersome,  
87 particularly when VRE endemicity is high. Due to the relatively low virulence of VRE, other  
88 facilities rely on standard precautions, predominantly ABHR usage, and only selectively perform  
89 ASC in high-risk areas such as Haematology and ICU (15). At Austin Health and Monash Medical  
90 Centre, two university teaching hospitals in Melbourne, Australia, patients are screened for VRE  
91 rectal colonization on-admission and weekly for all inpatients in defined high-risk clinical areas.  
92 VRE-colonized patients are cohorted and contact precautions (including strict adherence to ABHR  
93 guidelines) are used routinely (16).

94

95 In this current study, motivated by our observation that successive waves of new Efm clones that  
96 were driving increasing clinical infection, we commenced an investigation into the tolerance of  
97 more recent Efm isolates to the short chain alcohol (isopropyl alcohol) used in ABHR.

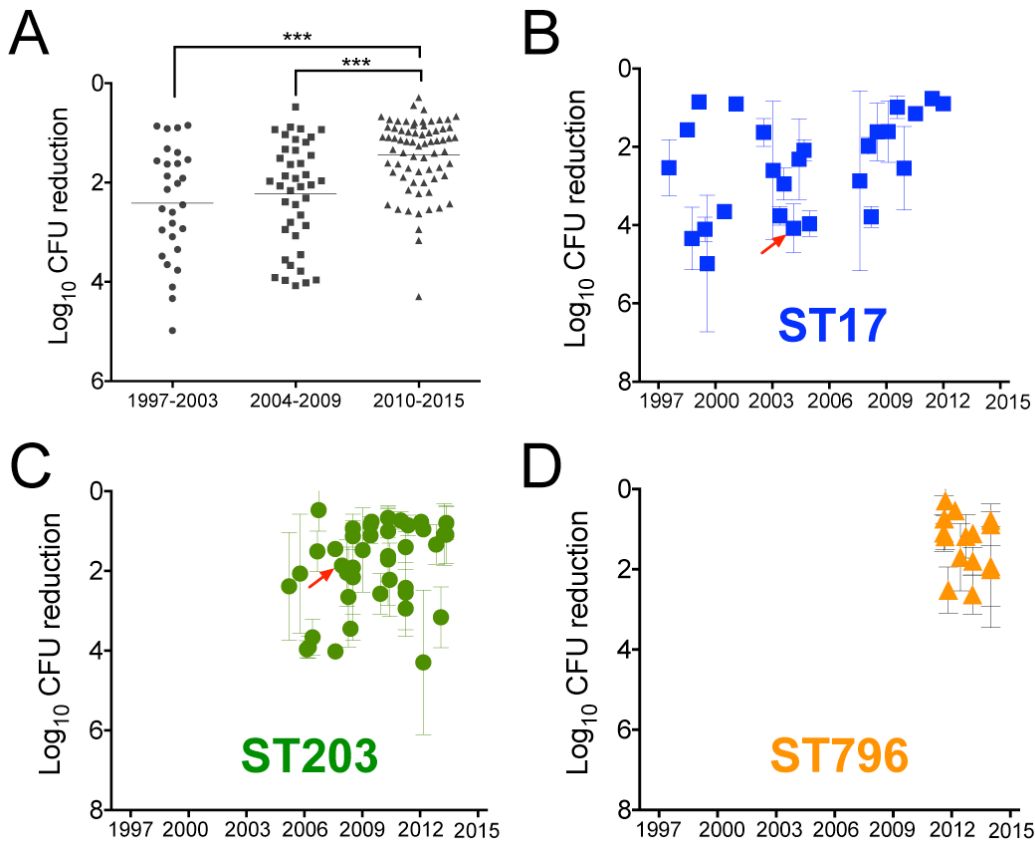
98

99 **Results:**

100 *Increasing isopropanol tolerance among hospital Efm isolates over time*

101 ABHR was systematically introduced to Australian health care facilities beginning at Austin Health  
102 in December 2002 (17-19). One consequence of this changed practice has been the substantial  
103 increase in the volume of ABHR products used by institutions. For instance, the volume of ABHR  
104 increased at Austin Health from 100L/month in 2001 to 1000L/month in 2015. We tested the  
105 hypothesis that Efm isolates are adapting to this changed environment and becoming more tolerant  
106 to alcohol exposure than earlier isolates. We assessed the isopropanol tolerance of 139 Efm isolates  
107 collected from two major Australian hospitals over 19 years. There was considerable variation in  
108 isopropanol tolerance, with a range of 4-7- $\log_{10}$  between isolates. These differences were  
109 independent of Efm sequence type (Data file S1), but we noticed that later isolates were more likely  
110 to be tolerant to isopropanol killing than earlier isolates (Fig. 1A), an observation that was  
111 supported by significantly different population mean tolerance when comparing pre-2004 with post-  
112 2009 isolates (0.97- $\log_{10}$  mean difference,  $p < 0.0001$ , Fig. 1A). There was genetic diversity among  
113 the Efm population across this time-period (discussed in more detail later) with two dominant CC17  
114 MLST types (ST17, and ST203) that each incrementally displayed increasing isopropanol tolerance  
115 (Fig. 1B,C). Isolates representing the most recently emerged clone (ST796) exhibited uniformly  
116 high isopropanol tolerance (n=16, median: 1.14- $\log_{10}$  reduction, Data file S1, Fig. 1D). There was  
117 no relationship between acquired vancomycin resistance and isopropanol tolerance. Exposure of a  
118 selection of Efm isolates to ethanol showed similar tolerance patterns as isopropanol, with ST796  
119 also significantly more ethanol tolerant compared to representatives of the other dominant Efm  
120 sequence types (fig. S1).

121



**Fig. 1:** Isopropanol tolerance phenotype variation among 139 *Efm* isolates over 18 years at two hospitals. (A) Changing isopropanol tolerance between 1998 and 2015. Plotted are the mean  $\log_{10}$  CFU reduction values for each *Efm* isolate obtained after exposure for 5 min to 23% isopropanol (v/v) plotted against specimen collection date and clustered in 5-6 year windows, showing significant tolerance increase of the population-mean over time. Un-paired Mann-Whitney test, two-tailed,  $p < 0.0001$ . Panels (B), (C), (D) show separately the mean  $\log_{10}$  CFU reduction values with range (at least biological triplicates) for each of the three dominant clones. The red arrows indicate isolates used in a previous hand-wash volunteer study (24).

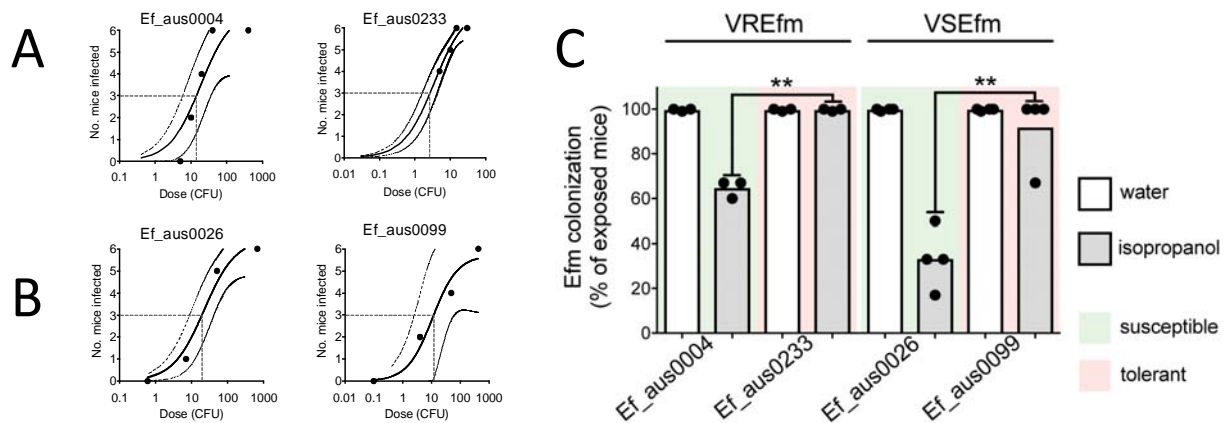
122

123 *Alcohol tolerance is a clinically relevant phenotype*

124 Our tolerance assay was based on exposure to 23% (v/v) isopropanol, as this concentration provided  
125 a discriminating dynamic range among the *Efm* isolates (refer methods). To assess the clinical  
126 relevance of these tolerance differences, we established an *Efm* contaminated surface transmission  
127 model, and compared the impact between two VRE $_{Efm}$  isolates on transmission of an intervention

128 using 70% isopropanol impregnated surface wipes of a contaminated surface. We employed a  
129 mouse gastrointestinal tract (GIT) colonisation model, first establishing that the colonising dose-50  
130 (CD50) among the four Efm isolates was not significantly different (Fig. 2). We selected a 2012,  
131 alcohol tolerant isolate (Ef\_aus0233, 0.45- $\log_{10}$ ) and a 1998, reduced tolerance isolate (Ef\_aus0004,  
132 4.34- $\log_{10}$ ). Groups of six BALB/c mice, pre-treated for seven days with vancomycin, were dosed  
133 by oral gavage with decreasing doses of each isolate. The CD50 for each isolate was low and not  
134 significantly different (Ef\_aus0004: 14 CFU, 95% CI 6-36 CFU, compared to Ef\_aus0233: 3 CFU,  
135 95% CI 1-6 CFU) (Fig. 2A, B). We then coated the floor of individually vented cages (IVC) with  
136 approximately  $3 \times 10^6$  CFU of each Efm isolate and subjected each cage to a defined disinfection  
137 regimen wiping with either water or a 70% v/v isopropanol solution. Groups of six BALB/c mice  
138 were then released into the treated IVCs for one hour, before being rehoused in individual cages for  
139 seven days and then screened for Efm gastrointestinal colonisation. Across three independent  
140 experiments, we then assessed the percentage of mice from each experiment colonised by Efm. The  
141 alcohol tolerant Efm isolate (Ef\_aus0233) was significantly better able to withstand the 70%  
142 isopropanol disinfection and colonise the mouse GIT than the more alcohol susceptible, Ef\_aus0004  
143 ( $p < 0.01$ , Fig. 3C). We then repeated the experiment, but this time using a pair of VSEfm isolates  
144 (Ef\_aus0026 and Ef\_aus0099) also with opposing alcohol tolerance phenotypes, but a much closer  
145 genetic association than the first pair (see below for selection criteria). Each isolate had a low CD50  
146 (Ef\_aus0026: 19 CFU, 95% CI 9-41 CFU, compared to Ef\_aus0099: 12 CFU, 95% CI 3-62 CFU)  
147 (Fig. 3B). Ef\_aus0099 was 4.4-fold more isopropanol tolerant than Ef\_aus0026 with a core genome  
148 difference of only 29 SNPs (Data file S2). Across four independent experiments, a significantly  
149 greater number mice were colonized by the isopropanol tolerant Efm isolate (Ef\_aus0099) than the  
150 more susceptible, Ef\_aus0026 ( $p < 0.01$ , Fig. 3C).

151



**Figure 2: Isopropanol tolerant *E. faecium* resist disinfection.** Transmission model of *E. faecium* using a murine gastrointestinal colonization model. (A) Establishing the colonizing dose-50 ( $CD_{50}$ ) for (A) VREfm and (B) VSEfm. Dotted lines indicate  $CD_{50}$ . (C) Contaminated cage-floor experiment. Depicted are the percentage of mice colonized with Efm after standardized cage-floor cleaning with either 70% isopropanol or sterile water. Isopropanol tolerant Efm are significantly more likely to be spread. Shown are the results of at least three independent experiments, based on six mice per experiment. The null hypothesis (no difference between sensitive vs tolerant isopropanol) was rejected for  $p < 0.05$ , unpaired *t*-test with Welch's correction.

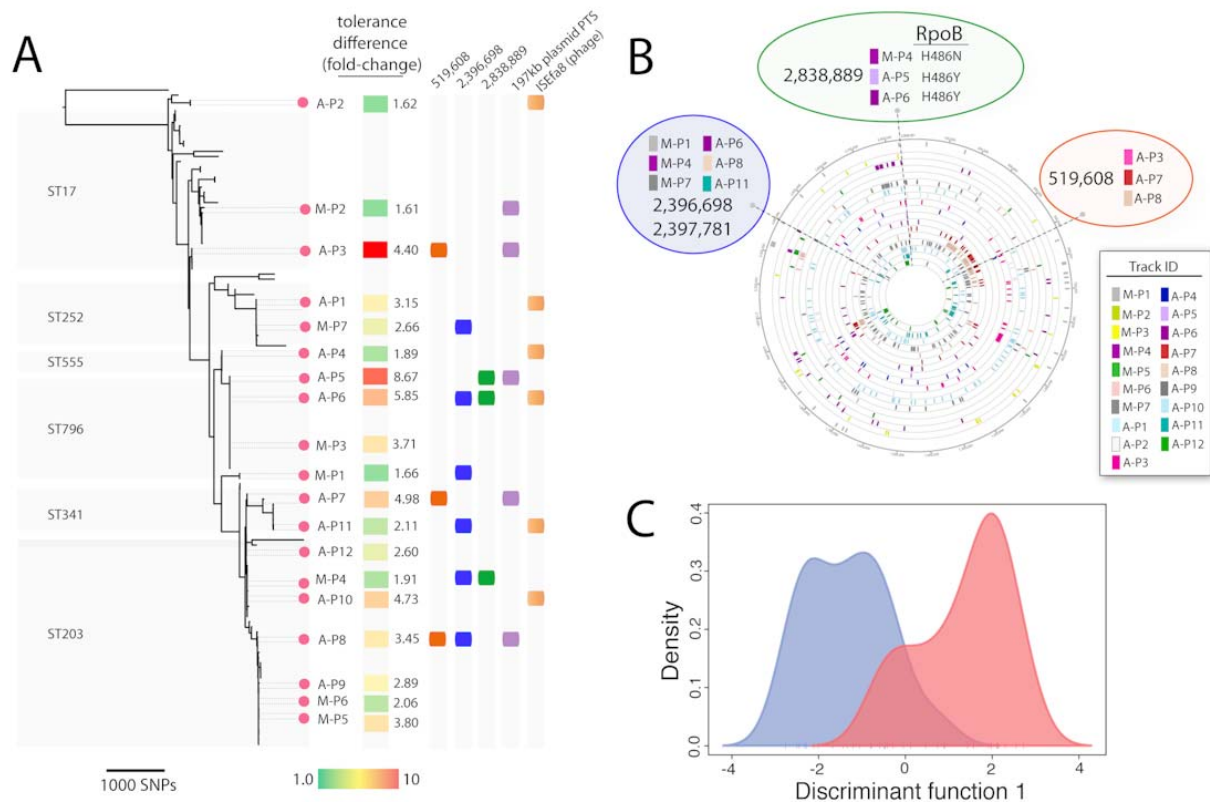
## 152 Population structure of Efm

153 To look for signatures of genetic adaptation that associated with alcohol tolerance we first  
 154 compared the genomes of 129 of the 139 Efm isolates (10 isolates failed sequencing). A high-  
 155 resolution SNP-based phylogeny was inferred from pairwise core-genome comparisons and  
 156 Bayesian analysis of population structure (BAPS) that stratified the population into seven distinct  
 157 genetic groups coinciding with previous MLST designations (Fig. 3A). The population had a  
 158 substantial pan-genome, comprising 8739 protein coding DNA sequences (CDS) clusters,  
 159 underscoring the extensive genetic diversity of this Efm population (fig. S2). There was also a  
 160 temporal pattern to the appearance of each genetic group. Beginning with the previously described  
 161 displacement of ST17 with ST203 in 2006 through to the emergence of ST796 in 2012, we  
 162 observed the introduction to the hospital at different times of distinct Efm clones, with each clone  
 163 exhibiting increasing alcohol tolerance (Fig. 1).



164 *Identifying bacterial genetic factors linked to alcohol tolerance*

165 High tolerance was observed within distinct Efm lineages, suggesting that multiple genetic events  
 166 have occurred leading to isopropanol tolerance (Fig. 3). We began the search for the genetic basis  
 167 of tolerance by evolutionary convergence analysis, to identify regions of the Efm genome that are  
 168 potentially harbouring genes or mutations linked to alcohol tolerance. We identified pairs of Efm



**Fig. 3: Population structure of *E. faecium* and identification of tolerant/sensitive pairs.** Alcohol tolerance is a polygenic trait. (A) Population structure of the 129 Efm isolates subjected to WGS and alcohol tolerance testing in this study. The phylogeny was inferred using maximum likelihood with RaxML and was based on pairwise alignments of 18,292 core genome SNPs against the *Ef\_aus0233* reference genome (filtered to remove recombination). Previous MLST designations are indicated. A heat map summary of the log<sub>10</sub> kill values is given for each taxon, with green least tolerant and red most tolerant. (B) Analysis of convergent SNP differences among phylogenetically-matched pairs. The prefix ‘A’ indicates Austin Hospital, ‘M’ indicates Monash Medical Centre. Three homoplastic mutations conserved in direction and presence among multiple pairs are highlighted and annotated. (C) Probabilistic separation of sensitive (blue) and tolerant (red) isolates according to a DAPC model built using accessory genome variation.

169 isolates that exhibited greater than 1.5-fold alcohol tolerance difference, and with less than 1,000  
170 core genome SNP differences. With these criteria, there were 19 pairs identified across the 129  
171 isolates (Fig. 3A, Data file S2).

172

173 We then searched for core genome mutations that occurred in different pairs but at the same  
174 chromosome nucleotide position and in the same direction of change (*i.e.* homoplasies). After  
175 applying these criteria, three loci were identified (Fig. 3B). One of these loci was the *rpoB* gene,  
176 encoding the beta subunit of RNA polymerase. The H486N/Y RpoB substitution seen in three pairs  
177 was associated with reduced alcohol tolerance (Fig. 3). Mutations in this region of *rpoB* are known  
178 to cause resistance to the antibiotic rifampicin, and it is exposure to this drug rather than an  
179 evolutionary response to alcohol, that likely selects these mutations. Nevertheless, the *rpoB*  
180 mutations serve as additional support for the approach and its capacity to detect homoplastic  
181 mutations associated with a changed alcohol tolerance phenotype. The two additional loci detected  
182 spanned an amino acid substitution in a putative symporter in three Efm pairs at chromosome  
183 position 519,608 and two mutations in six Efm pairs in a putative phage region (around position  
184 2,396,698) (Fig. 3A,B).

185

186 In addition to SNP variations, we also compared the presence/absence patterns of CDS between  
187 sensitive and tolerant Efm isolates in each of the 19 pairs. Here, we first used a supervised statistical  
188 learning approach called Discriminant Analysis of Principal Components (DAPC) to build a  
189 predictive model and identify CDS that contribute to the separation of pairs based on their  
190 isopropanol tolerance values. Using only the first 25 principal components, the model showed good  
191 separation of sensitive and tolerant isolates, with the resulting loading values used to guide the  
192 ranking of CDSs that associated with the alcohol tolerant phenotype (Data file S3). This analysis  
193 suggested that there is a genetic basis for the tolerance phenotype, with significant separation of the  
194 alcohol tolerant/sensitive populations (Fig. 3C). We then ranked CDS according to (i) their

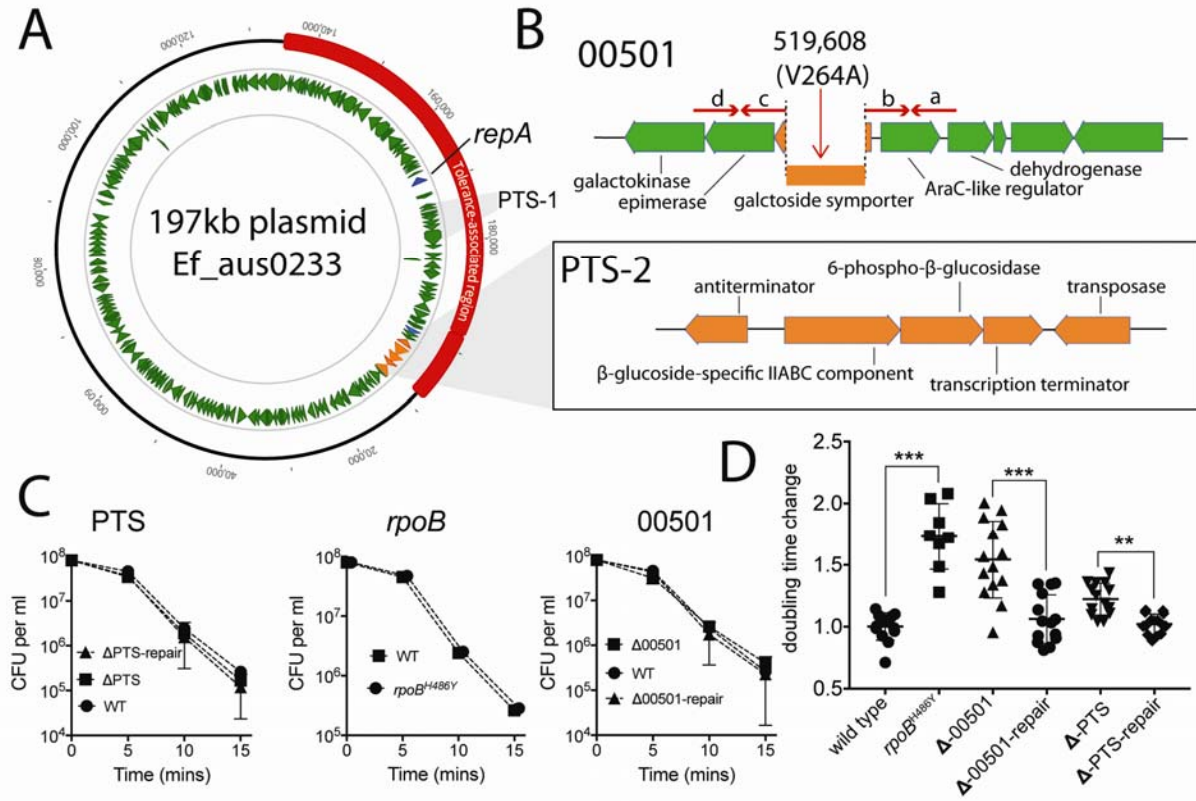
195 contribution to DAPC separation of the phenotypes, (ii) the frequency of CDS presence/absence  
196 among the 19 pairs and (iii) the direction of the CDS presence/absence (*i.e.* always present in  
197 tolerant isolates, Data file S3). This analysis identified two high-scoring loci, a copy of ISEfa8  
198 inserted adjacent to a putative prophage region around chromosome position 953,094 and a 70kb  
199 region of a 197kb plasmid that spans CDS encoding several hypothetical proteins, a predicted  
200 LPXTG-motif cell wall protein and two PTS systems, named PTS-1 and PTS-2 (Fig. 4A).

201

### 202 *Validation of bacterial genetic factors linked to alcohol tolerance*

203 To test the validity of the predictions based on convergence analysis and DAPC, we used allelic  
204 exchange to make targeted mutants in the isopropanol tolerant ST796 reference isolate,  
205 Ef\_aus0233. Given the reported role of PTS systems in solvent tolerance (20), we focused first on  
206 one of the plasmid associated PTS regions, deleting a 6.5kb region of PTS-2, a putative glucoside-  
207 specific PTS (Fig. 4A). We also made a deletion mutant of the CDS (locus\_tag 00501) encoding a  
208 putative galactoside symporter (Fig. 4B), where there was a specific V264A aa substitution  
209 associated with isopropanol tolerance. An *rpoB* mutant (H486Y) was also made, as this locus was  
210 also identified in the genome convergence analysis and so should present an altered tolerance  
211 phenotype. An absence of unintended second-site mutations was confirmed by WGS, and the PTS  
212 and 00501 mutations were also repaired. Screening these three mutants and their repaired versions  
213 by our isopropanol exposure killing assay showed no change in tolerance (Fig. 4C). To further  
214 explore the sensitivity of the two mutants to isopropanol we also conducted growth curve assays in  
215 the presence of 3% isopropanol, a concentration we determined provided useful discrimination  
216 among our Efm collection. All mutants showed significant increases in their doubling time  
217 compared to wild type, a phenotype restored in the repaired mutants (Fig. 4C). The mutants showed  
218 no growth defect in the absence of isopropanol (fig. S3). These experiments confirm predictions  
219 from convergence testing and DAPC that these loci are involved in promoting isopropanol  
220 tolerance. Loss of individual loci however did not impact sensitivity to isopropanol killing,

221 suggesting that isopropanol tolerance is a polygenic phenotype, with multiple genetic changes  
 222 across different loci likely to have occurred in tolerant Efm strains.  
 223



**Fig. 4.** Functional confirmation of genes associated with isopropanol tolerance. (A) Map of the 197kb Efm plasmid, showing the 70kb region associated with isopropanol tolerance (red) and the two PTS loci, including 6.3kb PTS-2 locus (orange) deleted by allelic exchange in the ST796 reference strain Ef\_aus0233. (B) Layout of the region around Ef\_aus0233 chromosome position 519,608, showing the region deleted by allelic exchange in 00501, encoding a putative symporter. Red arrows indicate position of primers used to generate the recombination substrate for mutant construction (Primer positions: a: 521,394 - 521,371; b: 520,396 - 520,420; c: 518,946 - 518,918; d: 517,961 - 517,985). (C) Impact of the mutations on Ef\_aus0233 to isopropanol exposure. Shown are the means and SD for biological triplicate experiments, with no difference between mutants and wild type. (D) Growth phenotypes in the presence of 3% (v/v) isopropanol of the 197kb glucoside  $\Delta$ PTS locus, *rpoB*<sup>H486Y</sup> and  $\Delta$ 00501-symporter mutants. Shown is fold change difference in doubling time for each mutant compared to WT. Depicted also are the phenotypes relative to WT of the repaired mutants. The null hypothesis (no difference between mean doubling time differences of mutant and repaired mutant or WT) was rejected for  $p < 0.01$ , unpaired Mann-Whitney test. Error bars depict standard deviation. All data points are shown for at least 3x biological duplicates and minimum of 3x technical replicates for each condition.

## 224 Discussion

225 In 2005, we published a 3-year study describing a progressive decline in rates of hospital  
226 acquired methicillin resistant *S. aureus* and Gram-negative infections following the introduction  
227 and promotion of ABHR (21). Similar programs were progressively rolled out to all major hospitals  
228 in Australia and compliance with ABHR is now a nationally reportable key performance indicator  
229 (19). The 2015 Australian National Hand Hygiene program report shows increasing and high  
230 (>80%) compliance rates in health care facilities across the country ([www.hha.org.au](http://www.hha.org.au)) and  
231 staphylococcal infection rates have declined nationally (18, 22). However, coincident with the  
232 introduction of ABHR and high compliance, there has been a paradoxical nationwide increase in  
233 VREfm and vancomycin susceptible *Enterococcus faecium* (VSEfm) infections (23).

234 In this study, we have shown recent clinical Efm isolates were significantly more alcohol-  
235 tolerant than their predecessors, and using our *in vitro* alcohol tolerance assay, date of isolation  
236 rather than genotype is a better predictor of Efm survival. To obtain a practical dynamic range and  
237 allow meaningful comparison between isolates, the tolerance assay used concentrations of alcohol  
238 lower than the usual 70% v/v of most ABHR products. However, with our mouse gut colonization  
239 model we were able to demonstrate that differences detected by this *in vitro* assay translated to an  
240 increased likelihood of transmission for tolerant strains when subject to a full 70% isopropanol  
241 surface disinfection intervention (Fig. 2). As tolerance increases, we hypothesise that there will be  
242 skin surfaces in contact with ABHR or inanimate surfaces in contact with other alcohol-based  
243 cleaning agents that do not receive the maximum biocide concentration or contact time required for  
244 effective bacterial killing. This idea is supported by our own previous clinical research using full  
245 concentration ABHR in 20 human volunteers and two strains of VREfm (one ST17, one ST203,  
246 Fig. 1B, C), and where we identified a mean 3.6-log<sub>10</sub> reduction in VREfm on the hands of test  
247 subjects, but very large inter-subject variance (24). For two volunteers, the reduction of VREfm was  
248 less than 1.6-log<sub>10</sub>, suggesting that some host factors might not only result in VREfm containment

249 failure (or even “super-spreading”), but also enhance the clinical likelihood for selection of Efm  
250 alcohol tolerance (24).

251       Until now there has been no assessment of alcohol tolerance in clinical *E. faecium*, but there  
252 has been growing interest in tolerance to other biocides such as chlorhexidine, a second active agent  
253 sometimes added to ABHR products (25, 26), including attempts to identify tolerance mechanisms  
254 through mutagenesis screens that have pinpointed a specific two-component regulator (27). Alcohol  
255 tolerance has been reported in other clinically relevant bacteria. For example, studies have reported  
256 the enhanced growth of *Acinetobacter baumannii* when exposed to low, non-lethal concentrations  
257 of alcohol and ABHR, and increased pathogenicity following the addition of ethanol (14, 28).

258       Research on alcohol tolerance mechanisms employed by enterococci is scant. Data in this  
259 field has been largely derived from studies of Gram-positive bacteria associated with spoilage of  
260 sake, in particular the lactic acid bacteria that are known to survive and grow in ethanol  
261 concentrations of greater than 18% (v/v) (29). The increase in tolerance over time displayed by  
262 isolates in our study is consistent with the accumulation of mutations and genes that have shifted  
263 the phenotype. Stepwise alcohol adaptation has been observed in laboratory experiments with a  
264 related Gram-positive bacterium, *Clostridium thermocellum*, that eventually tolerated up to 8%  
265 (w/v) ethanol (30). For bacteria in general, short chain alcohols such as ethanol and isopropanol are  
266 thought to kill by disrupting membrane functions (31, 32). The penetration of ethanol into the  
267 hydrocarbon components of bacterial phospholipid bilayers causes the rapid release of intracellular  
268 components and disorganisation of membranes (33). Metabolic engineering of solvent tolerant  
269 bacteria has uncovered major mechanisms of tolerance, showing that membrane transporters are  
270 critically important (31). For solvents like ethanol and isopropanol, potassium and proton  
271 electrochemical membrane gradients are a general mechanism that enhances alcohol resistance  
272 tolerance (34).

273       Our phylogenetic convergence and DAPC analyses in genetically independent Efm  
274 populations identified changes in several genetic loci likely contributing to alcohol tolerance.

275 Specific mutagenesis for three regions confirmed these predictions, showing that multiple mutations  
276 are required and loci involved in carbohydrate transport and metabolism are likely under selection.  
277 No one mutation showed a change in a killing assay after exposure to 25% (v/v) isopropanol (Fig.  
278 4C), but significant differences were observed on growth rate in the presence of 3% (v/v)  
279 isopropanol. The CDS (00501) encodes a putative major facilitator superfamily (MFS) galactoside  
280 symporter and the SNP at position 519,608 (V264A) occurs within one of its 12 transmembrane  
281 regions (Fig. 4B). We speculate that mutations such as V264A might help alter the membrane  
282 proton gradient to favour a tolerant state (34). In Gram-negative bacteria, transport systems are  
283 known to be upregulated or required in response to exposure to short-chain alcohols (35, 36) and  
284 bacterial MFS transporters specifically such as 00501 are frequently identified in screens for  
285 proteins linked to increased solvent tolerance. However, the specific mechanisms by which they  
286 promote tolerance are not understood (31). The enrichment in alcohol tolerant Efm strains for PTS  
287 loci is also noteworthy (Fig. 4). PTS are bacterial phosphoenolpyruvate (PEP) carbohydrate  
288 PhosphoTransferase Systems (37). They catalyse the phosphorylation and transport of different  
289 carbohydrates into the bacterial cell. However, there is a growing understanding that their various  
290 regulatory roles are equally important as their sugar uptake functions (37). Interestingly, they have  
291 also been implicated in solvent and stress tolerance. In *E. coli*, up regulation of a mannose-specific  
292 PTS led to increased organic tolerance to n-hexane exposure (20) and in *E. faecalis* PTS loci appear  
293 to be important for survival against low pH and oxidative stresses (38). It's also noteworthy that  
294 PTS loci are enriched in health care-associated *E. faecium* lineages, with specific systems  
295 associated with GIT colonization, biofilm formation, and serum survival (39-42).

296 It is possible that the significant positive relationship between time and increasing alcohol  
297 tolerance we report here (Fig. 1) is a response of the bacteria to increased exposure to alcohols in  
298 ABHR and that the more tolerant strains are able to displace their less alcohol tolerant predecessors.  
299 However, it is also conceivable that Efm are responding to another factor. For instance, modified or  
300 acquired transport systems might be conferring acid tolerance, leading to improved survival during

301 passage through the gastrointestinal tract. Secondary phenotypes like alcohol tolerance are then co-  
302 selected, passenger phenomena, that together multiply the environmental hardiness of the pathogen.

303         Whatever the drivers, the development of alcohol tolerant strains of Efm has the potential to  
304 significantly undermine the effectiveness of ABHR-based standard precautions, and may partly  
305 explain the increase in VRE infection that is now widely reported in hospitals in Europe, Asia, the  
306 Americas and Australia. ABHR remains an important general primary defence against cross-  
307 transmission of most microbial and some viral pathogens in health care settings. In hospitals with  
308 endemic VRE, it would seem prudent to optimise adherence to ABHR protocols to ensure adequate  
309 exposure times and use of sufficient volumes of ABHR product each time a healthcare worker  
310 cleans their hands. In addition, consideration may need to be given to the use of various  
311 formulations of ABHR (e.g. foams and gels) since they are known to have variable (generally  
312 reduced) efficacy compared to solutions (43). Furthermore, extending active surveillance cultures  
313 outside high risk areas of the hospital and return to strict contact precautions during outbreaks with  
314 new emergent strains of VRE may be required to prevent widespread cross-contamination.

315

## 316 **Materials and Methods**

### 317 *Bacterial isolates.*

318 Data file S1 in the supplementary appendix lists the 139 Efm isolates investigated in this study that  
319 were randomly selected within each year from predominantly blood culture isolates obtained at the  
320 Austin Hospital and Monash Medical Centre between 1998 and 2015. Isolates were stored at -80°C  
321 in glycerol. Sixty-six of the isolates were vancomycin resistant (60 *vanB*-type, 6 *vanA*-type). Some  
322 of these isolates have been described in a previous study on the epidemiology of Efm at the hospital  
323 between 1998 and 2009 (16) and included recently emergent epidemic clones ST203 and ST796.  
324 Six ST341, one ST414 and four ST555 isolates from an Australian-wide enterococci sepsis  
325 screening program conducted by the Australian Group on Antimicrobial Resistance (AGAR) were  
326 also included, as they were noted emergent clones in other Australian states but were only rarely



327 isolated at our hospitals (44). Isolates were grown in on brain heart infusion (BHI) media at 37°C  
328 unless otherwise stated.

329

330 *Alcohol tolerance assays and analysis.*

331 In preliminary experiments, various concentrations of alcohol and Efm inoculum sizes were  
332 assessed. At ‘full strength’ isopropanol (70%), killing was complete and resulted in greater than 8-  
333 log<sub>10</sub> reductions in broth culture and an inability to detect differences between isolates. However, by  
334 lowering the alcohol concentration in a stepwise fashion, we were able to identify a dynamic range  
335 in which we observed marked differences in the time-kill curves between isolates. Guided by these  
336 experiments and published literature (45) we then measured Efm survival after exposure to 23.0%  
337 (v/v) isopropanol. Overnight cultures were grown in 10 mL of BHI medium (Difco, BD). After  
338 overnight growth, each strain was diluted to an optical density at 600 nm (OD<sub>600nm</sub>) value of 0.5  
339 using PBS. To 1 mL of the diluted culture, either 23% isopropanol (v/v) or 23% PBS was added  
340 and samples were vigorously vortexed, followed by a 5-min incubation at room temperature.  
341 Immediately prior to sampling, each culture was again vortexed for 5 seconds and samples were  
342 serially diluted between 10-1000 fold in 7.5% Tween80 in PBS (v/v) to inactivate alcohol killing  
343 and to give a countable number of colonies on each plate (46). An automatic spiral plater (Don  
344 Whitley) was used to plate 50 µl aliquots of an appropriate dilution of each strain in triplicate onto  
345 BHI agar plates. Plates were incubated overnight at 37°C and colonies were counted using an  
346 aCOLyte-3 colony counter (Synbiosis). The limit of detection with this technique was 6000  
347 CFU/ml. For later isopropanol tolerance experiments with mutants, the above killing assay was  
348 varied slightly such that that 1ml of 32.5% v/v (final concentration of 25% v/v) of isopropanol was  
349 added to 300 µL of cells equating to an OD<sub>600nm</sub> of 1.66 (~8x10<sup>7</sup> CFU/ml). These experiments  
350 were conducted as described above except spot plates (10µL of dilutions in triplicate) were  
351 conducted instead of spiral plating plus additional sampling points were added (10 min and 15 min).  
352 Biological replicates were performed for each isolate and average CFU values for cultures exposed

353 to isopropanol and those exposed to PBS (as a control) were obtained. From these data, a mean  
354  $\log_{10}$  CFU reduction was calculated for each isolate by subtracting the  $\log_{10}$  CFU remaining after  
355 exposure to isopropanol from the mean  $\log_{10}$  CFU of cultures treated with PBS. Differences in  
356 population means for Efm isopropanol tolerance were explored using a Mann-Whitney test with a  
357 two-tailed P-value. The null hypothesis (no difference between sample means) was rejected for  
358  $p < 0.05$ . Statistical analyses were performed using GraphPad Prism (v7.0b).

359

360 Growth assays in the presence of isopropanol were performed as follows. Single colonies of Efm  
361 were grown in BHI media overnight at 37°C with shaking. The bacterial cell culture concentration  
362 was then standardised to an optical density at 600 nm (OD600) of 3.5. Cells were diluted 10-fold in  
363 BHI and 10  $\mu$ L inoculated into 190  $\mu$ L of BHI broth with or without 3% (v/v) isopropanol. Cells  
364 were dispensed in 96-well plates, incubated at 37°C with agitation and the OD600 measured every  
365 10 min over 24 hours using EnSight™ Multimode Plate Reader. The maximum doubling time was  
366 determined by fitting local regression over intervals of 1 hour on growth curve data points and by  
367 taking the maximum value of the fitted derivative using the R package cellGrowth  
368 ([www.bioconductor.org/packages/release/bioc/html/cellGrowth.html](http://www.bioconductor.org/packages/release/bioc/html/cellGrowth.html)). The growth rate for each  
369 bacterial strain was determined from a minimum of three technical replicates for at least three  
370 biological triplicate experiments.

371

372 *Whole genome sequencing and bioinformatics analyses.*

373 Twenty-two of the isolates examined in the current study have been sequenced previously (47-49).  
374 Genomic analysis and comparisons were performed using established bioinformatics methods that  
375 involved assessing Efm population structure and defining core and accessory genomes. Whole  
376 genome DNA sequences were obtained using either the Illumina HiSeq or MiSeq platforms, with  
377 library preparation using Nextera XT (Illumina Inc). Resulting DNA sequence reads and existing  
378 sequence reads were analysed as previously described to define a core genome by aligning reads to

379 the 2,888,087 bp ST796 reference chromosome (Genbank: NZ\_LT598663.1) (50) using Snippy  
380 v3.1 (<https://github.com/tseemann/snippy>). The resulting nucleotide multiple alignment file was  
381 used as input for Bayesian analysis of population structure using hierBAPS v6.0 (51) and  
382 phylogenetic inference using RaxML v8.2.11 (52). Whole genome alignments generated by Snippy  
383 were used for subsequent assessment of recombination using ClonalFrameML (53). Pairwise SNP  
384 differences were calculated using a custom R script ([https://github.com/MDU-](https://github.com/MDU-PHL/pairwise_snp_differences)  
385 [PHL/pairwise\\_snp\\_differences](https://github.com/MDU-PHL/pairwise_snp_differences)). Genomes for each isolate were also assembled *de novo* using  
386 Velvet v1.20.10 (54), with the resulting contigs annotated with Prokka v1.10 (55). A pan-genome  
387 was generated by clustering the translated coding sequences predicted by Prokka using Proteinortho  
388 (56) and visualized with Fripan (<http://drpowell.github.io/FriPan/>).

389

390 In order to identify potentially causative variants while reducing the impact of lineage specific  
391 effects, pairs of Efm isolates that exhibited greater than 1.5-fold alcohol tolerance difference and  
392 less than 1,000 core genome SNP differences were examined. With these criteria, 19 pairs were  
393 identified across the 129 isolates. Separate core genome comparisons were undertaken for each the  
394 pair using Snippy. The resulting gff files of each within-pair comparison were intersected using  
395 bedtools v2.26.0 (57) and inspected on the Ef\_aus0233 chromosome in Geneious Pro (version  
396 8.1.8, Biomatters Ltd. [[www.geneious.com](http://www.geneious.com)]).

397

398 The potential role of gene content variation in the alcohol tolerant phenotype was examined by  
399 using a supervised probabilistic approach to assess the contributions of gene presence/absence at  
400 separating between sensitive and tolerant isolates. Here, an alignment of accessory genome  
401 orthologs was used as input for the generation of a Discriminant Analysis of Principal Component  
402 (DAPC) model using the R package adegenet v2.0.1 (58). DAPC is a linear discriminant analysis  
403 (LDA) that accommodates discrete genetic-based predictors by transforming the genetic data into  
404 continuous Principal Components (PC) and building predictive classification models. The PCs are

405 used to build discriminant functions (DF) under the constraint that they must minimize within group  
406 variance, and maximize variance between groups.

407

408 *Mouse models of Efm gut colonization.*

409 Animal experimentation adhered to the Australian National Health and Medical Research Council  
410 *Code for the Care and Use of Animals for Scientific Purposes* and was approved by and performed  
411 in accordance with the University of Melbourne AEC (Application: 1413341.3). Female Balb/C  
412 mice (6-8 weeks old) were used to develop the VREfm and VSEfm gut colonization models. For  
413 VREfm colonization, mice were provided drinking water *ad libitum* containing 250mg/L  
414 vancomycin for 7 days before VREfm exposure. For VSEfm colonization, after dosing with  
415 vancomycin as above, mice were then provided drinking water with ampicillin containing  
416 (250mg/L) for a further 7 days. Before exposure of the animals to VREfm or VSEfm, fecal pellets  
417 were collected from each mouse to check their Efm status. Briefly, at least two fecal pellets from  
418 each mouse were collected and cultured in 10 mL tryptone soy broths (TSB) in a 37-degree shaker  
419 for overnight. The cultured broths were then inoculated onto VRE-chrome agar plates for VREfm  
420 screening or enterococcosel agar plates for enterococci screening. After 1 week of antibiotic pre-  
421 treatment, the mice were dosed by oral gavage with a 200  $\mu$ L volume of Efm. The bacteria were  
422 prepared by culturing overnight in TSB at 37°C with shaking. Bacteria were harvested by  
423 centrifugation and washed 3x with sterile distilled water and diluted in sterile distilled water as  
424 required before use.

425

426 The Efm IVC cage cross-contamination assays were performed in a blinded manner. Bacterial  
427 suspensions prepared as described above, were normalized to an OD<sub>600</sub> 0.37 ( $\sim 1 \times 10^8$  CFU/mL) and  
428 diluted with sterile distilled water to  $\sim 1 \times 10^6$  CFU/mL. Each cage was then completely flooded with  
429 10 mL of diluted Efm suspension. Seven millilitres of the suspension were removed from the  
430 inundated cage floor. The contaminated cages were left in the biosafety cabinet for 1.5 hours to dry.

431 The dried cage floors (150 x 300mm) were wiped with 40 x 40mm sterile filter paper soaked in 850  
432  $\mu$ L of freshly prepared 70% (v/v) isopropanol in a consistent manner, with 8 vertical wipes and 24  
433 horizontal wipes in one direction using the same surface of the filter. Each wiping movement  
434 partially overlapped the previous. After the isopropanol cage floor treatment, six naïve mice were  
435 released into the cage for one hour. Each animal was then relocated to a fresh cage, singly housed  
436 and provided with appropriate antibiotics in the drinking water. Fecal pellets were collected from  
437 each mouse after 7 days to check the Efm colonization status as described above.  $CD_{50}$  values were  
438 calculated by interpolation using the non-linear regression and curve-fitting functions in GraphPad  
439 Prism (v7.0b).

440

#### 441 *Allelic exchange mutagenesis in Efm.*

442 To delete a plasmid encoded region encoding a PTS system (6.5kb) and a symporter from the  
443 chromosome (1kb), first deletion constructs were PCR amplified (Phusion polymerase - New  
444 England Bioabs) (Table S1) from Ef\_aus0233 genomic DNA. The construct included 1kb of DNA  
445 up/downstream of the region to be deleted and was joined by SOE-PCR. Gel extracted amplimers  
446 were cloned into pIMAY-Z (59) by SLiCE (60). Electrocompetent cells of Ef\_aus0233 were made  
447 using the method of Zhang *et al* (61). Purified plasmid (1  $\mu$ g) was electroporated, with cells  
448 selected on BHI agar containing chloramphenicol 10  $\mu$ g/ml at 30°C for 2-3 days. Allelic exchange  
449 was conducted as described (59) except cells were single colony purified twice pre (30°C) and post  
450 (37°C) integration. While Efm exhibit intrinsic beta-galactosidase activity, cells containing pIMAY-  
451 Z could be differentiated from pIMAY-Z cured cells after 24h at 37°C. To complement the  
452 symporter deletion mutant, the wild type allele for the symporter (amplified with the A/D primers  
453 and cloned into pIMAY-Z) was recombined into the symporter deletion mutant. All mutants and  
454 complemented strains were whole genome sequenced to ensure no secondary mutations cofounded  
455 the analysis.

456

457 *Isolation of spontaneous rpoB mutants in Ef\_aus0233.*

458 An overnight BHI culture of Ef\_aus0233 was concentrated 10-fold and 100 µL was spread plated  
459 onto BHI agar containing 200µg/ml of rifampicin. A total of three potential *rpoB* mutants were  
460 screened by Etest for stable rifampicin resistance. All were resistant to above 32 µg/mL rifampicin.  
461 The strains were subjected to whole genome sequencing and single mutations were identified in the  
462 *rpoB* gene with one mapping to aa position 481, representing the H481Y substitution.

463

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616

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622 MMCL, SAB conducted laboratory experiments. SAB, MLG, AAM, EAG, DK, TMK, GWC, JOR  
623 contributed isolates and obtained metadata. TPS, AHB, AGdS, TS performed bioinformatic  
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625 drafted the manuscript.

626 **Competing interests:** The authors have no competing interests to disclose.

627 **Data and materials availability:** DNA sequence reads are available from Genbank under study  
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#### 629 **SUPPLEMENTARY MATERIALS:**

630 **Fig. S1.** Tolerance of *E. faecium* to ethanol exposure

631 **Fig. S2.** Core and pan genome analysis of 129 *E. faecium* genomes

632 **Fig. S3.** Growth curves of mutants

633 **Table S1:** Oligonucleotides used in this study

634 **Data file S1:** Strain list

635 **Data file S2:** Pairwise comparisons of high-low alcohol tolerant *E. faecium*

636 **Data file S3:** DAPC analysis based on ortholog comparisons versus alcohol tolerance

637