Increasing tolerance of hospital *Enterococcus faecium* to hand-rub alcohols

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Running title: *Enterococcus faecium* alcohol tolerance

Summary: We observed increasing tolerance over time of the hospital pathogen *Enterococcus faecium* to the alcohols used in alcohol-based hand rubs (ABHR). The progressive gain in tolerance helps explain the failure of standard precautions that rely on ABHR to control cross-transmission.

Abstract:

Background: Alcohol-based hand rubs (ABHR) are a pillar of infection control, recommended by the World Health Organization. The introduction of ABHR and hand hygiene to Australian hospitals from 2002 has been associated with a decrease in *Staphylococcus aureus* infections. However, this decrease has been paralleled by a nationwide increase in vancomycin resistant *Enterococcus faecium* (VRE Efm) infections. Here, we tested the hypothesis that recent clinical isolates of Efm are more tolerant than stored historical isolates to the alcohols now used extensively in hospital hand hygiene programs.

Methods: We studied 77 stored clinical isolates of Efm, 66 randomly selected by year from our hospital over 18 years (1998-2015), and 11 obtained recently from other Australian hospitals with sequence types not encountered locally. We measured $\log_{10}$ reduction in colony forming units after five minutes exposure to isopropyl alcohol, stratified by year of isolation and multi-locus sequence type.

Results: There was a highly significant increase in Efm alcohol tolerance over time observed across all sequence types tested in this assay, with differences of up to 4.6 $\log_{10}$ kill at five minutes between invasive isolates from 1998 compared to those from 2015 ($\log_{10}$ reduction after alcohol exposure by year of isolation; Spearman Rank $r = 0.43$, $p < 0.0004$).

Conclusions: Recent isolates of Efm obtained from bloodstream infections were significantly more tolerant to alcohol than the earliest isolates tested. The progressive gain in alcohol tolerance partly explains the failure of standard precautions that rely on alcohol-based hand rubs to control VRE cross-transmission.

Introduction

Enterococcus faecium (*E. faecium*) has become a leading cause of hospital acquired blood stream infections (BSI) worldwide [1]. Most of these infections are due to hospital-adapted clonal-complex 17, clade A-1 *E. faecium* strains that are typically resistant to ampicillin, aminoglycosides and quinolones. [2] Hospital-adapted *E. faecium* may also become highly vancomycin resistant through the acquisition of mobile DNA elements harbouring *van* operons. US estimates of VRE colonization on hospital admission range from 10-14% [3]. A survey of European countries performed in 2014 reported that 8% of *E. faecium* isolates overall from patients with BSI were vancomycin-resistant. Contemporary surveys in the UK, Ireland and Australia revealed even higher incidences of vancomycin-resistance with rates of 21%, 45% and 51% respectively [4, 5].

In Australia there have been systematic improvements in health-care worker hand-hygiene compliance in hospitals since the introduction and promotion of alcohol-based hand-rubs (ABHR) under the guidance of Hand Hygiene Australia [6] and the Australian Commission on Safety and Quality in Health Care (ACSQHC) [7]. These programs have been linked to progressive reductions in hospital-acquired infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) [8] so it is somewhat of a paradox that Australia appears to now have a higher proportion of BSIs caused by vancomycin resistant *E. faecium* (VRE) than most other comparable countries [5, 9].

One possibility to explain this paradox is partial failure of universal precautions to prevent cross-transmission through the development of tolerance to alcohols widely used either in hand hygiene products or alcohol impregnated wipes used to clean shared equipment in hospital wards. [10] Although these vary between hospitals, most ABHR products contain 70% (v/v) isopropyl or ethyl alcohol [11-13]. Waterless disinfection with ABHR for 30 seconds has better efficacy and is much quicker than traditional approaches with soap and water, more than meeting the required 3·5 $\log_{10}$ reduction in bacterial counts considered to indicate effectiveness [13, 14]. The presence of high-concentration alcohol in ABHR is responsible for rapid killing of almost all bacterial pathogens. However, some bacteria are relatively resistant to alcohol at lower concentrations. This

phenomenon, referred to as alcohol tolerance, has been described across several bacterial genera [12, 15, 16]. In the current study, motivated by our observation that successive waves of new *E. faecium* clones were driving increased clinical infection in our hospital despite much improved health care worker hand hygiene compliance, we investigated tolerance to alcohol in clinical isolates of *E. faecium* collected at the Austin hospital, a University of Melbourne teaching hospital, over an 18-year period.

Materials and Methods

Bacterial isolates.

Table 1 lists the 66 *E. faecium* isolates investigated in this study that were randomly selected within each year from predominantly blood culture isolates obtained at the Austin Hospital between 1998 and 2015. Thirty-seven of the isolates were vancomycin resistant (31 *vanB*-type, 6 *vanA*-type) and included recently emergent epidemic clones ST203 [9] and ST796 [17]. Some of these isolates have been described in a previous study on the epidemiology of *E. faecium* at the hospital between 1998 and 2009 [9]. Isolates from other hospitals around Australia, representing different *E. faecium* outbreak genotypes but not seen frequently at the Austin Hospital, were included to investigate whether any changes we might observe were linked to particular STs or particular hospitals only. Therefore we included one *E. faecium* ST341, one ST414 and four ST555 isolates from an Australian-wide enterococci sepsis screening program conducted by the Australian Group on Antimicrobial Resistance (AGAR) [5].

Alcohol tolerance assays.

In preliminary experiments, various concentrations of alcohol and *E. faecium* inoculum sizes were assessed (data not shown). At 'full strength' isopropyl alcohol (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 [18] we selected 23.0% (v/v) isopropyl alcohol for the majority of experiments. Overnight cultures were grown at 37°C in 10 mL of BHI medium (Difco, BD). After overnight growth, each strain was diluted to an OD_{600nm} value of 0.5 using PBS. To 1 mL of the diluted culture, either 23% (v/v) isopropyl alcohol or PBS was added and samples were vigorously vortexed, followed by a 5-minute incubation at room temperature. Immediately prior to sampling, each culture was again vortexed for 5 seconds and samples were serially diluted between 10-1000 fold in 7.5% Tween80 in PBS (v/v) to inactivate alcohol killing and to give a countable number of colonies on each plate [19]. An automatic spiral plater (Don Whitley Scientific) was used to plate 50 ul 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.

Colonies were counted following overnight incubation at 37°C and an average colony forming units (CFU) value was calculated. Biological replicates were performed for each isolate and average CFU values for cultures exposed to isopropyl alcohol 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 5 minutes of exposure to isopropyl alcohol from the mean $\log_{10}$ CFU of cultures treated with PBS. Differences in population means for *E. faecium* isopropyl alcohol 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 $p<0.05$. The Spearman rank test with two-tailed P-value was used to assess the correlation between *E. faecium* isopropyl alcohol and tolerance isolation date. Statistical analyses were performed using GraphPad Prism (v6.0f).

Results:

Increasing isopropyl alcohol *tolerance among hospital E. faecium isolates over time*

We first assessed the isopropyl alcohol tolerance for the 66 Austin Hospital *E. faecium* isolates derived from blood cultures by measuring bacterial survival after exposure of 10^8 CFU to 23% (v/v) isopropyl alcohol for 5 minutes. There was considerable variation in survival, with a difference of up to 4·6-$\log_{10}$ between isolates. This difference was independent of sequence type but we noticed that isolates collected earlier in time were likely to be more sensitive to isopropyl alcohol killing than recent isolates, an observation that was supported by significantly different population mean tolerance when comparing pre-2006 with post-2009 isolates (1·2-$\log_{10}$ mean difference, $p<0·001$) (Figure 1A). Comparing all 66 isolates from 1998 to 2015 revealed a significant, continuous increase in isopropyl alcohol tolerance with time, again suggesting that hospital *E. faecium* populations might be under the same selective pressure (Figure 1B). The positive correlation between isolation date and resistance to isopropyl alcohol killing was highly statistically significant ($r=0·4250$, $p<0·0004$, Figure 1B). There was considerable genetic diversity among the *E. faecium* population across this time period with two dominant MLST types (ST17 and ST203) that each incrementally displayed increasing isopropyl alcohol tolerance (Figure 1C, 1D). Isolates representing the most recently emerged clone (ST796, a new ST first recognised in 2012) exhibited uniformly high isopropyl alcohol tolerance (n=10, median: 0·92-$\log_{10}$ reduction, Table S1, Figure 1E). Exposure of a selection of *E. faecium* isolates to ethyl alcohol showed similar tolerance patterns as isopropyl alcohol, with ST796 also significantly more ethyl alcohol tolerant compared to representatives of all other *E. faecium* sequence types (data not shown). There was no relationship between vancomycin resistance and isopropyl alcohol tolerance.

Isopropyl alcohol tolerance - a nationwide phenomenon

We obtained 11 *E. faecium* isolates representing other contemporary clone types (predominantly ST341 and ST555) causing hospital outbreaks in other Australian states [5] and found that these

isolates also exhibited significant alcohol tolerance, indistinguishable from the Austin Health *E. faecium* isolates obtained from the same period (Table 1, Figure 2).

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 following the introduction and promotion of alcohol based hand rubs (ABHR) [10]. Similar programs were progressively rolled out to all major hospitals in Australia and compliance with ABHR has become a nationally reportable key performance indicator [20]. In 2015 Hand Hygiene Australia reported >80% compliance in health care facilities across Australia (www.hha.org.au) and this program has been linked to a national reduction in hospital acquired methicillin resistant *Staphylococcus aureus* infections [8, 21]. However, coincident with the introduction of ABHR and steadily improving health care worker compliance, there has been a paradoxical nationwide increase in colonization and infection with vancomycin resistant enterococci in Australian hospitals, first observed from 2005 [22, 23]. At first we associated change in VRE epidemiology with the appearance of a new sequence type, ST203, which was identified initially as vancomycin sensitive *E. faecium* in blood cultures but then acquired *vanB* vancomycin resistance operons and caused a nationwide outbreak of VRE colonisation and infection [5, 9]. However ST203, while still present, is being pushed aside now by new waves of different *E. faecium* STs – ST555 in Western Australia (both vancomycin sensitive and resistant), ST796 in Victoria (virtually all vancomycin resistant *vanB*) [5], *vanA* ST203 and ST80 in Victoria and ST80 and ST17 *vanA* in New South Wales and Western Australia (unpublished). Rapid change in the *E. faecium* population can be partly explained by the inherent propensity for frequent recombination in *E. faecium* [24] [25] but other factors are likely to be driving this rapid evolution. As each new *E. faecium* ST appears we do not observe increased antibiotic resistance or increased likelihood of carriage of known virulence genes [5, 9, 25] yet Australia now has the highest proportion of VRE of any comparable country [5]. We wondered

whether this phenomenon - at least in part – could be explained by the acquisition of resistance or tolerance by *E. faecium* to the constituents of ABHR products.

In this study we have demonstrated that recent isolates of both vancomycin sensitive and resistant *E. faecium* that are causing contemporary hospital acquired blood stream infections are much more likely to be tolerant to alcohol at a concentration of 23% (v/v) than the earliest available isolates obtained 18 years ago. For example, one of our recent *E. faecium* BSI isolates from 2014, an ST796 *vanB* VRE, is more than 4-logs more tolerant than ST17 *E. faecium* BSI isolates obtained in 1998. Time of isolation is strongly linked to this phenomenon; it is not restricted to any particular sequence type or to isolates obtained from just one hospital or just one Australian state.

At present we have only established an association between time, increasing alcohol tolerance and increasing incidence of infection with *E. faecium*, particularly VRE, in Australian hospitals. We acknowledge that we have not established causation and may have discovered an epiphenomenon that is not itself involved in the increased rates of colonisation and infection. In the tolerance assay we implemented, concentrations of alcohols were set lower than the usual 70% v/v of most ABHR products to obtain a practical dynamic range for meaningful comparisons between isolates. However, in our own previous clinical research using full concentration ABHR in 20 human volunteers with two strains of *E. faecium* VRE (one ST17, one ST203, Figure 1C, D), we identified a mean 3·6-$\log_{10}$ reduction in vancomycin resistant *E. faecium* on the hands of test subjects, but the variance in these experiments was large. For two volunteers, the reduction of vancomycin resistant *E. faecium* was less than 1·6-$\log_{10}$, suggesting that some host factors might not only result in containment failure (or even “super-spreading”), but also enhance the clinical likelihood for selection of *E. faecium* alcohol tolerance [26]. As tolerance increases, we hypothesise that there will be skin surfaces in contact with ABHR or inanimate surfaces in contact with alcohol-based cleaning agents that do not receive the correct biocide concentration or contact time required for effective bacterial killing. Increasing tolerance to alcohol may allow some *E. faecium* cells to exploit suboptimal use of ABHR and alcohol impregnated wipes in hospitals. It is not hard to

imagine an increased survival advantage for bacteria transiently present at the edge of the "clean zone" when doctors quickly rub their hands with alcohol between patients or busy nurses incompletely wipe down a trolley with alcohol wipes between care episodes. We therefore propose that the simplest explanation for the positive association between time and increasing alcohol tolerance we report here (Figure 1B) is that the bacteria are responding to increased exposure to alcohols and that the more tolerant strains are able to displace their less alcohol tolerant predecessors. It is also possible that *E. faecium* are responding to another factor, and alcohol tolerance is a passenger phenotype. Nevertheless, regardless of whether links are direct or indirect, increasing tolerance has consequences for our approach to infection control.

For bacteria in general, short chain alcohols such as ethanol and isopropyl alcohol are thought to kill by disrupting membrane integrity [27]. The penetration of ethanol into the hydrocarbon components of bacterial phospholipid bilayers causes the rapid release of intracellular components and disorganisation of membranes [28]. There has been very little research on mechanisms employed by medically important organisms such as Enterococci to evade the effects of alcohol. Data in this field has been largely derived from studies of Gram-positive bacteria associated with spoilage of sake, in particular the lactic acid bacteria that are known to survive and grow in high ethanol concentrations of greater than 18% (v/v). [29] However, there are some examples of biocide tolerance and other biocide induced pleiotropic effects in medically important bacteria. These include the findings that growth and virulence in *Acinetobacter baumannii* is enhanced by exposure to low non-lethal concentrations of ABHR solution, [15, 30] and that sub-lethal exposure of VRE *E. faecium* to chlorhexidine increases expression of vancomycin resistance genes of the *vanA* but not the *vanB* genotype [31].

ABHR remains an important general primary defence against cross transmission of most microbial and some viral pathogens in hospitals. However, in hospitals with endemic vancomycin resistant enterococci, it would seem prudent to optimise adherence to ABHR protocols to ensure adequate exposure times and use of sufficient volumes of ABHR product each time healthcare

231 workers clean their hands. In addition, consideration may need to be given to the use of foam or gel
232 formulations of ABHR since they are known to have variable (generally reduced) efficacy
233 compared to liquid ABHR solutions [32]. It would also seem prudent to implement contact
234 precautions during outbreaks with new emergent strains of VRE as horizontal control measures
235 such as universal standard precautions based on ABHR do not appear to be effective in controlling
236 VRE in hospitals [17]. The underlying genetic determinants of alcohol tolerance in *E. faecium* and
237 whether we have identified causation or just association are important topics for future research.

238

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240

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243

244 **Conflicts of interest**: The authors have no conflicts to disclose.

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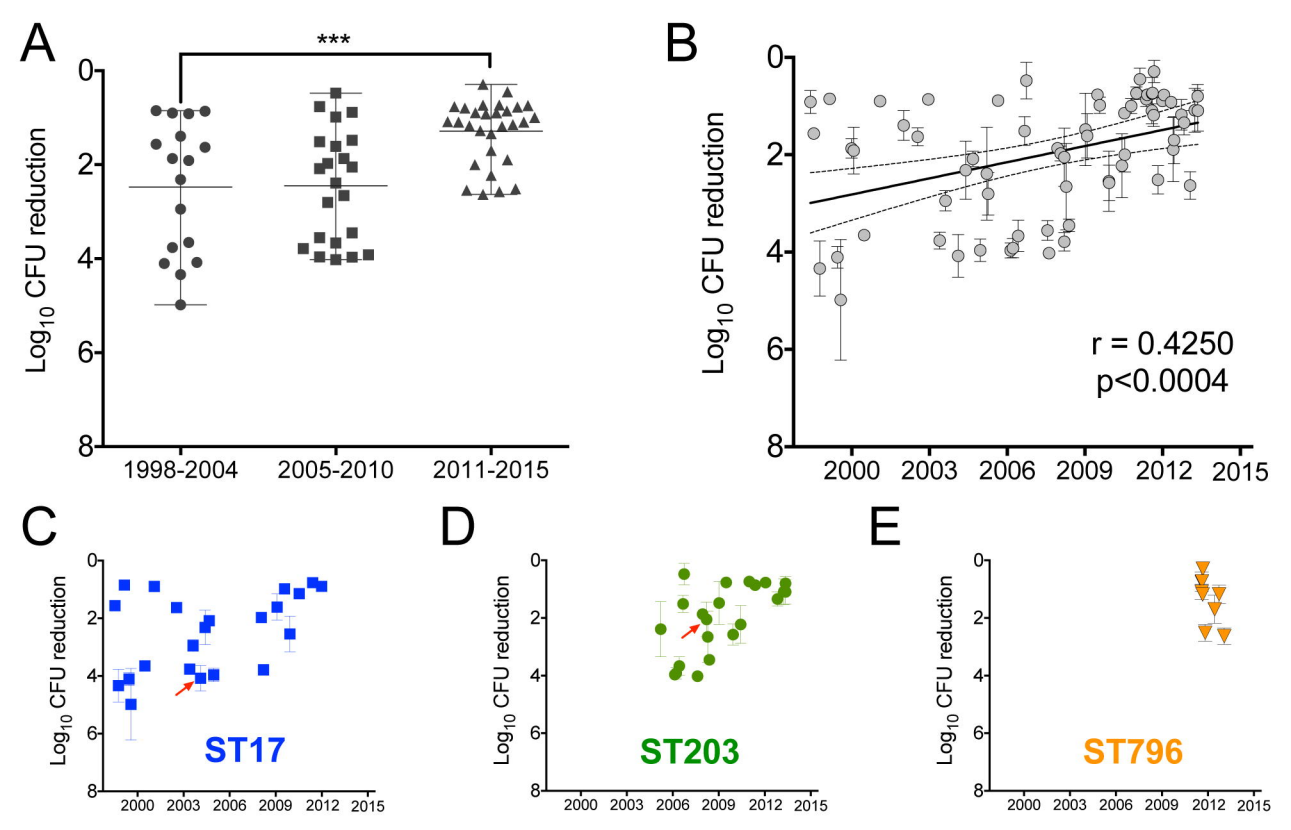
331 **Figure legends:**

332 **Fig. 1.** Single institution isopropyl alcohol tolerance phenotype variation among 66 *E. faecium*
333 isolates over 18 years. (A) Changing Isopropyl alcohol tolerance among the hospital *E. faecium*
334 population across three time periods between 1998 and 2015. Plotted are the mean $\log_{10}$ CFU
335 reduction values for each *E. faecium* isolate obtained after exposure for 5 min to 23% Isopropyl
336 alcohol (v/v), with population mean and range within each time period indicated. (B) Depicted also
337 are the mean $\log_{10}$ CFU reduction values with SEM for each *E. faecium* isolate plotted against
338 specimen collection date and highlighting the significant positive correlation of increasing isopropyl
339 alcohol tolerance with time. Trend line with 95% CI shown. Panels (C), (D), (E) show separately
340 the mean $\log_{10}$ CFU reduction values with SEM for each of the three dominant clones. The red
341 arrows indicate isolates used in a previous hand-rub volunteer study [26].

342

343 **Fig. 2.** Isopropyl alcohol tolerance among 11 non-Austin hospital *E. faecium* isolates. Plotted are
344 the mean $\log_{10}$ CFU reduction values for each *E. faecium* isolate obtained after exposure for 5 min
345 to 23% isopropyl alcohol (v/v), with population mean and range within each time period indicated.
346 A low and high tolerance *E. faecium* isolate were tested in parallel for comparison.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***
Log10 CFU reduction
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2
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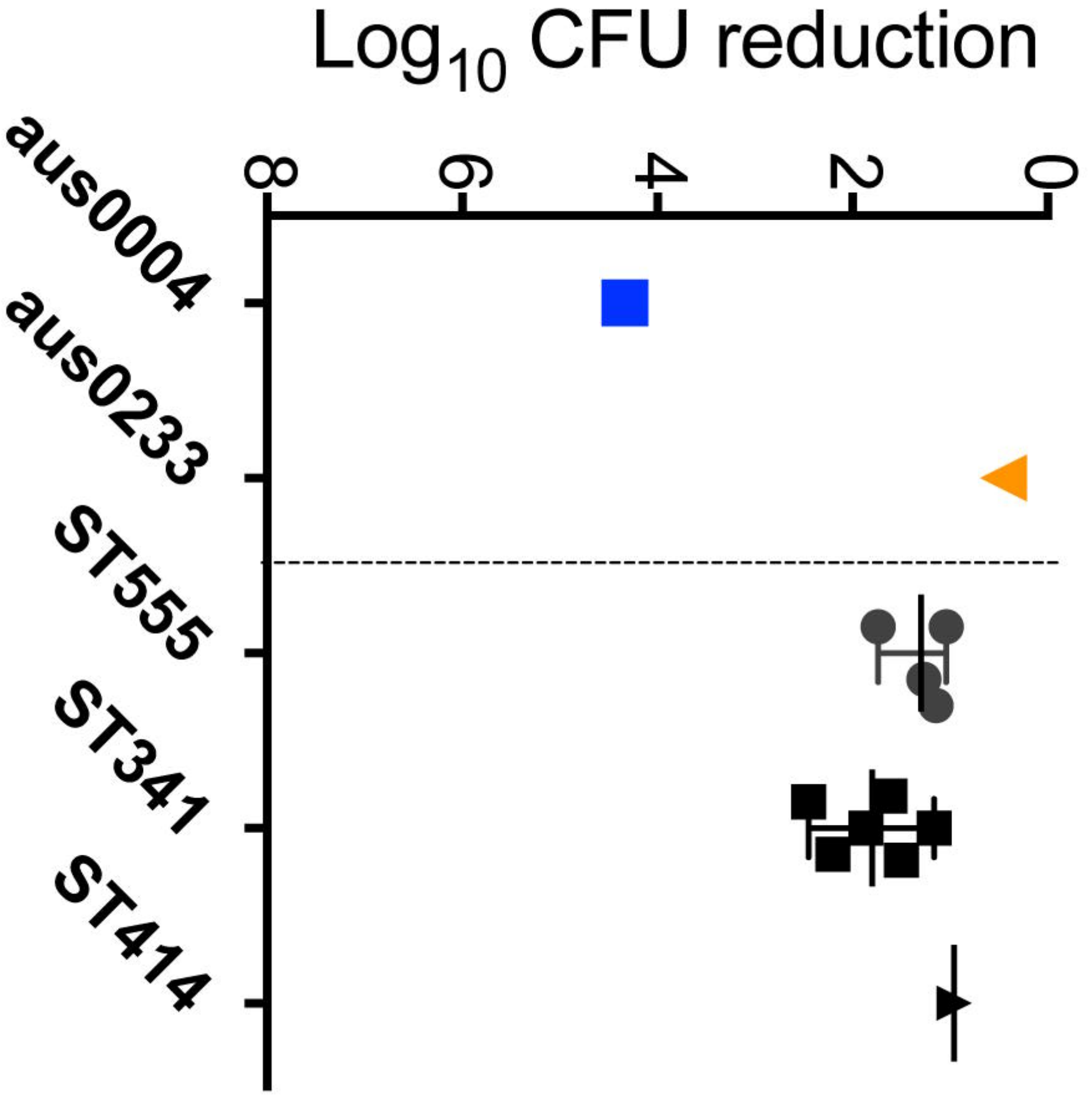
1998-2004
2005-2010
2011-2015
B
Log10 CFU reduction
r = 0.4250
p<0.0004
2000 2003 2006 2009 2012 2015
C
Log10 CFU reduction
ST17
2000 2003 2006 2009 2012 2015
D
Log10 CFU reduction
ST203
2000 2003 2006 2009 2012 2015
E
Log10 CFU reduction
ST796
2000 2003 2006 2009 2012 2015


Log10 CFU reduction
8
6
4
2
0
aus0004
aus0233
ST555
ST341
ST414