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Global spread of three multidrug-resistant lineages of *Staphylococcus epidermidis*

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Author contributions

B.P.H. and T.P.S. conceived the project, which was supervised by B.P.H., T.P.S. and I.R.M. J.Y.H.L. performed all experimental work, with assistance from I.R.M. J.Y.H.L., A.G.d.S., T.S., T.P.S. and B.P.H. analysed data, including analysis of genome sequence data. K.Y.L.C., A.K., R.H., N.W., M.D.B., B.S., F.L., M.D., A.D., R.P., A.R.L. and T.M.K. established and analysed clinical and reference isolate data sets and performed susceptibility testing. J.Y.H.L., I.R.M., B.P.H. and T.P.S. drafted the manuscript. All authors reviewed and contributed to the final manuscript.

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Competing interests

The authors declare no competing interests.

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Abstract

Staphylococcus epidermidis is a conspicuous member of the human microbiome, widely present on healthy skin. Here we show that *S. epidermidis* has also evolved to become a formidable nosocomial pathogen. Using genomics, we reveal that three multidrug-resistant, hospital-adapted lineages of *S. epidermidis* (two ST2 and one ST23) have emerged in recent decades and spread globally. These lineages are resistant to rifampicin through acquisition of specific *rpoB* mutations that have become fixed in the populations. Analysis of isolates from 96 institutions in 24 countries identified dual D471E and I527M RpoB substitutions to be the most common cause of rifampicin resistance in *S. epidermidis*, accounting for 86.6% of mutations. Furthermore, we reveal that the D471E and I527M combination occurs almost exclusively in isolates from the ST2 and ST23 lineages. By breaching lineage-specific DNA methylation restriction modification barriers and then performing site-specific mutagenesis, we show that these *rpoB* mutations not only confer rifampicin resistance, but also reduce susceptibility to the last-line glycopeptide antibiotics, vancomycin and teicoplanin. Our study has uncovered the previously unrecognized international spread of a near pan-drug-resistant opportunistic pathogen, identifiable by a rifampicin-resistant phenotype. It is possible that hospital practices, such as antibiotic monotherapy utilizing rifampicin-impregnated medical devices, have driven the evolution of this organism, once trivialized as a contaminant, towards potentially incurable infections.

Staphylococcus epidermidis is universally present on human skin[1]. The shift in medicine towards invasive procedures has favoured its emergence as a significant nosocomial pathogen, particularly in the setting of prosthetic devices[2]. The ability of the bacterium to form biofilms over foreign bodies, in which bacteria are protected from antibiotics and the immune system, is key in the evolution of disease[3]. *S. epidermidis* and other coagulase-negative staphylococci (CoNS) are leading causes of surgical site and central-line-associated bloodstream infections[4], with major economic implications[5]. In spite of this impact, relatively little is understood about the mechanisms of pathogenesis and optimal treatment of

S. epidermidis. Many of the clinical decisions made when treating this species are based on assumptions from studies in *Staphylococcus aureus*.

A single *S. epidermidis* lineage, multilocus sequence type (MLST) 2, dominates in the hospital environment. MLST eBURST analysis of a global strain collection found 74% of nosocomial isolates belong to clonal complex 2, for which ST2 is the founder[6]. The *S. epidermidis* population structure, as assessed by MLST, comprises six genetic clusters (GCs)[7,8]. Isolates from GC5 (encompassing ST2 and ST23) are almost exclusively nosocomial[8] and significantly enriched for antibiotic resistance and biofilm production, suggesting hospital adaptation[7]. Methicillin resistance in *S. epidermidis* has been reported to be as high as 70–92% in some institutions[9], and is frequently associated with co-resistance to other antibiotic classes[10,11].

We recently reported the first complete genome and methylome of BPH0662, an ST2 *S. epidermidis* exhibiting near pan-drug resistance, including intermediate heteroresistance to vancomycin, responsible for a serious post-neurosurgical infection[12]. Vancomycin intermediate heteroresistance is characterized by the presence of bacterial subpopulations capable of growth within the intermediate range, despite testing as vancomycin-susceptible by standard laboratory methods. Few studies have described the phenomenon in *S. epidermidis*[13,14] or CoNS collectively[15–17]. The diagnostic definitions and clinical implications of *S. epidermidis* vancomycin heteroresistance are poorly understood, and resistance mechanisms remain unknown.

Ongoing cases of similarly pan-resistant *S. epidermidis* infections in unrelated patients within our hospital suggested dissemination of a multidrug-resistant lineage. We began investigations using comparative and functional genomics to characterize the molecular epidemiology and resistance mechanisms of *S. epidermidis* linked to clinically significant infections, initially within our institution and then globally.

Results

Increasing prevalence of multidrug-resistant *S. epidermidis*.

To address the hypothesis that a multidrug-resistant strain was spreading at Austin Health (an 800-bed tertiary hospital in Melbourne, Australia), the antibiotic susceptibilities of clinical *S. epidermidis* isolates from 2007 to 2013 were explored (see Methods for clinical definition). We observed an increase in teicoplanin resistance from 3.6% in 2007 to 35.3% in 2013 (Fig. 1a). The proportion of isolates that exhibited dual resistance to rifampicin and fusidic acid also progressively rose from 10.9% in 2007 to 31.4% in 2013 (Fig. 1b). Rifampicin resistance correlated with teicoplanin resistance. Like our previously described *S. epidermidis* BPH0662 index case isolate[12], these isolates were also resistant to β-lactams, macrolides, quinolones, aminoglycosides and sulfonamides, indicating the emergence of multidrug-resistant *S. epidermidis* (MDRSE) within our institution. Using rifampicin and fusidic acid resistance as markers for MDRSE, 52 clinically significant MDRSE isolates were identified, of which 33 were available for further testing. Thirty-five clinically significant non-MDRSE comparator strains, two non-MDRSE reference strains (ATCC 12228[18] and RP62a[19]), BPH0662 and BPH0663 (subsequent isolate from the index case)

were also included in analyses (full antibiograms are shown in Supplementary Fig. 1). In *S. aureus*, specific rifampicin resistance mutations in *rpoB* result in vancomycin heteroresistance[20,21]. We postulated that MDRSE vancomycin heteroresistance might also be explained by rifampicin resistance mutations. Macromethod Etests (METs) are a sensitive method for the detection of reduced glycopeptide susceptibility in staphylococci[22]. METs performed on these 72 isolates revealed a significant association with heteroresistance to vancomycin (Fig. 1c) and teicoplanin (Fig. 1d) for MDRSE compared to non-MDRSE ($P < 0.0001$ for both).

International clonal expansion of endemic, multidrug-resistant, ST2 and ST23 *S. epidermidis* lineages resulting in clinical disease.

To investigate the relationship between clinically significant isolates of MDRSE and non-MDRSE at our institution, whole-genome comparisons were made of the 72 isolates plus two additional published, finished *S. epidermidis* genomes. A maximum-likelihood phylogeny was inferred from an alignment of 54,493 core genome single nucleotide polymorphisms (SNPs) (Fig. 2). Bayesian analysis of population structure (BAPS), derived from core-SNP analysis, categorized the 74 genomes into five groups (named A–E). The largest (group E) represented ST2, accounting for 59% of isolates and consisted of two dominant sublineages, one MDRSE ($n = 27$), to which the BPH0662 reference belonged, the other predominantly non-MDRSE ($n = 16$), of which two were MDRSE (henceforth referred to as the ST2-mixed lineage). A third cluster of five MDRSE isolates belonging to the ST23 lineage (BAPS group C) was also identified (Fig. 2). The presence of isolates from three distinct lineages across at least six years indicated that each was endemic within our institution, resulting in repeated, clinically significant, nosocomial infections.

We next sought to determine the broader distribution of these three MDRSE lineages across Australia and globally. A further 32 rifampicin-resistant clinical *S. epidermidis* isolates from 13 institutions in Australia, and 121 global isolates from Austria ($n = 1$), Belgium ($n = 18$), Denmark ($n = 38$), France ($n = 18$), Germany ($n = 21$), Republic of Ireland ($n = 1$), United Kingdom ($n = 12$) and the United States ($n = 12$) originating from 61 institutions were collected, totalling 227 isolates (Supplementary Table 1a).

A maximum-likelihood phylogeny for the 227 isolates was inferred from an alignment of 56,756 core genome SNPs and revealed the same basic population structure as before, with the majority of isolates clustered into three MDRSE lineages (Fig. 3 and Supplementary Fig. 2a). Overall, core-SNP-based comparisons suggested high genomic identity within the BPH0662 lineage, with a median pairwise SNP difference of 62 SNPs (interquartile range (IQR) 41–82). In contrast, the median difference within the ST2-mixed clade was 281.5 SNPs (IQR 190.25–378), indicating more genetic diversity among ST2-mixed isolates. Compared to one another, the two ST2 lineages were distinct with a median of 1,669 SNPs (IQR 1,649–1,703) between them (Fig. 3b,c). The ST23 MDRSE also demonstrated high intra-lineage identity with a median difference of 103 SNPs (IQR 88–116), while the ST5 lineage was composed of heterogeneous isolates with a median intra-group difference of 374 SNPs (IQR 141–587) (Fig. 3c).

The BPH0662 ST2 MDRSE lineage, dominant in Australia, was also identified from 25 institutions in four countries (Australia, Belgium, Denmark and the United Kingdom) and persisted within two Australian sites for at least 8 years, while the ST2-mixed lineage was identified in 33 institutions within seven countries (Australia, Belgium, Denmark, France, Germany, Ireland and the United Kingdom). The ST23 lineage was present in 17 institutions across seven countries (Australia, Austria, Belgium, Denmark, France, Germany and the United States); this lineage persisted up to 17 years in Denmark and 15 years at a single site in the United States. Co-circulation of all three clones was observed in two of the European sites (BEL-A and DEN-G). While isolates from the same country tended to cluster within each of the three MDRSE lineages, genotype intermingling was also observed across countries, consistent with the international dissemination of three individual successful clones (Fig. 3 and Supplementary Fig. 2).

Pan-genome analysis identified 5,692 orthologue gene clusters.

Minimal variability in gene content was observed within the BPH0662 ST2 clade (Supplementary Fig. 2c). The accessory gene content specific to this lineage was the same as previously characterized for BPH0662, and clustering based on genome content aligned strongly with core genome phylogeny (Supplementary Fig. 2d), the MDRSE ST2-mixed lineage contained similar accessory gene content (Supplementary Fig. 2c). All ST2 and subclustered ST188 (n = 133) and ST23 (n = 50) isolates possessed the *icaRADBC* operon, which was not present in any of the ST5 or subclustered ST87 (n = 15) isolates. The *ica* operon was highly conserved among 193 isolates (Supplementary Fig. 2c). Orthologue clustering clearly demonstrated that RP62a (ST10), a reference isolate frequently used in laboratory experiments, is an outlier compared to other clinical isolates (Supplementary Fig. 2d).

Evolutionary phylogeny suggests independent coevolution of two ST2 and one ST23 multidrug-resistant *S. epidermidis* lineages.

To model the evolutionary trajectory of *S. epidermidis* and date the emergence of the drug-resistant ST23 and ST2 lineages, we used core genome SNP diversity and year of isolation to infer a time-scaled phylogeny in a Bayesian framework (Supplementary Fig. 3). Overall, this analysis suggested that each of the three drug-resistant lineages emerged independently in the 1980s (BPH0662 ST2 clade: 1982 (95% highest posterior density interval (HPDI) 1972–1991); the major rifampicin-resistant subclade of ST2-mixed: 1987 (95% HPDI 1978–1998); ST23 clade: 1984 (95% HPDI 1974–1992)). This analysis also suggested the first substantial diversification within the ST2 lineage around 1958 (95% HDPI 1940–1974).

Genetic determinants of antibiotic resistance.

Ninety-nine percent of the 133 ST2 isolates possessed *mecA* (Supplementary Table 1d), which correlated with the antibiogram data (Supplementary Fig. 1). Among these, 106 possessed the full *mecA*, *mecR1* and *mecI* complex as a typical class A *mec*. The *fusB* gene was present in 139 of the 143 fusidic-acid-resistant isolates, in keeping with previous reports[23,24], the majority in chromosomal phage resistance islands: SeRI$_{fusB\text{-}704}$ (accession no. JF808725) or SeRI$_{fusB\text{-}5907}$ (accession no. JF777506) both attached downstream of *groEL* (n = 121); or SeRI$_{fusB\text{-}7778}$ (JF808726), attached downstream of *rpsR* (n = 13).

Eighteen linezolid-resistant isolates were identified, all European (Germany *n* = 14, France *n* = 4, Ireland *n* = 1). At least one of three recognized genetic determinants of linezolid resistance were identified for each isolate, including acquisition of *cfr*; the G2576T substitution in domain V of 23S rRNA (equivalent to G2602T in *S. epidermidis*)[25,26], or insertion of a glycine residue ($_{71}$GR$_{72}$ to $_{71}$GGR$_{72}$) in ribosomal protein L4[26] (Supplementary Table 2). All five of the *cfr*-positive isolates were from Germany, three of which had acquired *S. epidermidis* plasmid p12–02300 (accession no. KM521837) previously associated with an outbreak of linezolid resistance in German hospitals[25], one had a previously undescribed plasmid. Twelve isolates were resistant to daptomycin, two of which (AUS14 and FRA09) had an MprF S295L substitution, known to cause daptomycin resistance[27]. Due to the pleiotropic nature of daptomycin resistance, other potential causative mutations were not identified.

Two co-occurring mutations in RpoB, D471E and I527M, explained rifampicin resistance for 163 of the 187 isolates in this study. Two isolates had alternative D471 substitutions. The remaining had at least one RpoB substitution occurring at site H481 (*n* = 7), S486 (*n* = 13), V135 (*n* = 2) and N353 (*n* = 1) (Supplementary Figs. 2 and 4). Based on alignment with the known sequence defined for other bacterial species, the rifampicin resistance determining region (RRDR) in *S. epidermidis* spanned amino-acid positions 462 to 488 (inclusive). Three critical positions known to form covalent bonds with rifampicin in other species (at which most rifampicin-resistant mutations occur)[28,29] were determined to correspond to D471, H481 and S486. Although outside the RRDR, sites V135 and I527 aligned with positions also known to be associated with rifampicin resistance in other bacterial species[28,29]. Glycopeptide susceptibility testing found the majority of isolates with the dual RpoB D471E and I527M mutations (96.9%) or single RpoB H481D/L/R/Y substitutions (100%) were vancomycin heteroresistant (Fig. 3 and Supplementary Fig. 2).

Dual D471E and I527M substitutions are the most common RpoB mutations in *S. epidermidis* worldwide.

To confirm that the dominant RpoB mutations observed in our study were representative of other *S. epidermidis* globally, we performed an analysis of all publicly available *S. epidermidis* sequence data. Using the selection/ exclusion criteria outlined in the Methods, a curated list of 160 clinical isolates (see Supplementary Table 1 for full metadata) downloaded from NCBI Sequence Read Archive (SRA) were assembled, and the *rpoB* genes analysed in conjunction with the four NCBI reference strains and the 222 isolates from this study (BPH0663 was excluded as an in vivo serial isolate of BPH0662); data from a Swedish study by Hellmark et al.[30] in which the *rpoB* gene of 33 *S. epidermidis* strains isolated from prosthetic joint infections were sequenced and correlated with phenotype were also included. Of the 419 isolates analysed, 251 were identified as having at least one of 40 putative amino-acid substitutions in RpoB, occurring across 32 sites. The dual D471E and I527M substitutions were the most common, present in 187 of the isolates, accounting for 73.9% of the observed mutations, and 86.6% of the mutations occurring within the RRDR of RpoB (Supplementary Fig. 4). Interestingly, neither D471E nor I527M were observed as solitary substitutions. Analysis of metadata indicated that isolates possessing the dual mutations originated from 68 different institutions in 11 countries, and in silico MLST

demonstrated ST2 (62.9%) and ST23 (33.1%), accounted for 96.0% of isolates with the dual D471E and I527M substitutions.

Dual D471E and I527M mutations in RpoB confer co-resistance to rifampicin and vancomycin in *S. epidermidis*.

To confirm a causal link between these mutations and vancomycin heteroresistance we swapped the D471E and I527M *rpoB* allele from BPH0662 into four rifampicin-susceptible *S. epidermidis* backgrounds—two clinical, non-MDRSE ST2 strains, BPH0676 and BPH0736; and reference strains RP62a (ST10) and ATCC 12228 (ST8)—using plasmid artificial modification and allelic exchange. Acquisition of rifampicin resistance enabled positive screening of mutants. Whole-genome sequencing confirmed the presence of only the intended two substitutions in *rpoB*, with the exception of 12228-*rpoB*662, which acquired an additional spontaneous SNP leading to a M305L substitution in a hypothetical regulatory protein (locus_tag SE2129). To the best of our knowledge, this is the first successful report of allelic exchange in ST2 *S. epidermidis*.

Compared to the four wild-type (WT) parental strains—BPH0676, BPH0736, RP62a and ATCC 12228—all *rpoB*662 mutants acquired high-level rifampicin resistance equivalent to that of BPH0662 (64 μg ml^{-1}). The mutants were not identified as glycopeptide resistant using standard testing with vancomycin broth microdilution (BMD) or Vitek 2. To determine the role of these mutations in the evolution of glycopeptide heteroresistance, extended glycopeptide testing by vancomycin gradient assay (VGA), vancomycin and teicoplanin METs and a vancomycin population analysis profile to area under the curve ratio (PAP:AUC) was performed for each of the WT and *rpoB*662 mutant pairs. Experiments for the BPH0676-WT and BPH0676-*rpoB*662 pair are shown in Fig. 4. Compared to its parental strain, the BPH0676-*rpoB*662 mutant had a statistically significant increase in mean ratio to BPH0662 for VGA ($P = 0.0039$) and PAP:AUC ($P = 0.0023$). Furthermore, the gains observed in both vancomycin and teicoplanin MET MICs for the BPH0676-*rpoB*662 mutant achieved the criteria for vancomycin heteroresistance. Statistically significant increases in glycopeptide resistance were also observed for the other *rpoB*662 mutants compared with their respective parental strains (Supplementary Fig. 5). Having independently validated each mutant compared to their parental strain, the pairs were then analysed collectively. For each phenotypic testing method, a statistically significant increase in mean glycopeptide resistance was observed for the *rpoB*662 mutant group compared to their paired parental strains (Fig. 5). These experiments show that D471E and I527M RpoB substitutions cause the vancomycin-heteroresistant phenotype.

Mutants containing dual D471E and I527M RpoB substitutions outcompete WT *S. epidermidis* in the presence of vancomycin.

To assess the advantage conferred by the dual D471E/I527M RpoB substitutions in the presence of vancomycin, 1:1 competition assays for BPH0736-WT and its *rpoB*662 mutant were conducted. In the absence of vancomycin, the D471E/I527M RpoB allele posed a substantial fitness cost, with a significant shift to the WT population at 48 h (Fig. 6). However, with the introduction of vancomycin the population dynamics reversed and at 48 h with 4 μg ml^{-1} vancomycin, the population was 100% *rpoB*662 mutant (Fig. 6). Individual

growth curves for each of the WT and *rpoB*662 mutant pairs were not significantly different in the absence of antibiotics (Supplementary Fig. 6). These experiments confirm that the D471E and I527M RpoB substitutions in *S. epidermidis* were capable of generating both high-level rifampicin resistance as well as vancomycin heteroresistance in *S. epidermidis* strains from divergent backgrounds.

Discussion

We have uncovered here the emergence of three genetically distinct MDRSE lineages, with common dual D471E and I527M RpoB substitutions, that have evolved within the hospital environment to exhibit near pan-drug resistance. The presence of the same mutations in multiple phylogenetically distant lineages is consistent with independent emergence of these mutations with subsequent fixation, presumably due to their favourable antibiotic-resistant phenotype. The international prevalence of these dual mutations, as determined in this study, suggests that a high proportion of rifampicin-resistant clinical *S. epidermidis* isolates possess mutations that also confer glycopeptide heteroresistance, not detectable by standard diagnostic methods. Although dominance may vary by region, the same three lineages, with low intra-group genomic diversity, were demonstrated to be endemic and co-circulate within multiple institutions worldwide, suggesting their unrecognized presence in other establishments. The diminishing pool of antibiotics available for the treatment of infections due to such isolates, and the increasing catalogue of complex genetic interactions that contribute to antimicrobial resistance between unrelated drug classes, reinforces the need for the judicious utilization of remaining therapeutic options.

In particular, this research has implications for how rifampicin is utilized in clinical practice. Device impregnation with rifampicin is of particular concern, as low-level rifampicin monotherapy released in this situation could predispose colonizing *S. epidermidis* to develop rifampicin resistance and concomitant vancomycin heteroresistance. To avoid generating resistance with monotherapy, combination therapy is recommended when using rifampicin for the treatment of staphylococci[31,32]. Historical studies describing dual therapy with vancomycin and rifampicin for *S. epidermidis* prosthetic valve endocarditis[33] have informed current staphylococcal clinical guidelines[31]. However, the emergence of rifampicin resistance when using this treatment combination for serious infections has been reported for both *S. epidermidis*[34] and *S. aureus*[35–37]. Our results suggest that co-prescription of vancomycin and rifampicin will promote the generation of resistance to both agents, although additional experiments are required to test this hypothesis. Widespread loss of vancomycin as a first-line treatment option against *S. epidermidis* would have significant clinical impact, especially in view of the limited available substitutes. While alternative agents such as daptomycin, ceftaroline, lipoglycopeptides and linezolid exist, staphylococcal resistance to all has been described[36]. Considered in this context, our confirmation of a causal association between rifampicin resistance mutations and vancomycin heteroresistance in *S. epidermidis*, together with clinical reports demonstrating lack of cross-protection, suggest that current clinical recommendations warrant review. Alarmingly, our study has identified that near untreatable isolates already exist, with two German vancomycin heteroresistant isolates, one ST2-mixed (GER16) the other an ST23 clone (GER15), also

testing as resistant to both daptomycin and linezolid, with tetracycline the only antibiotic option remaining (Supplementary Fig. 1).

Surveillance is not routinely performed for *S. epidermidis*, and consequently there are no robust data on the prevalence of rifampicin resistance in this species. Reference laboratories may be sent MDRSE isolates of clinical concern, but no denominator data exist to determine the background number of susceptible isolates. The data from individual centres contributing to this study suggested local variation in the annual prevalence of *S. epidermidis* rifampicin resistance at each institution between 2012 and 2017: 10% increasing to 20% for Erasme Hospital, Belgium; 10% for Monash Health, Australia; 6% for Hvidovre Hospital, Denmark; and 3% for Mayo Clinic, Rochester, MN, USA. These rates included all *S. epidermidis* isolated from clinical samples for each institution, and almost certainly underestimated the prevalence in clinically significant isolates. This was highlighted by our Australian data, which demonstrated that despite only 10.8% of all *S. epidermidis* isolated from blood cultures in the state of Victoria over the month of March 2017 testing rifampicin-resistant, 30.0% of the clinically significant isolates were rifampicin-resistant (all of which were vancomycin-heteroresistant). In view of the ubiquitous nature of *S. epidermidis* and difficulties distinguishing colonizing from clinically significant isolates, determining the true prevalence of these MDRSE lineages is likely to remain a challenge. Therefore, rifampicin resistance is a potentially useful marker for identifying these clinical *S. epidermidis* clones.

The retrospective nature of the isolate collection we used, which was biased towards the acquisition of multidrug-resistant *S. epidermidis*, is a limitation of this study, and sampling may have missed some less prevalent genotypes. Furthermore, we did not have access to data regarding the treatment received or clinical outcomes of the patients from whom isolates were obtained. Notwithstanding these limitations, we obtained a good representation of the international diversity of rifampicin resistance in clinical *S. epidermidis*, which was the main focus of this study. Having identified the existence of three MDRSE *S. epidermidis* lineages with international presence, future research could include more geographically diverse, prospective sampling of isolates with associated clinical data to include treatment and outcomes, which could better delineate the global extent of these clones and offer insight into the clinical impact of these lineages. Our findings indicate that similar glycopeptide resistant isolates are likely to be present within other institutions but unrecognized by standard phenotypic testing methods, highlighting a need for clinical vigilance. These data challenge current assumptions that cross-protection is afforded by the co-prescription of rifampicin and vancomycin for the treatment of staphylococci, indicating that a review of clinical guidelines is warranted to avoid the further selection of near untreatable clones.

Methods

Media and reagents.

S. epidermidis and *S. aureus* were routinely cultured at 37 °C in BBL brain heart infusion (BHI) broth (Becton Dickson). For electroporation, *S. epidermidis* was cultured in B medium (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose). *Escherichia coli* was routinely cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5%

NaCl). BMD minimum inhibitory concentrations (MICs) were performed in cation-adjusted Mueller Hinton (MH) broth (Difco). For growth on agar, BHI or L broth were solidified with 1.5% agar, to yield BHIA and LBA, respectively. The following antibiotics were purchased from Sigma Aldrich and used at the specified concentrations: chloramphenicol (Cm) 10 μg ml^{-1} for *S. epidermidis*, 15 μg ml^{-1} for *E. coli*; ampicillin (Amp) 100 μg ml^{-1} for *E. coli*; kanamycin (Kan) 50 μg ml^{-1} for *E. coli*. The following antibiotics were used at variable concentrations for susceptibility testing: rifampicin (Sigma Aldrich) and vancomycin (Hospira).

Oligonucleotides were purchased from Integrated DNA Technologies and are listed in Supplementary Table 3. Genomic DNA was isolated with DNeasy Blood and Tissue Kit (Qiagen). To weaken the cell wall of *S. epidermidis* before DNA extraction, harvested cells were washed with PBS. Lysostaphin (Ambi) was added to the Gram-positive lysis buffer (final concentration 100 μg ml^{-1}) and incubated at 37 °C for 30 min. Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen). PCR products and gel extractions were purified using QIAquick Gel Extraction Kit (Qiagen). Restriction enzymes and Phusion DNA polymerase were purchased from New England Biolabs. Phire Hotstart DNA polymerase was purchased from Thermofisher. Colony PCR was performed as previously described[38].

Bacterial isolates.

Bacterial strains used in this study are listed in Supplementary Table 3. Comparator strains consisted of four reference strains (*S. epidermidis* ATCC 12228, ATCC 35984 [RP62a]; *S. aureus* ATCC 700698 [Mu3], ATCC 700699 [Mu50]); 69 clinically significant *S. epidermidis* isolates (34 MDRSE; 35 non-MDRSE) collected at Austin Health, Victoria, Australia, from 1 January 2007 to 31 December 2013 (all available MDRSE were included as comparators; for non-MDRSE comparators, five strains were randomly selected for each year, with the exception of 2010, for which only four clinically significant non-MDRSE were available); 10 clinically significant MDRSE collected from 1 January 2010 to 31 December 2013 originating from three hospitals within the Monash Health network in Victoria, Australia; 22 rifampicin-resistant blood culture isolates originating from 12 different hospitals submitted to the Microbiological Diagnostic Unit Public Health Laboratory, Victoria, Australia, collected during the month of March 2017 as part of a state-wide *S. epidermidis* survey. For Australian isolates, clinical significance was defined as repeated culture (≥2 occasions) of isolates with identical antibiograms as determined by Vitek 2 (bioMérieux), from the same patient, from bloodstream or sterile site samples. International comparator strains consisted of 18 rifampicin-resistant blood culture isolates collected between 2015 and 2017 at Erasme Hospital, Brussels, Belgium; 27 rifampicin-resistant blood culture isolates collected between 2012 and 2017 submitted to the Department of Clinical Microbiology, Hvidovre University Hospital, Copenhagen, Denmark, originating from four institutions; 11 rifampicin-resistant clinical isolates submitted to Statens Serum Institut, Copenhagen, Denmark in 2001, originating from six institutions (including Hvidovre Hospital); 18 rifampicin-resistant clinical isolates submitted to the Institute for Infectious Agents Department of Bacteriology French National Reference Centre for Staphylococci, Lyon, France, between 2004 and 2016 originating from 18 different hospitals; 22 clinically significant rifampicin-resistant isolates submitted to the

National Reference Centre for Staphylococci and Enterococci, Robert Koch Institute, Wernigerode, Germany from 2012 to 2017, originating from 20 different institutions in Germany and one from Austria; 13 rifampicin-resistant clinically significant isolates submitted to the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National Infection Service, Public Health England, between November 2015 and November 2016, originating from 10 different hospitals in the United Kingdom and the Republic of Ireland; 12 rifampicin-resistant clinically significant isolates collected between 2000 and 2016 at the Mayo Clinic, Rochester, MN, United States. To maximize recruitment, rifampicin resistance alone was used as a marker for MDRSE since prescription of fusidic acid is subject to regional practice and use in the United States is still undergoing trials, seeking Food and Drug Administration approval.

Antibiotic susceptibility testing.

The background antibiogram of *S. epidermidis* for Austin Health from 2007 to 2013 was determined by collating all routine isolates that had susceptibility testing performed (interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria), excluding duplicate strains obtained from the same patient. Comparator isolates underwent susceptibility testing with a Vitek 2 Gram Positive ID card (AST-P612; bioMérieux) on a Vitek 2 (bioMérieux) according to the manufacturer's instructions. BMD MICs for vancomycin and rifampicin were performed as recommended by CLSI[39]. Rifampicin Etests (bioMérieux) were performed according to the manufacturer's instructions. Extended glycopeptide susceptibilities were determined by MET with vancomycin and teicoplanin Etest strips (bioMerieux) using a 2.0 McFarland inoculum and prolonged incubation time of 48 h. *S. aureus* criteria for vancomycin heteroresistance were used (vancomycin and teicoplanin MET of >8; teicoplanin MET alone of >12), as no criteria have been defined for *S. epidermidis*. Vancomycin PAPs were performed as previously described[40], but overnight *S. epidermidis* cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.4. For VGAs, 50 ml of BHIA supplemented with vancomycin 4 μg ml^{-1} was set in a 10 cm square Petri dish with one side elevated at a 5° angle (to form a two-dimensional gradient); the Petri dish was then placed level and a second 50 ml BHIA layer was set over the vancomycin layer. Plates were used within 48 h. Overnight cultures of tested strains were standardized to an OD_{600} of 0.08 in BHI and a 10 μl loop was used to streak the strain across the plate from vancomycin 0 to 4 μg ml^{-1}. Distance of growth was measured in mm after 48 h incubation at 37 °C, and expressed as a ratio to growth of control strain, BPH0662. With the exception of Vitek 2 and Etests, all antibiotic susceptibility testing of strains was performed in biological triplicate. Interpretation of all phenotypic testing was blinded, with the exception of VGAs due to the pairing of mutant and parental isolates on the same gradient plate to ensure consistent experimental conditions.

Genome sequencing.

Draft genomes of the 222 clinical comparator strains, the isolates of RP62a and ATCC 12228 used to create mutant strains, four *rpoB* mutant strains and Ec_Se662I-II *E. coli* were sequenced on either an Illumina MiSeq or NextSeq platform, as indicated in Supplementary Table 1c, with Nextera XT libraries, according to the manufacturer's instructions.

Genome analysis.

Illumina paired-end reads were trimmed using Trimmomatic v0.36[41], and overlapping pairs merged with PEAR v0.9.10[42]. Draft genomes were de novo assembled with SPAdes v3.7.1[43] using the unassembled R1 and R2, and merged read pairs as input. Genome annotation was performed with Prokka v1.12[44]. An alignment of core genome SNPs across the *S. epidermidis* isolates was determined using Snippy v3.2[45] using the closed BPH0662 genome as a reference. iqtree v1.5.3[46] using model finder[47] and ultrafast bootstrap[48] was then used to produce a maximum-likelihood phylogenetic tree. Pairwise SNP analysis was performed using pairwise_snp_differences[49]. For pan-genome analysis, protein orthologue clustering was performed using Proteinortho v5.11[50], alignment of the resultant CDS orthologues to BPH0662 and visualization of the pan-genome were performed using FriPan[51]. De novo assemblies were genotyped using in silico MLST[52], and the resistome and virulome were characterized with abricate[53] screened against the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (NCBI BARRGD, PRJNA313047) and Virulence Factors of Pathogenic Bacteria Database (VFDB)[54], respectively. The tree was clustered using a hierarchical Bayesian Analysis of Population Structure (hierBAPS) model[55]. To infer the evolutionary phylogeny of ST2 *S. epidermidis*, regions of phage insertion and recombination were masked to ensure modelling was only informed by spontaneous SNPs within the core genome. Five (BPH0704, BPH0697, BPH0677, BPH0737 and SEI) of the 227 isolates were omitted from final analyses based on their extreme divergence, which suggested either excessive natural selection, thereby violating the assumption of evolutionary neutrality used in the model, or separation of these lineages so remotely in the past as to be beyond the abilities of this method to analyse correctly. Two historical, non-MDRSE Australian clinical isolates (BPH0823 isolated in 1989 and BPH0825 isolated 1990) were included to improve temporal sampling depth. Snippy v3.2[45] was used to generate an alignment of the 224 relevant isolates to the full BPH0662 chromosome. Phage elements in the BPH0662 reference were identified using PHASTER[56] and masked from the alignment with BEDtools[57]. Gubbins v2.2.0[58] was used to filter recombination and identify core-SNPs from filtered alignment, with RAxML v8.2.10[59] for tree building. A final maximum-likelihood tree was produced with iqtree v1.5.3 using model finder and ultrafast bootstrap. BEAST v2.4.3[60] was used to generate a timed phylogeny (chronogram) assuming a relaxed clock and using coalescent tree prior. The assumption of a molecular clock was tested using root-to-tip distance in R v3.4[61] using the Analysis of Phylogenetics and Evolution (APE) package v4.1[62].

All publicly available *S. epidermidis* genome raw reads in NCBI SRA were downloaded (4 November 2016) and assembled as described above. Exclusion criteria were as follows: sequencing performed on a Roche 454 or Ion Torrent platform (per Genbank metadata); single end reads; sequencing depth of <30×; isolates sequenced as part of this study; mutagenesis isolate pairs; organism not *S. epidermidis* or sequencing of mixed bacterial species as determined by Kraken v0.10.15beta[63]; >300 assembled contigs; raw reads not associated with publications and lacking metadata. When isolates were identified as exact genetic replicates, a single representative was used in analysis. To determine the diversity of mutations in RpoB, the RpoB translation for ATCC 12228 (accession no. NP_763861) was used as reference for a tblastn query on the combined NCBI SRA and study isolate

assemblies using BLAST+ v2.6.0[64], the output of which was aligned with MUSCLE v3.8.1551[65]; this was then manually collated with the RpoB mutations reported in ref. [30]. To determine the RRDR of RpoB for *S. epidermidis* the RpoB translations for ATCC 12228 and BPH0662 were aligned with the published 27-amino-acid RRDR defined for *E. coli* and *M. tuberculosis*[28,29] and *S. aureus*[29]. Using the above method, the following reference nucleic acid/gene translations from ATCC 12228 were used to screen for the presence of mutations conferring linezolid resistance: 23S rRNA (GeneID 3190203), L3 (NP_765379), L4 (NP_765378) and L22 (NP_765374). For Mprf, the gene translation from RP62a was used as the reference (WP_002456532).

Bacterial transformation.

Overnight cultures of *S. epidermidis* grown in 10 ml of B medium were diluted to an OD_{600} of 0.5 in 100 ml of fresh, prewarmed B medium. Cultures were then reincubated for 40 min and chilled in an ice slurry for 10 min. All subsequent steps were performed as previously described[38], with the exception of post-electroporation recovery in which cells were incubated in 1 ml of BHI supplemented with 500 mM sucrose (filter sterilized) at 28 °C for 1 h before plating on BHIA Cm10.

Construction of *E. coli* Ec_Se662I-II for transformation of ST2 *S. epidermidis*.

The *E. coli* Ec_Se662I-II mutant expressing both BPH0662 type I RM systems in a DC10B background was constructed using a previously described protocol and primers[12]. The DC10B-MS1 mutant with *hsdMS1* integrated between *essQ* and *cspB* was subjected to a second recombination event to integrate *hsdMS2* between *ybbD* and *ylbG*.

Allelic exchange of *rpoB* using pIMAY.

To construct pIMAY(*rpoB*662), pIMAY[38] was linearized with KpnI and gel extracted; the vector backbone was then amplified from within the multiple cloning site (MCS) with primers IM1/IM2. The entire *rpoB* gene of rifampicin-resistant, reference strain BPH0662 was amplified using primers rpoB3/rpoB4 (incorporating flanking KpnI sites), and the product gel extracted. Seamless ligation cloning extract (SLiCE) was used to insert the *rpoB* construct into the pIMAY vector backbone, as previously described[66,67]. After transformation into *E. coli* Ec_Se662I-II, the *rpoB* insert was verified by Sanger sequencing and the vector pIMAY(*rpoB*662) subsequently electroporated into four rifampicin-susceptible *S. epidermidis* strains: BPH0676, BPH0736, RP62a and ATCC 12228. Transformants were selected for on BHIA plus Cm after 2 days at 28 °C. Integration and excision of pIMAY(*rpoB*662) was performed as previously described[38], with the exception of concurrent (rather than sequential) colony PCRs, for loss of replicating plasmid (with MCS primers IM3/IM4), and chromosomal integration of the plasmid upstream (*rpoB* out forward/IM3) and downstream (IM4/rpoB out reverse). Plasmid excision in favour of mutant creation was selected for on BHIA plus ATc1 (Sigma) with rifampicin at 4, 8, 16 or 32 μg ml^{-1}, large colonies were patch-plated on BHIA plus Cm10 and BHIA plus rifampicin at 4, 8, 16 or 32 μg ml^{-1} and grown at 37 °C overnight, with successful mutants bearing a Cm^S, Rif^R phenotype. Potential mutants were selected from the plate containing the lowest rifampicin concentration. Stability of Rif^R mutants was confirmed by overnight growth on BHIA without antibiotics, with subsequent restreaking on BHIA plus rifampicin 32. Draft

genomes of successfully created mutant strains (BPH676-*rpoB*662, BPH0736-*rpoB*662, RP62a-*rpoB*662 and 12228-*rpoB*662,) and the parental strains from which they were derived (BPH0676, BPH0736, RP62a and ATCC 12228, respectively) were sequenced to determine if any unintended mutations were present. Variant calling between the *rpoB*662 mutants to their respective WT parental strains was cross-checked by two methods, using Snippy[45] and Nesoni[68]. Mutants and their corresponding parental strains were subjected to phenotypic testing to determine the role of rifampicin resistance mutations (D471E and I527M) in RpoB on the evolution of glycopeptide heteroresistance in *S. epidermidis*.

Growth curves and vancomycin competition assay.

For growth curves, overnight cultures were diluted to an OD_{600} of 0.05 in 50 ml of prewarmed BHI and grown at 37 °C with aeration at 200 r.p.m. The OD_{600} values were measured hourly for 12 h. Growth curves were performed in biological triplicate. The doubling time for each strain was calculated as previously described69, using the 2 and 5 h exponential phase time points. For vancomycin co-culture assays, overnight cultures of WT and the corresponding *rpoB*662 mutant strain were diluted to an OD_{600} of 0.5 in BHI, 0.5 ml of each culture was added to 49 ml of BHI containing vancomycin at 0, 2 or 4 μg ml^{-1} (starting OD_{600} of co-cultures = 0.01). Cultures were incubated at 37 °C with aeration at 200 r.p.m. For vancomycin 0 and 2 μg ml^{-1}, co-cultures reached saturation by 24 h and the medium was refreshed with a 1/1,000 dilution of the 24 h co-culture in 50 ml of BHI containing vancomycin at 0 and 2 μg ml^{-1}, respectively. Cultures were incubated at 37 °C with aeration at 200 r.p.m, for a further 24 h (48 h total). The vancomycin 4 μg ml^{-1} co-culture did not reach saturation until 48 h, so the medium was not refreshed at 24 h. Serial dilutions of each co-culture were performed at times 0, 24 and 48 h, plated on BHIA and incubated at 37 °C overnight; colonies were then patched onto BHIA plus rifampicin 32 μg ml^{-1} to determine the proportion of WT to *rpoB*662 mutant.

Statistical analysis.

Etest MICs were log2-transformed to normalize the distribution before statistical analysis. All statistical analyses (Mann–Whitney *U* test, unpaired or paired Student's *t*-test, two-way ANOVA with Tukey's test) were performed with GraphPad Prism 7 for Mac OS X (GraphPad Software), with the exception of the pairwise SNP analysis which was performed using pairwise_snp_ differences[49] in R[61].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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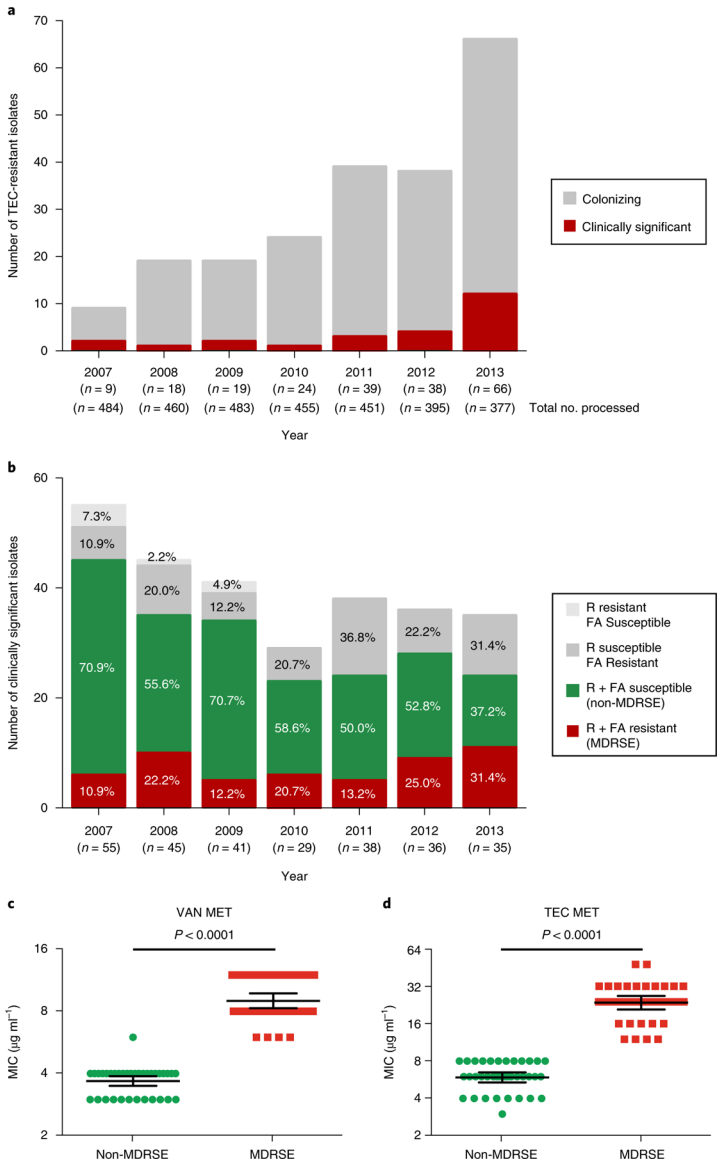

Fig. 1. Increasing prevalence of multidrug-resistant *S. epidermidis* (MDRSe).

The prevalence of antibiotic-resistant *S. epidermidis* isolates at a single Australian institution over a seven-year period is shown. Susceptibilities were performed on a Vitek 2 (bioMérieux) platform and interpreted using CLSI criteria. **a**, Prevalence of teicoplanin (TEC)-resistant isolates. **b**, Prevalence of clinically significant isolates exhibiting rifampicin (R) and fusidic acid (FA) resistance. **c**,**d**, Vancomycin (VAN) (**c**) and teicoplanin (TEC) (**d**) METs were performed on 70 clinically significant *S. epidermidis* strains (35 rifampicin- and fusidic acid-susceptible (non-MDRSE); 35 rifampicin-plus fusidic acid-resistant (MDRSE)) plus two non-MDRSE reference isolates (RP62a and ATCC 12228); $n = 72$. The y axis is plotted on a log2 scale. Error bars represent geometric mean + 95% confidence interval (CI). Null hypothesis (no difference between means) was rejected for $P < 0.05$ (two-tailed Mann–Whitney U test of log2 transformed MICs).

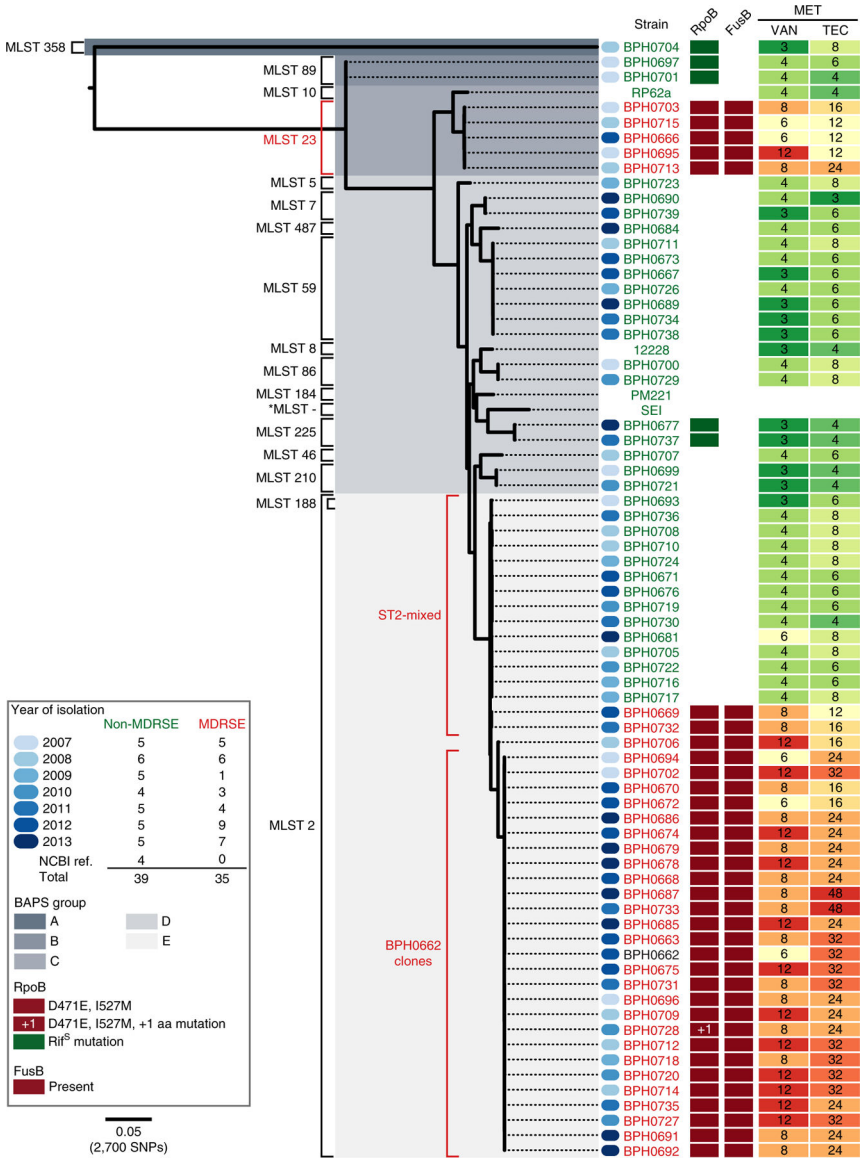

Fig. 2. Clonal expansion of endemic, multidrug-resistant ST2 and ST23 *S. epidermidis* lineages resulting in clinical disease within a single institution.

Maximum-likelihood core-SNP-based phylogeny for 69 clinically significant strains collected from 2007 to 2013 (34 multidrug *S. epidermidis* (MDRSE); 35 non-MDRSE); four closed, published *S. epidermidis* genomes; and the closed BPH0662 genome from the MDRSE index case (used as the reference strain); $n = 74$. Overlaid are the results of in silico MLST, BAPS, the determinants of rifampicin (RpoB mutations) and fusidic acid (*fusB*) resistance, and heatmap of vancomycin heteroresistant phenotype testing, as determined by VAN and TEC METs. The scale bar indicates number of nucleotide substitutions per site (bold), with an approximation of SNP rate (in parentheses). *Published reference strain SEI was not classifiable by the existing MLST scheme.

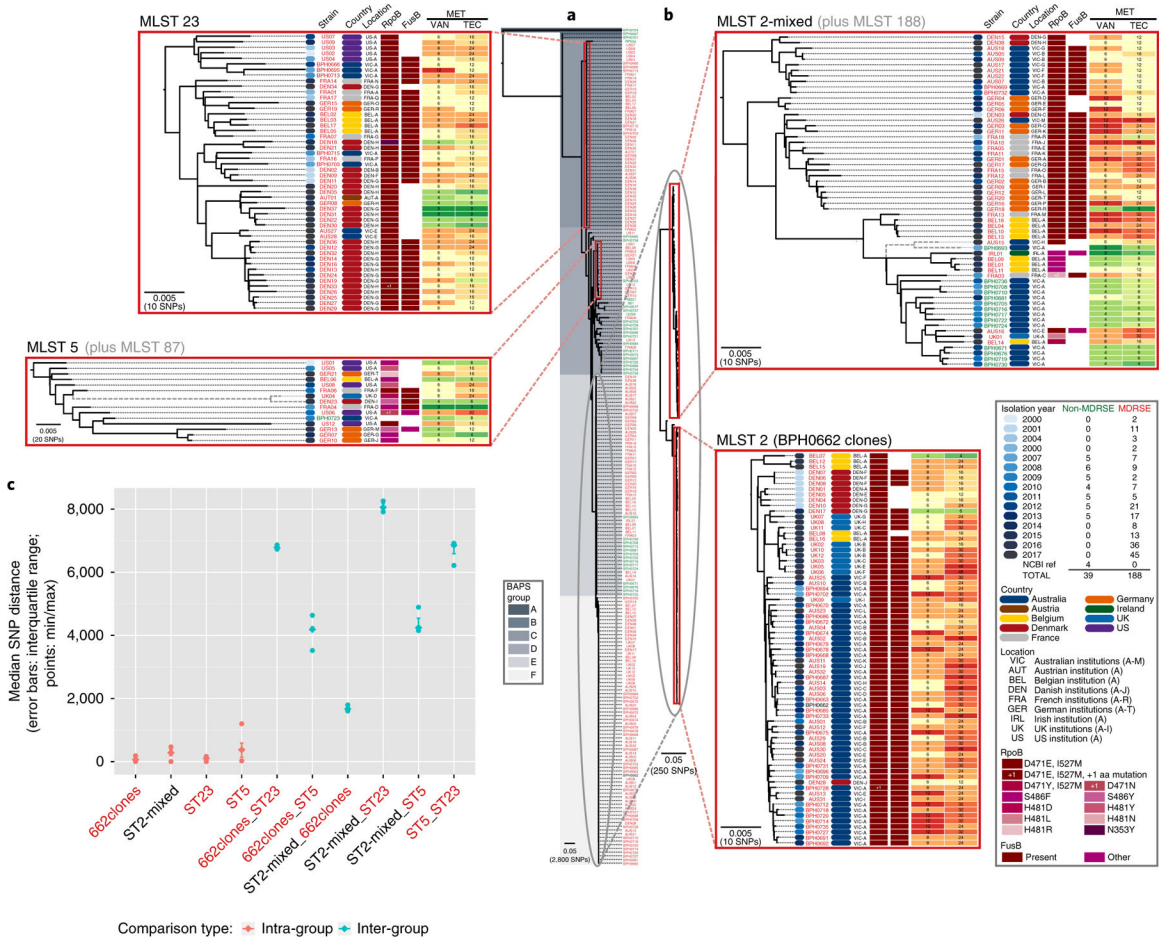

Fig. 3. International clonal expansion of endemic, multidrug-resistant ST2 and ST23 *S. epidermidis* lineages resulting in clinical disease.

a, Maximum-likelihood, core-SNP-based phylogeny of 227 clinical isolates originating from 77 institutions in 10 countries, using BPH0662 as the reference genome, with BAPS groups overlaid. **b**, Subset analyses of the 133 ST2 isolates (plus ST188, a single locus variant of ST2). Insets show subset analyses for each of the four main MDRSE lineages: ST2-mixed (n = 60); ST2 BPH0662 clones (n = 71); ST23 (n = 50); ST5 (n = 15). Overlaid are the results of the determinants of rifampicin (RpoB mutations) and fusidic acid (*fusB*) resistance, and heatmap of vancomycin heteroresistant phenotype testing, as determined by VAN and TEC METs. BPH0662 was used as reference for all core-SNP subset analyses. Scale bars indicate number of nucleotide substitutions per site (bold), with an approximation of SNP rate (in parentheses). **c**, Pairwise-SNP analyses of the four main MDRSE lineages (sample sizes as in **b**).

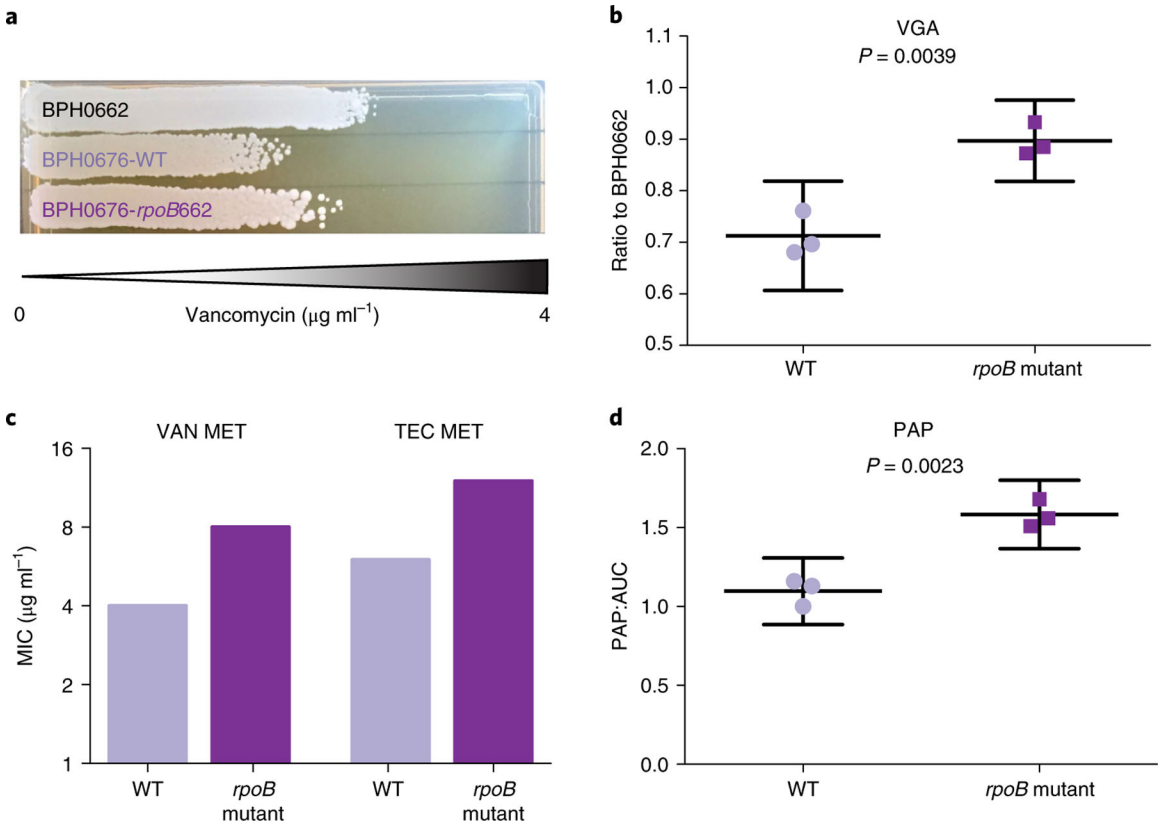

Fig. 4. RpoB mutations confer vancomycin heteroresistance in *S. epidermidis*.

a, An example of a VGA for BPH0676-WT, BPH0676-*rpoB*662 and the BPH0662 control strain. **b**, Biological triplicate data for the BPH0676-WT and BPH0676-*rpoB*662 mutant pair compared by VGA. Error bars represent mean ± 95% CI of three independent experiments. Null hypothesis (no difference between means) was rejected for $P < 0.05$ (two-tailed, unpaired Student's *t*-test). **c**, Data for a single set of VAN and TEC METs for BPH0676-WT and the BPH0676-*rpoB*662 mutant pair. The *y* axis for METs is plotted on a $\log_2$ scale. **d**, Biological triplicate data for the BPH0676-WT and BPH0676-*rpoB*662 mutant pair compared by vancomycin PAP. Error bars represent mean ± 95% CI of three independent experiments. Null hypothesis (no difference between means) was rejected for $P < 0.05$ (two-tailed, unpaired Student's *t*-test).

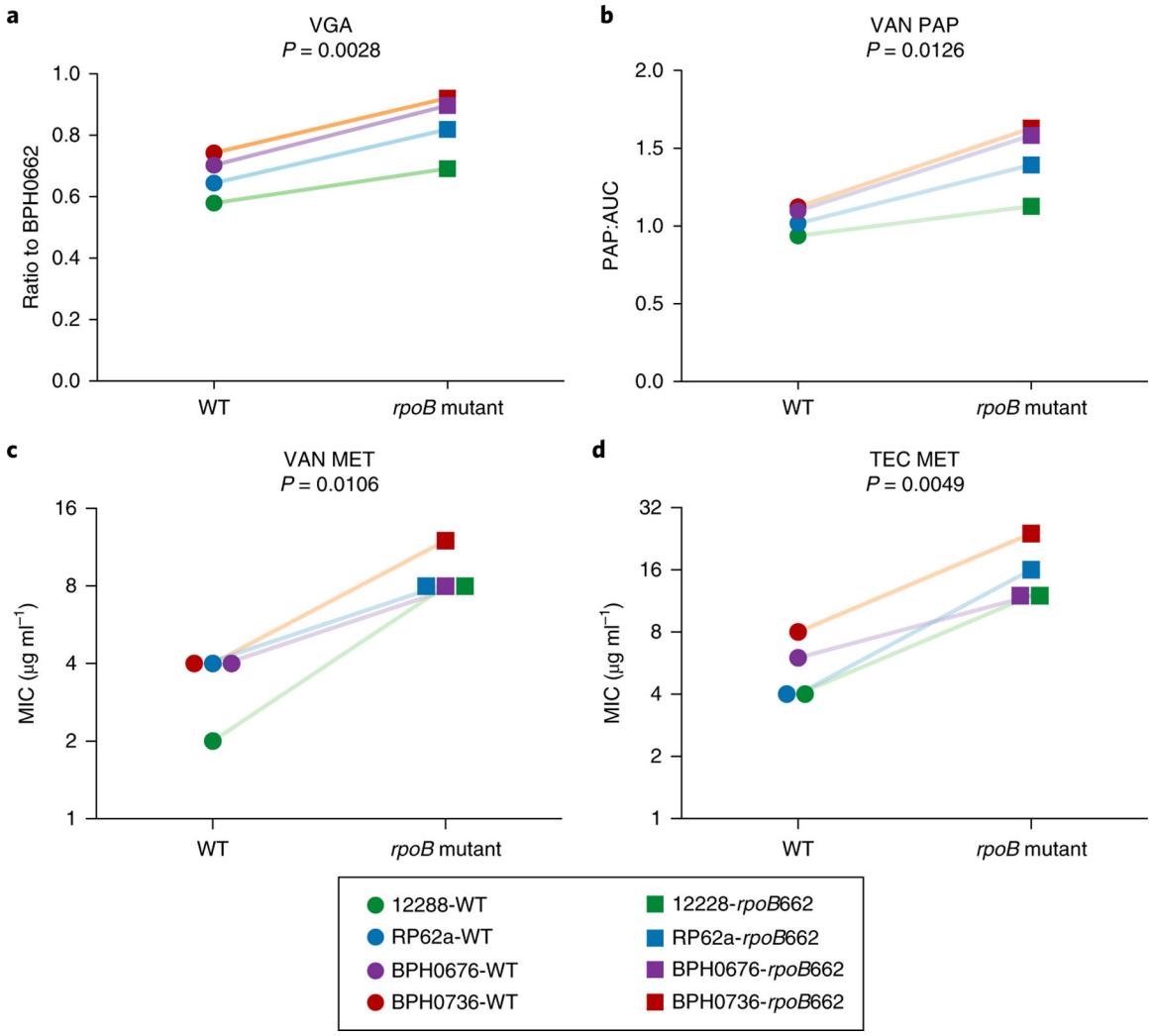

Fig. 5. RpoB D471e and I527M mutations cause vancomycin heteroresistance in four different *S. epidermidis* backgrounds.

a–d, RpoB D471E and I527M dual mutations were introduced into four different, rifampicin-susceptible *S. epidermidis* strains: two clinically significant ST2 strains, BPH0676 and BPH0736, and reference strains RP62a (ST10) and ATCC 12228 (ST8). The gains in vancomycin tolerance of these *rpoB*662 mutants compared to their respective WT parental strains were individually validated (Supplementary Fig. 4), then summary data analysed. Three different phenotypic methods for the detection of vancomycin heteroresistance were used: VGA (**a**), VAN PAP (**b**), and VAN (**c**) and TEC (**d**) METs. The *y* axis for METs is plotted on a $\log_2$ scale. Data points for VGA and VAN PAP represent the mean of three independent experiments for each strain. Null hypothesis (no difference between means) was rejected for $P < 0.05$ (two-tailed, paired Student's *t*-test).

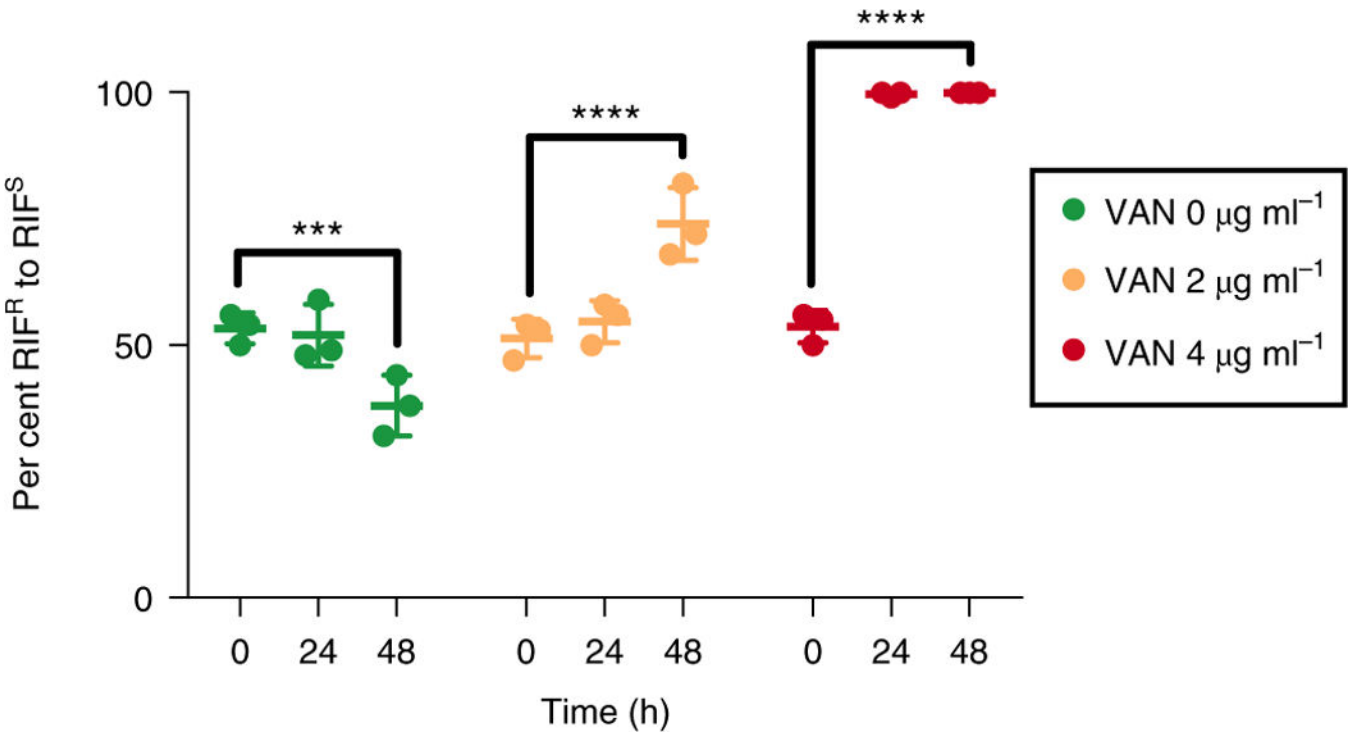

Fig. 6. Mutants containing dual D471e and I527M RpoB substitutions outcompete WT *S. epidermidis* in the presence of vancomycin.

Competition assays for the BPH0736-WT and *rpoB*662 mutant pair performed in the presence of VAN 0, 2 and 4 μg ml^{-1}, with the percentage of rifampicin resistant (RIF^R) to rifampicin susceptible (RIF^S) isolates determined by plate count. All data points for biological triplicate experiments are displayed. Horizontal lines depict mean and error bars show standard deviation. Null hypothesis (no difference between means) was rejected for $P < 0.05$. Differences assessed using two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons. ***$P < 0.001$, ****$P < 0.0001$.